Supraspinal And Spinal Mechanisms Of Morphine-Induced Hyperalgesia

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SUPRASPINAL AND SPINAL MECHANISMS OF
MORPHINE-INDUCED HYPERALGESIA

by
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Abstract

SUPRASPINAL AND SPINAL MECHANISMS OF
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by

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Morphine is the most prominent pharmacological treatment for moderate to severe pain in both acute and chronic paradigms. However, morphine notoriously elicits a paradoxical state of increased pain sensitivity known as hyperalgesia that complicates its use in clinical application. Research over the past three decades has reported that morphine-induced hyperalgesia is dose- and sex-dependent, and likely involves the synchronous activity of several neural networks beyond the opioid system. Whereas systemic, supraspinal, and spinal administration of morphine all cause hyperalgesia that is differentially reversible by N-methyl-D-aspartate receptor (NMDAR) antagonists or melanocortin-1 receptor (MC1R) antagonists, it is unknown as to whether or not these non-opioid systems that contribute to this state are located supraspinally or spinally. The current studies were performed with the goal of elucidating the precise location of regulatory action of this sex- and dose- dependent state of morphine hyperalgesia.

In all studies, outbred CD-1 male and female mice were pretreated with the general opioid receptor antagonist, naltrexone (NTX) 24 hours prior to morphine treatment. All mice were subsequently implanted with osmotic pumps, continuously dispensing a low (1.6mg/kg/24h) or
high dose of morphine (40mg/kg/24h). As noted previously, mice of both sexes were hyperalgesic by Day 4 of continuous infusion of either morphine dose, a state that persisted through Day 6 of infusion. The first series demonstrated that NMDAR and MC1R systems that mediate this morphine-induced hyperalgesic state are located supraspinally, as intracerebroventricular injections of MK-801 and MSG606, respectively successfully reversed hyperalgesia during a one-hour testing period. A second series of studies investigated possible involvement of spinal systems. Whereas intrathecal MK-801 significantly reversed hyperalgesia in males at both doses, and females at the low morphine infusion dose, spinal administration of MSG606 significantly reduced hyperalgesia in females following continuous high dose morphine infusion. This indicates that the sex-dependent mechanism involved in morphine-induced hyperalgesia is located supraspinally and spinally, and either locus can independently modulate female-typical hyperalgesia.

A third series of studies investigated hormonally-regulated mechanisms involved in morphine-induced hyperalgesia. Ovariectomized females displayed male-typical patterns of hyperalgesia after i.c.v. and i.t. antagonist injection paradigms following continuous infusion of either dose of morphine on Day 4. On Day 6, NMDAR and MC1R antagonist injections were preceded by an acute systemic progesterone injection in ovariectomized female mice, and intact male mice. Following continuous morphine infusion, ovariectomized females displayed male-typical patterns of hyperalgesic reversal. However, following progesterone administration, hyperalgesia elicited by high doses of morphine was reversed by i.c.v. injection of MK-801 and MSG606 in both males and ovariectomized females. Conversely, following i.t. injections the data show that ovariectomized females are able to recruit the NMDAR or MC1R system, while males
exclusively used the NMDAR system to mediate hyperalgesia. The current studies indicate that in terms of modulating morphine-induced hyperalgesia, there are both supraspinally- and spinally-regulated sex-dependent effects that mediate morphine-induced hyperalgesia.
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CHAPTER 1.

INTRODUCTION

The use of opium, primarily for recreational purposes, has been documented as early as 3000 to 2500 B.C. During this time, period the Sumerians were amongst the first to cultivate poppies, naming them “hul gil” or “plant of joy”. The Sumerians were also documented to have isolated opium from the seed capsules of poppies, giving it the name “gil”, meaning “joy”. Around 1500 B.C., descriptions of medicinal administration of opium surfaced; however, physicians at the time were hesitant to use such methods due to opium’s unpredictable potency. By the thirteenth century A.D., the use of opium was believed to be widespread across Europe, and it is around this period that manuscripts describing abuse and addiction to the drug surfaced. Despite the apparent negative side effects of opium, efforts to ban the substance were unsuccessful (Christup, 1997; Brownstein, 1993).

Although the extensive use of and experimentation with opium over the previous millennia had already been cited, Friedrich Sertürner is credited with the discovery of the active ingredient in opium now known as morphine, in 1806. Sertürner successfully isolated the alkaloid compound from the dried juice of the unripe seedpods of the poppy plant, and named the substance “morphium” after the god of dreams, Morpheus. Subcutaneous intravenous administration of morphine for surgical procedures and the management of pain began in the 1850s; however, it was also becoming increasingly clear that morphine had negative and potentially harmful side effects such as addiction, respiratory depression, and analgesic tolerance which would require an increase in dosage (Brownstein, 1993; Mao, Sung, Ji, and Lim, 2002). At this point, it became
imperative to discover either a natural or a synthetic substance that could offer the positive, pain relieving effects of morphine without the unwanted side effects. Use of codeine, heroin, meperidine, and methadone all became common practice in the search for a safer, alternative opioid (Brownstein, 1993). Nonetheless, morphine is still regarded as one of the most effective analgesics for treatment of both acute and chronic pain (Mao et al., 2002; Brownstein, 1993, Lee et al., 2011; Ossipov, Lai, King, Vanderah, Malan, et al., 2004). In a never-ending effort to elucidate the precise pharmacokinetics and pharmacodynamics of morphine, studies within the last three decades have provided evidence that while morphine is a potent analgesic, it also produces a state of increased sensitivity to pain, or hyperalgesia (Woolf, 1981; Crain & Shen, 2001; Vaughan & Connor, 2003; Chieng, Hallberg, Nyberg, & Christie, 2005; Galeotti, Stefano, Guarna, Biacnhi, & Ghelardini, 2006; van Dorp, et al., 2009). This hyperalgesic state is seemingly evoked by both acute and continuous infusion doses of morphine (Juni, Klein, and Kest, 2006).

Hyperalgesia is a puzzling side effect of morphine. While morphine is well known for its analgesic properties, the drug also produces a paradoxical state of heightened nociception after both acute and long-term use (Woolf, 1981; Crain & Shen, 2001; Vaughan & Connor, 2003; Chieng, Hallberg, Nyberg, & Christie, 2005; Galeotti, Stefano, Guarna, Biacnhi, & Ghelardini, 2006; van Dorp, et al., 2009). Hyperalgesia is defined as an increased sensitivity to pain as well as allodynia, which is pain evoked by a stimulus that is not under normal circumstances considered painful (Heger, Mair, Otter, Helwig, & Suttrop, 1999). Such a phenomenon is usually evident following administration of titrated doses of opioids, which is almost always necessary after the inevitable development of tolerance (Pasero & McCaffery, 2012). Although
morphine is particularly infamous for its likelihood to evoke hyperalgesia, other short-acting opiates such as fentanyl (Waxman, Arout, Caldwell, Dahan, & Kest, 2009) and remifentanil (Cooper, Lindsay, Ryall, Kokri, Eldabe, & Lear, 1997; Guignard, Bossard, Coste, Sessler, Lebrault, et al., 2000; Hansen, Duedahl, Romsing, Hilsted, & Dahl, 2005) also induce the same phenomenon. While hyperalgesia can be predictably elicited in animals (Woolf, 1981; Mao et al., 1994; Ossipov, Lai, Vanderah, & Porreca, 2003; Waxman et al., 2009; Juni et al. 2010; Waxman et al., 2010), in humans this paradoxical state appears to be less predictable and a quite serious complication of clinical opioid treatment (Compton, 2008; De Conno et al., 1991; Ossipov et al., 2004; Sjøgren et al., 1994; Sjøgren et al., 1998).

There are several prominent hypotheses of opioid-induced hyperalgesia. One common theory of hyperalgesia is that this state is a causative factor in analgesic tolerance. Specifically, the increased pain sensitivity that one experiences after opioid treatment causes the need for increasing doses of opioids, thus indicative of tolerance (Vanderah et al., 2001). A second popular hypothesis is that hyperalgesia is an adaptive response that serves as a systems-level opponent process in response to morphine analgesia after prolonged treatment. That is, as the body is always striving to maintain homeostasis, one’s natural response to exogenously elicited analgesia is to create an opposing state of hyperalgesia in attempt to regain physiological equilibrium (Simonnet & Rivat, 2003; Ossipov et al., 2003; Ossipov et al., 2004; Li, 2012). A third theory that is gaining popularity is that hyperalgesia is a result of a glial cell-regulated immune response (Hutchinson et al., 2009; Lewis et al., 2010; Watkins, Wiertelak, Goehler, Mooney-Heiberger, Martinez, et al., 1994; Watkins, Hutchinson, Rice, & Maier, 2009). With that said, multiple systems have been implicated in morphine-induced hyperalgesia. For
example, Crain & Shen (1990, 2000) review evidence suggesting that although opioids are reliably inhibitory, they can also exert excitatory effects on sensory neurons to produce the aversive side effects of opioids. Furthermore, it is postulated that hyperalgesia is served by a cellular mechanism by which opioids sensitize spinal neurons, thereby increasing pain sensitivity. Specifically, while opioids are thought to produce analgesia via action at inhibitory Gi/Go-coupled opioid receptors, Gs-coupled excitatory opioid receptor activity could be the cellular mechanism responsible for hyperalgesia. Specifically, Wu et al. (1998) report that opioid receptors can switch between Gi/Go-coupled and Gs-coupled receptors following alterations in GM1 ganglioside, a compound that affects neuronal plasticity as well as the activity of neurotrophins.

At the receptor level, morphine hyperalgesia has been demonstrated to be dose- and sex-dependent. For example, infusing lower morphine doses produces no analgesia, but evokes hyperalgesia within hours of administration. This hyperalgesia resolves within one week in males, but persists for minimally 14 days in females. Alternatively, higher continuous infusion doses of morphine cause analgesia that lasts approximately two to three days, followed by a hyperalgesic period that persists through day 11 in both males and females (Juni, Klein, & Kest, 2006; Waxman et al., 2010). Additionally, there is no cross-adaptation between high and low morphine doses (Juni et al., 2006). Furthermore, whereas the N-Methyl-D-aspartate receptor (NMDAR) antagonist MK-801 can reverse morphine hyperalgesia in mice of both sexes following the low infusion dose, it does so only in males – not females – undergoing continuous infusion of the larger dose (Juni et al., 2008; Waxman et al., 2010). Furthermore, ovariectomy in females causes the recruitment of exclusively a NMDAR modulatory mechanism akin to males,
while estrogen replacement in these same females restores female-typical patterns (Juni et al., 2008). This suggests that females possess functional male-typical hyperalgesic mechanisms, but their use is prevented by circulating ovarian hormones (Juni et al., 2008). The melanocortin-1 receptor (MC1R) antagonist MSG606 has been found to reverse hyperalgesia in exclusively females infused with a high dose of morphine, indicative of minimally two sexually dimorphic, dose-dependent hyperalgesic systems (Juni, Cai, Stankova, Waxman, Arout, et al., 2010).

The mechanisms mediating opioid hyperalgesia are numerous and complex. Although a majority of the literature has investigated chronic morphine treatment, the sites of action and mechanisms underlying sex- and dose-dependent chronic morphine-induced hyperalgesia still remain nebulous. Therefore, a thorough analysis of hyperalgesia during continuous morphine infusion in both supraspinal and spinal circuits of males and females is necessary to contribute to our current understanding of the molecular mechanisms, organismic factors, and neurocircuitry underlying this phenomenon.

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

1) Opioid Pharmacology
   a. Discovery of the opioid receptors
   b. Opioid receptor neuroanatomical distribution
   c. Opioid receptor modulation of nociception

2) Prevalence of Opioid-Induced Hyperalgesia
   a. Human Studies
b. Animal Studies

3) Mechanisms of Opioid-Induced Hyperalgesia
   a. Opioid-related mechanisms
   b. Cellular sensitization
   c. Morphine metabolites

4) Opioid-Induced Hyperalgesia and the N-Methyl-D-aspartate Receptor (NMDAR) System
   a. NMDAR distribution
   b. NMDAR-mediated opioid hyperalgesia
   c. Role of glutamate transporters in opioid-induced hyperalgesia

5) Opioid-Induced Hyperalgesia and the Melanocortin-1 Receptor (MC1R) System
   a. MC1R distribution
   b. Sex differences in opioid-induced analgesia and hyperalgesia

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

1a. Discovery of the opioid receptors. In the 1940s, scientists began rigorous research on the effects that various agonists and antagonists have on the nervous system. It was found that certain compounds such as nalorphine and naloxone had diverse pharmacokinetic effects on opioid activities and responses. For example, being a mixed agonist-antagonist, nalorphine was found to block the effects of morphine while also emitting its own analgesic potency (Brownstein, 1993). However, the pure opioid antagonist naloxone has been found to only abolish the analgesic effects of morphine and thus quickly induce withdrawal, while not causing any effects on its own (Brownstein, 1993). Studies using agonists and antagonists have been immensely useful in defining the pharmacokinetics and pharmacodynamics of morphine, such that their usage led to the discovery of endogenous opioids. In 1973, three independent groups of investigators concurrently reported the finding of endogenous receptors for opioids, referring mainly to the μ receptor (Pert & Snyder, 1973; Simon, 1973; Terenius, 1973). It was then reasoned that if the human body has preexisting and naturally occurring receptors that respond to compounds not found in the body such as naloxone (Pert & Snyder, 1973), then there must be a compound of similar chemical structure that is produced within the body that is meant to bind these endogenous receptors. Consequently, it was reasoned that this compound likely serves as the body’s natural pain reliever (Pert & Snyder, 1973).

Such findings led to a distinction of two primary classes of opioid receptor ligands: opiates and opioids. Consequently, this led to a distinction in terminology, as the terms “opiate” and “opioid” were and still are often used in a transposable manner. Scientifically speaking, the term “opiate” applies to substances extracted from the juices of the opium poppy Papaver

somniferum, which therefore concerns the first two drugs that were found in these juices: morphine and codeine. The latter term “opioid” refers to all substances, whether endogenous or exogenous, which bind to opioid receptors. Exogenous opioids can be natural (i.e. morphine), semisynthetic (i.e. heroin), or synthetic (i.e. fentanyl). In addition to the discovery of the aforementioned exogenous opioid substances, four classes of endogenous opioid peptides that bind to opioid receptors were also revealed by the early 1980s: endorphins, enkephalins, dynorphins, and deltorphins (Brownstein, 1993).

Following the discovery of an endogenous opioid system, researchers sought to further characterize opioid receptors. Martin et al. (1967) were amongst the first to suggest the existence of not one, but several opioid receptor subtypes after their extensive studies of the neurophysiological and behavioral effects of several opioid compounds (Brownstein, 1993). Through these studies, Martin and his colleagues observed three independent behavioral syndromes in response to three different opioid agonists. Subsequently, three different receptors were named after the agonist that was first found to bind to each one: mu was named for its ability to bind morphine, kappa was named for ketocyclazocine, and sigma was named for SKF-10,047. Following this, the delta receptor was discovered and named for its differential binding of [Met⁵]enkephalin and β-endorphin against [³H][Leu⁵]enkephalin and [³H]naloxone (Lord, Waterfield, Hughes, & Kosterlitz, 1977; Waterfield, Leslie, Lord, Ling, & Kosterlitz, 1979; Waterfield, Lord, Hughes, & Kosterlitz, 1978, Brownstein, 1993). Another receptor, termed the epsilon receptor, was proposed for a short time as an additional opioid receptor for its ability to bind β-endorphin. However, ensuing advances in the ability to clone receptors as well as the introduction of molecular technology led to the confirmation of only three opioid receptors: mu
(µ), which preferentially binds morphine; delta (δ), which primarily binds enkephalins and delorphins, and kappa (κ), which preferentially binds benzomorphans and dynorphins (Brownstein, 1993; Mollereau, Parmentier, Mailleux, Butour, Moisand, et al., 1994; Ossipov et al., 2004). More recently, an opioid-like receptor has been found and termed ORL₁, and is now considered a fourth opioid receptor (Mollereau, Simons, Soularue, Liners, Vassart, et al., 1996). Interestingly, although ORL₁ is both structurally and functionally similar to the other three opioid receptor subtypes, it does not bind typical opioid agonists or antagonists and has an endogenous ligand of nociceptin, or orphanin FQ. All four opioid receptors are classified as G protein-coupled receptors that activate the following G-proteins: Gᵢ to inhibit adenylyl cyclase, Gₖ to stimulate K⁺ channels, and Gₒ to inhibit Ca²⁺ channels (Mollereau et al., 1994). The investigation into the existence of other opioid-like receptors and the validity of such receptors in the opioid system is still ongoing.

1b. Opioid receptor neuroanatomical distribution. The anatomical distribution of the three main opioid receptors (µ, δ, and κ) varies rostrocaudally with each subtype (Gouardères, Cros, & Quirion, 1985). Pert and Snyder (1973) first reported a concentration of opioid receptors in the nervous tissue of both the mammalian brain and the intestine of the guinea pig. Distribution and anatomical localization of each of the opioid receptor subtypes has been investigated via in vitro autoradiography, radioligand binding, highly selective-ligand binding, in situ hybridization of mRNA for each receptor subtype, and immunohistochemistry (Ossipov et al., 2004). In terms of spinal sites of action, opioid receptors are concentrated in lamina I, II, and III of the dorsal horn (the marginal zone, the substantia gelatinosa, and the nucleus proprius, respectively) and dorsal root ganglia in the spinal cord (Quirion, 1984; Gouardères et al., 1985). These outer laminae are
the principal spinal sites of action of morphine on the transmission of nociceptive signals (Besse et al., 1990). Specifically, the µ-opioid receptor is highly concentrated and is the primary subtype in the outer laminae of the dorsal horn in the cervical to lumbosacral portion of the spinal cord, making up approximately 70% of opioid receptors found in this region (Quirion, 1984). The δ-opioid receptor is more diffusely distributed throughout the dorsal horn, accounting for approximately 23% of opioid receptors in the spinal cord, though it is found in dense concentrations in the outer laminae of the cervical and thoracic segments. The κ-opioid receptor is present in the smallest foci of the three subtypes (approximately 7%), and is concentrated in the outer laminae of the dorsal horns of the lumbosacral region of the spinal cord. It receives nociceptive inputs primarily from the viscera of the body (Quirion, Zajac, Morgat, & Roques, 1983; Quirion, 1984; Besse, Lombard, Zajac, Roques, & Besson, 1990; Besse, Lombard, & Besson, 1991; Ossipov et al., 2004).

ORL₁’s distribution is discrete yet widespread, as its transcripts are found in many areas of the central nervous system including the limbic system, hypothalamus, brainstem, and spinal cord. Additionally, ORL₁ does not appear to have an affinity for any one particular area, making it likely that its functions are not be limited solely to pain transmission, which supports Mansour et al.’s (1987) earlier speculation of a more functionally diffuse opioid receptor (Mollereau et al., 1994). In addition to antinociceptive functions, ORL₁ has displayed a role in other central functions such as emotions, regulation of neuroendocrine secretion and immune functioning, food intake, instinctive behaviors, spatial memory, and anxiety (Mollereau et al., 1994; Bertorelli, Corradini, Rafiq, Tupper, Calò, et al., 1999; Barlocco, Cignarella, Giardina, & Toma, 2000; Evans et al., 2009). Although aspects of its functioning and purpose are still under
investigation, lack reliability, and are generally in utter disagreement, ORL₁ has been found to elicit hyperalgesia and is at least partially colocalized with µ-opioid receptors in synapses (Rossi, et al., 1997; Evans, et al., 2009). ORL₁ is similar to traditional opioid receptors in that its hyperalgesia is insensitive to opioid receptor antagonists, and it mediates analgesia readily reversed by opioid antagonists. However, ORL₁’s endogenous ligand, orphanin FQ/nociceptin (OFQ/N) has a poor affinity for all other opioid receptors (Rossi et al., 1997; Bertorelli et al., 1999), and importantly, the receptor itself has low affinity for classic opioids (Rossi, Leventhal, & Pasternak, 1996; Barlocco, Cignarella, Giardina, & Toma, 2000).

There are similarities between the classic opioid receptors and ORL₁, both structurally and functionally. However, while ORL₁ activation via nociceptin does inhibit cAMP accumulation (Peluso, LaForge, Matthes, Kreek, Kieffer, et al., 1998; Fawzi, Zhang, Weig, Hawes, & Graziano, 1997), activates K⁺ channels, and inhibits Ca²⁺ currents similar to the other opioid receptors (Lou, Zhang, Ma, & Pei, 1998), differences arise in in terms of effect. For example, studies have found that ORL₁ hyperalgesia is immediately evident after intracerebroventricular injection of nociceptin in mice that is insensitive to naloxone, and persists for minimally 50 minutes post-injection, demonstrating a slightly different dose-response curve than that of morphine (Reinscheid, Nothacker, Bourson, Ardati, Henningsen, et al., 1995; Hara, Minami, Okuda-Ashitaka, Sugimoto, Sakai, et al., 1997; Meunier, Mollereau, Toll, Suaudeau, Moisand, et al., 2002; Suaudeau, Florin, Meuier, & Costentin, 2009). Others have found that intrathecal injection of nociceptin causes paralysis in mice (Reinscheid et al., 1995). However, some studies have found that intrathecal injections cause potent antinociception that is reversed by naltrexone (King, Rossi, Chang, Williams, & Pasternak, 1997), while others find strong hyperalgesia.
(Okuda-Ashitaka, Tachibana, Houtani, Minami, Masu, et al., 1996; Hara, Minami, Okuda-Ashitaka, Sugimoto, Sakai, et al., 1997). Other groups have demonstrated naloxone-reversible analgesia following a period of rapid onset hyperalgesia after nociceptin injection, indicating likely mediation by an unidentified ORL₁ subtype (Rossi, Leventhal, & Pasternak, 1996). Due to these unreliable and often contradictory results in terms of analgesia and particularly hyperalgesia, ORL₁ and its ligand nociceptin are unlikely mediators of opioid-induced hyperalgesia.

Spinal µ-opioid receptors are the primary subtype found on central terminals of afferent neurons, suggested after autoradiographic studies following dorsal root rhizotomy. The remaining subtypes reside on interneurons or second order neurons that transmit nociceptive information to higher order supraspinal sites (Ossipov et al., 2004). Such findings are in support of pharmacological studies of opioid receptor binding in the spinal cord; intrathecal injections of µ-receptor agonists illustrated a superior analgesic effect, with δ- and κ-receptor ligands producing less potent or nonexistent effects via spinal mechanisms (Leighton et al., 1988; Besse et al., 1990). However, other studies have illustrated an ability of agonists for all three subtypes to induce an analgesic effect when administered spinally, lending further credence to the notion of differentially distributed opioid receptor subtypes at the various levels of the spinal cord (Quirion, 1984). For example, δ-opioid receptor agonists have been found to inhibit the transmission of pain signals from primary sensory afferents in laminae I and II to projection neurons in the spinothalamic tract, contributing to evidence of a greater concentration of δ-opioid receptors in the outer laminae of the dorsal horn relative to the deeper layers. Morphine and [Met5]-enkephalin have also been shown to exert dose-dependent, naloxone reversible analgesia
after intrathecal administration in rat (Yaksh, Huang, & Rudy, 1977a). Interestingly, it has been shown that transection of the spinal cord causes a loss in potency of systemic morphine in response to thermal nociceptive stimuli, while the effect of spinally administered morphine remains intact. Such evidence supports the prevailing notion that opioids act directly on spinal sites to modulate nociceptive inputs (Advokat & Burton, 1987). Additional support for this notion can be found in studies showing that the functional blockade of spinal opioid receptors leads to a marked reduction in the analgesic potency of opioids (Hara, et al., 1999).

**1c. Opioid receptor modulation of nociception.** In addition to having a differential distribution in the spinal cord, each subtype has been shown to modulate a specific class of nociceptive perceptions. While μ-opioid receptor agonists appear to be equally active in both thermal and visceral nociception, thermal and visceral nociception have been shown to be processed respectively by δ- and κ- opioid receptor agonists (Quirion, 1984). All three subtypes are expressed primarily in afferent nociceptive C- and Aδ fibers of the dorsal root ganglia (DRG) cells as shown by immunohistochemical methods (Dado, Law, Loh, & Elde, 1993; Arvidsson, et al., 1995). Nociceptive signals are carried from peripheral terminals to the dorsal horn by the slow, unmyelinated C fibers, where they are communicated to the second-order projection neurons of the spinothalamic tract. The first phase of pain, commonly associated with sensations of immediate, sharp painful sensations is modulated by the fast-conducting Aδ fibers, while duller, longer lasting pain sensations are a result of the involvement of slower conducting C fibers; physiological changes in these latter fibers are often the culprit in the hyperalgesia and allodynia experienced by post-herpetic neuralgia patients (Fields, Rowbotham, & Baron, 1998; Staud et al., 2007).
Descending inhibitory projections from supraspinal sites that are activated by opioid receptors also regulate nociceptive signals entering the spinal cord. In addition to spinal loci, studies have shown significant levels of opioid receptor mRNA in cortical, diencephalic, and brainstem regions (Quirion, 1984). With regard to cranial distribution, the \( \mu \)-opioid receptor is densely disseminated in the anterior cingulate cortex, neocortex, amygdala, hippocampus, ventral dentate gyrus, presubiculum, nucleus accumbens, thalamus, habenula, interpeduncular nucleus, the periaqueductal gray, both the superior and inferior colliculi, and the raphe nuclei, with particularly dense concentrations in the caudate putamen. The \( \delta \)-opioid receptor is not quite as diffuse; it can be found in the anterior cingulate cortex, neocortex, amygdala, olfactory tubercle, nucleus accumbens, the superior and inferior colliculi, the raphe nuclei, and the caudate putamen, and is particularly dense ventrolaterally. Finally, \( \kappa \)-opioid receptors are densely distributed throughout the amygdala, olfactory tubercle, nucleus accumbens, caudate putamen, medial preoptic area, hypothalamus, median eminence, periventricular thalamus, and the interpeduncular nucleus, and are particularly concentrated ventromedially (Mansour, Khachaturian, Lewis, Akil, & Watson, 1987). All three opioid receptor subtypes are found pre- and postsynaptically, and are often present within the same neuron (Ossipov et al., 2004).

Descending control of pain arises from multiple supraspinal sites. Generally, opioid receptors can also be found throughout the frontal cortex, hippocampus, thalamus, hypothalamus, periaqueductal gray (PAG) and the rostroventral medulla (RVM). The latter two brain regions are most commonly associated with opioid-mediated antinociception, and share reciprocal connections in terms of their role in this process (Quirion et al., 1983; Quirion, 1984; Ossipov,
2004). Specifically, opioids activate cells in the PAG that in turn excite neurons in the RVM.

It is speculated that opioid-induced antinociception is modulated, at least in part, by descending pathways originating from the PAG, indicated by studies using microinjection of morphine into this area. It has been shown that antinociception initiated in the PAG is at least partially mediated by noradrenergic neurons, which are not present in the PAG (Fang & Proudfit, 1998; Bajic & Proudfit, 1999). Intracerebroventricular microinjection of morphine has been shown to produce antinociception in rodents (Porreca et al., 1984); additionally, microinjection into the ventrolateral PAG has also been shown to appease the activity of projection neurons in the dorsal horn in response to peripheral nociceptive stimuli (Bennett & Mayer, 1979; Lewis & Gebhart, 1977a, 1977b). In addition to the RVM, the PAG is also heavily interconnected with the hypothalamus and limbic forebrain structures including the amygdala, anterior cingulate cortex, and medial prefrontal cortex (Heinricher, Tavares, Leith, & Lumb, 2009).

While opioids activate neurons in the PAG, these projections directly interact with the RVM, resulting in excitation (Basbaum, Clanton, & Fields, 1978; Basbaum & Fields, 1978, 1984). The RVM is also a critical region to bidirectional nociceptive modulation, as it receives inputs from the dorsal horn of the spinal cord and additional rostral sites, and projects diffusely to both superficial and deep laminae of the dorsal horn (Zhuo & Gebhart, 1997). The RVM is the region of the medulla including the nucleus raphe magnus, the nucleus gigantocellulararius pars alpha, and surrounding reticular neurons ventral to the nucleus gigantocellulararius and extending between the caudal facial nucleus and the inferior olivary complex (Ossipov et al., 2004). Ablation of the raphe magnus and raphe pallidus of the RVM has resulted in decreased
nociceptive thresholds, as well as a weakened analgesic response to morphine (Proudfit, 1980). Thus, the PAG-RVM system plays a large role in inhibitory control and serves to suppress nociceptive inputs (Morgan, Whittier, Hegarty, & Aicher, 2008).

Analysis of molecular circuitry of the RVM through electrophysiological studies has revealed populations of specialized neurons termed “on”, “off”, and “neutral” cells. These cells are considered to be the neural basis for the bidirectional control that the RVM has in nociception (Fields & Heinricher, 1985; Fields, Heinricher, & Mason, 1991; Heinricher & Morgan, 1992; Neubert, Kincaid, & Heinricher, 2004; Ossipov et al., 2004; Heinricher et al., 2009). The off-cells continuously discharge in a tonic manner in response to the initiation of nociceptive input, and pause immediately before an animal’s behavioral response to a noxious stimulus. At this point, on-cell firing accelerates. Therefore, the on-cells are associated with the modulation of behavioral reflexes involved in pain (Heinricher, Barbaro, & Fields, 1989; Ossipov et al., 2004). In other words, behavioral responses to noxious thermal stimuli such as the tail flick or paw withdrawal are marked by a shift in the activity of RVM-cell populations, such that as the on-cells are activated, the off-cells become silent (Heinricher et al., 1989). It is now generally believed that off-cells function to produce a net inhibitory effect on nociception. Additionally, Boyer, Morgan, and Craft (1998) found that when morphine was microinjected into the RVM, male rats demonstrated superior antinociception when compared to female rats (and they hypothesized this sex difference to be at least partly regulated by the RVM itself). However, it has more recently been proposed that in addition to inhibiting pain, this system may also facilitate nociception with on-cells likely having a large role in this process as the mediators of said descending facilitative information (Neubert et al., 2004; Heinricher et al., 2009). For
instance, neurotensin is a tridecapeptide known to elicit hyperalgesia at low doses, whereas microinjection into the RVM has been shown to induce analgesia at high doses (Buhler, Proudfit, & Gebhart, 2008). In rats, neurotensin has been shown to selectively activate on-cells when low doses are given, resulting in enhanced nociception, or hyperalgesia. Furthermore, higher doses of neurotensin additionally activate off-cells and elicit antinociception (Zhou & Gebhart, 1997; Urban & Gebhart, 1999).

Additional studies using microinjections of capsaicin into the PAG induced hyperalgesic states as measured by the tail flick test, followed later by an analgesic state, that were correlated with bursts of RVM on-cell and off-cell activity, respectively (McGaraughty et al., 2003). It is therefore believed that on-cells play a significant role in the facilitation of nociception, enhanced sensitivity to noxious stimuli, and reduced sensitivity to analgesic drugs, while off-cells mediate descending antinociceptive inputs from the RVM (McGaraughty et al., 2003; Neubert et al., 2004). Neutral-cells were originally characterized by the absence of a reaction to deleterious stimuli (Ossipov et al., 2004), as they show no response to noxious stimuli; however, it is now believed that they may be recruited when necessary to assist on- or off-cells, as studies have shown such phenotypic conversions during chronic pain states. Studies have also shown an increase in both on- and off-cell populations and a corresponding decrease in neutral-cells 24 hours after inflammation when compared to naïve animals (Miki et al., 2002).

While the PAG predominately expresses the µ-opioid receptor subtype with little to no manifestation of the others, the RVM has been shown to contain all three subtypes, although still favoring populations of the µ subtype (Mansour et al., 1987; Ossipov et al., 2004). In addition,
the locus coeruleus has also been shown to have a role in the modulation of nociceptive inputs and also expresses opioid receptors. Finally, the serotinergic nucleus raphe magnus may function to relay nociceptive information from the PAG to descending pathways throughout the spinal cord. Overall, it appears that opioids exert their effects via synergistic interactions between activity at spinal and supraspinal loci (Ossipov, 2004). What remains nebulous is the interaction between these receptors, their corresponding pathways, their actions in various brain areas, and if or how all of these variables contribute to hyperalgesia following opioid treatment.

2. Prevalence of Opioid-Induced Hyperalgesia

2a. Human studies Although a majority of research on opioid-induced hyperalgesia (OIH) is comprised of animal studies, the obvious application of such research is the use of opioids in clinical settings involving treatment of pain, where this phenomenon inserts a complication. The success of pain treatment in humans is hindered by the appearance of hyperalgesia, where opioid titration and rotation become a potentially dangerous guessing game. Further complicating the issue is the inability to reliably predict or elicit opioid-induced hyperalgesia in clinical settings. That is, there is little literature to consistently support the existence of OIH under reliable circumstances (Lee et al., 2011). As opioids have become a cornerstone for treatment of pain related to health issues such as cancer and surgical procedures, understanding the mechanisms underlying OIH is imperative in order for clinicians to effectively address patient needs. As such, there have been studies sporadically conducted in the human population using observational case studies, as well as cross-sectional methodology. A majority of these studies focus on a few particular cohorts; for example, patients suffering from chronic illnesses such as cancer, those with acute pain following various surgical procedures, populations of opioid
addicts (both former and current), and healthy volunteers undergoing human experimental pain testing.

In one series of four case studies of patients with cancer (Sjøgren, Jensen, & Jensen, 1994), morphine-induced hyperalgesia disappeared after the cessation of morphine administration, or also when treatment with various other opioids was substituted for morphine. For instance, a young girl undergoing intravenous (i.v.) morphine infusion developed both hyperalgesia and myoclonus, which was obfuscated when the i.v. morphine was discontinued. Following the cessation of morphine, she was maintained on occasional doses of oral methadone instead and thus relatively free of hyperalgesic symptoms. In a second case, hyperalgesia evoked by a small dose of sustained-released morphine was obfuscated after the patient was alternatively given an oral regimen of ketobemidone. A third case describes a patient in which injections of high dose intramuscular (i.m.) morphine was given, where hyperalgesia was eliminated following the substitution of subcutaneous (s.c.) sufentanil. Finally, the case of a boy is described in which hyperalgesia was evident following a regimen of high doses of both oral and i.m. morphine. In his case, hyperalgesia subsided after morphine treatment was discontinued (Sjøgren, Jensen, & Jensen, 1994). In another case study (Mercadante & Acuri, 2005), a 48-year-old man with diagnosed chest sarcoma complained of severe pain in various regions, and was subsequently placed on a regimen of fentanyl patches with additional adjuvant doses of oral morphine in an unsuccessful attempt to treat the aforementioned pain. Additionally, the patient developed myoclonus as a result of opioid treatment. While reducing the fentanyl dose, an adjuvant prescription of methadone was given that reduced the patient’s pain completely within eight hours. Subsequently, this patient was maintained on oral morphine without issue.
In a study of ninety patients who had undergone laparoendoscopic urologic surgery, patients received high-dose remifentanil for the treatment of postoperative pain and subsequently developed hyperalgesia. However, it was found that a single oral dose of pregabalin, a gabapentinoid compound, effectively attenuated these symptoms (Lee, Lee, & Kim, 2013). In a controlled using a double-blind study on the use of perioperative analgesics performed by Chia, Liu, Wang, Kuo, and Ho (1999), the investigators found that post-operative consumption of fentanyl was of greater magnitude when intraoperative doses of the drug were higher, as compared to a low dose cohort. Specifically, 60 female patients undergoing abdominal hysterectomy were randomly assigned to either a group to receive 1µg/kg of fentanyl prior to surgery (low dose group), or a group receiving 15µg/kg prior to surgery and maintained on 100µg/hr during surgery (high dose group). Measured at both four and eight hours postoperatively, patients in the high dose group reported higher pain intensity and subsequently required more postoperative fentanyl to relieve their pain states. While the authors attribute this difference in pain intensity to the development of tolerance (Chia et al., 1999), it could very well be a clinical manifestation of hyperalgesia that is independent of the development of tolerance (Juni, Klein, & Kest, 2006), as fentanyl has been found to elicit this phenomenon in both acute and chronic paradigms in mice (Waxman et al., 2009).

Presumably attributable to its NMDAR-antagonistic properties, although weak, some practitioners report the successful use of adjuvant methadone to control opioid-induced hyperalgesia. This was the case in several of the above-mentioned case studies, and additionally in a case reported by Axelrod and Reville (2007). In this case, a young girl suffered from
malignant pain and was unsuccessfully treated with numerous other opioids. Nonetheless, there remains an abundance of unresolved conflict in the literature regarding clinical manifestations of OIH.

Doverty et al. (2001a) note the inconsistencies in the literature and attributed them to the array of pain modalities and tests used to assess opioid-induced hyperalgesia. In an effort to resolve the conflicting literature, Doverty et al. (2001a) conducted a study using 16 patients enrolled in the South Australian Public Methadone Maintenance Program. In their study, the investigators counterbalanced two pain induction methods: electrical stimulation and the cold pressor test. In order to induce pain via electrical stimulation, a cutaneous electrode was attached to one ear lobe and pulses lasting for 14ms were delivered. Beginning at 0 volts, electricity increased every 1.4 seconds by 2 volts, and participants verbally indicated the first perception of the stimulus as painful, and resultant increases in pain up until they could no longer tolerate it. The methodology for the cold pressor test was adapted from the procedures of Eckhardt et al. (1998, as cited in Doverty et al., 2001a), where patients essentially held their arm in ice water until they, again, could no longer tolerate the pain. Both measures were reliant on participant’s subjective reports of pain. Additionally, they accounted for fluctuations in plasma concentrations of methadone over time using liquid chromatographic methods. Doverty et al. (2001a) found that on tests of electrical stimulation, 30 minutes prior to their daily scheduled dose of methadone (0 hours), patients maintained on methadone had a lower tolerance for pain (i.e. they reported intolerance of the electrical stimulation at a significantly lower voltage) when compared to their control counterparts. However, three hours after the initial test (and thus 2.5 hours after their methadone dose), there were significant differences in the initial detection of pain (i.e.}
methadone maintenance patients reported the stimulus as painful at a lower voltage), as well as in the tolerance of said pain (i.e. methadone maintenance patients again reported intolerance of the electrical stimulation at a significantly lower voltage) when patients maintained on methadone were compared to the control patients not receiving methadone. Interestingly, on the cold pressor test, patients maintained on methadone reported both a decrease in detection and tolerance of pain at both 0 hours and three hours later when compared to their control counterparts, thus exhibiting significant hyperalgesia as a result of methadone maintenance. Additionally, Doverty et al. (2001a) found that at trough plasma concentrations of methadone, patients had similar threshold detection values as controls, and were less pain-sensitive than controls at their peak methadone plasma concentration. Finally, in a follow-up study, Doverty et al. (2001b) also found that methadone maintenance patients are cross-tolerant to the antinociceptive properties of morphine, such that attempting to treat acute pain in these patients using conventional doses of morphine is unsuccessful.

Another popular cohort of study in clinical applications of opioid-induced hyperalgesia includes healthy, non-opioid dependent volunteers undergoing acute opioid treatment. Compton, Athanasos, & Elashoff (2003) conducted a preliminary study using the cold pressor test to assess hyperalgesia after administration of three different opioid protocols (i.m. morphine, i.v. morphine, or i.v. hydromorphone) and subsequent naloxone-precipitated withdrawal. When compared to their placebo condition, the participants in this study had markedly and reproducibly reduced thresholds and tolerance to the cold-pressor test, sometimes as much as 70% of their initial baseline. However, the fact that this study employed a within-subjects design, although allowing for increased statistical power, creates a potentially serious and detrimental confound.
Participants’ responses could have differed based solely on the fact that they had previously received opioids in this very study, such that they are not truly opioid-naïve after participating in the first condition. Additionally, this study only used men and thus leaves an important area of investigation unfounded: gender differences in clinical applications of opioid-induced hyperalgesia. Finally, preclinical research has shown that hyperalgesia is not merely a side effect of opioid withdrawal, and can readily occur as its own independent phenomenon (Juni, Klein, & Kest, 2006). In short, reliable, non-confounded clinical applications of opioid-induced hyperalgesia have remained lacking.

In an attempt to address the limitations consistently present in the literature, including cross-sectional designs, failure to distinguish between tolerance and hyperalgesia, and the use of patients who have previously received opioids, Chu and colleagues conducted an observational study using opioid-naïve pain patients beginning chronic oral opioid therapy for persistent lower back pain (Chu, Clark, & Angst, 2006). Both analgesic tolerance and hyperalgesia using cold pressor and experimental heat pain paradigms were assessed after one month of oral morphine treatment. Patients demonstrated significant hyperalgesia and analgesic tolerance on the cold pressor test but not heat pain models, partially supporting previous findings in populations who have received chronic opioid treatment as well (Chu, Clark, & Angst, 2006; Doverty et al., 2001a; Doverty et al., 2001b). Such evidence supports the notion that hyperalgesia is specific to certain pharmacologic agents as well as pain modalities; such that one’s modality-specific hyperalgesic response after one opioid may differ vastly when tested using another nociceptive stimulus and a resulting different sensory modality (Doverty et al., 2001a).
2b. Animal studies  

Over the past few decades, there has been an increasingly growing interest in opioid-induced hyperalgesia and its underlying mechanisms. As seen above, research addressing clinical applications of this phenomenon is relatively limited. However, there is an abundance of available literature on this topic using animal models. In 2006, Angst and Clark 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, and electrical pain. Despite wide variations in methodology and animal species under study, the authors were surprised by the lack of agreement on a pathway mediating OIH, and instead found evidence for numerous pathways as contributors to pain processing. 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, and likely influenced by varying genetic backgrounds in animals as well (Angst & Clark, 2006; Mogil et al., 1999a; Mogil et al., 1999b).

In general, animal models of opioid-induced hyperalgesia follow two basic paradigms based on the duration of opioid exposure. Acute paradigms typically involve the systemic administration of a single opioid dose and the observation of resulting changes in behavioral responses. In such paradigms, a dose-dependent, biphasic role for morphine and other µ receptor agonists has been found, whereby animals demonstrate intense antinociceptive effects followed by periods of enhanced pain sensitivity typically lasting three to four hours (Ding & Bayer, 1993; Larcher, Laulin, Celerier, Le, & Simonnet, 1998; Laulin, Larcher, Celerier, Le, & Simonnet, 1998; Celerier, Laulin, Larcher, Le, & Simonnet, 1999; Crain & Shen, 2001; Borgland, 2001; Xu, Colpaert, & Wiesenfeld-Hallin, 2003).
The majority of studies investigating OIH use chronic administration paradigms, where animals are administered opioids for a range of three to fourteen days, using a variety of routes including multiple daily injections, subcutaneous pellet or osmotic pump implantation, or intrathecal catheters. Here too, biphasic responses are consistently observed, with said opioids eliciting antinociception generally lasting one to three days. Following this analgesic period, there is a gradual hyperalgesic onset (Laulin, Celerier, Larcher, Le, & Simonnet, 1999; Li, Angst, & Clark, 2001a; Li, Angst, & Clark, 2001b; Gardell et al., 2002; Bie & Pan, 2003; Ossipov, Lai, Vanderah, & Porreca, 2003; Kest, Palmese, Juni, Chesler, & Mogil, 2004; Coutaux, Adam, Willer, & Le, 2005; Juni et al., 2006). Furthermore, the finding of hyperalgesia in animal studies using uninterrupted opioid delivery paradigms (Celerier et al., 2000; Celerier, Laulin, Corcuff, Le, & Simonnet, 2001; Kest et al., 2002; Simonnet & Rivat, 2003; Ossipov, Lai, King, Vanderah, & Porreca, 2005; Vanderah et al., 2001a; Juni et al., 2006), supports the idea that nociception is not simply a consequence of opioid withdrawal, as could be the case in studies using multiple injection paradigms, but rather a direct consequence of opioid treatment. Thus, there is a growing body of evidence elucidating a dual-role for morphine as well as other opioids, whereby these agents produce coinciding activation of two paradoxical mechanisms; the first being an intense but short-lasting pain inhibitory phase (analgesia), and a weaker but longer-lasting pronociceptive period (hyperalgesia). Although concomitantly present during the analgesic phase, the hyperalgesic effect is often not observed immediately because it is allegedly masked by this simultaneous analgesia, and thus can only be evident after analgesia subsides. However, this initial hyperalgesia can be unmasked, as is the case in studies using either sub-analgesic doses of morphine (Vierck, costa-Rua, Nelligan, Tester, & Mauderli, 2002) or with
CXBK mice that show reduced analgesic responses due to their μ-opioid receptor deficiency (Li et al., 2001a). Altogether, this suggests that hyperalgesia may be a direct consequence of opioid exposure that is independent of prior analgesic processes.

3. Mechanisms of Opioid-Induced Hyperalgesia

3a. Opioid-Related Mechanisms  Pioneering research by Collier and colleagues (1974, 1981) uncovered the notion that hyperalgesia may not be a phenomenon elicited solely by the administration of exogenous opioids, but that this process may be modulated by the endogenous opioid system, particularly the kappa opioid receptor. Collier et al. (1974) first identified a “quasi-morphine withdrawal syndrome (QMWS)” and defined it as behaviors characteristic of an opioid-dependent animal experiencing actual morphine withdrawal, but present in an opioid-naïve animal given non-opioid drugs. Additionally, during a state of QMWS, the effects of opioids and their antagonists should be analogous to those undergoing true opioid withdrawal. For instance, Collier et al. (1981) found that QMWS is extraordinarily similar to naloxone-precipitated withdrawal seen in animals dependent on morphine. Such drugs that, when injected into naïve rodents, elicit QMWS (including hyperalgesia) are 3-isobutyl-1-methylxanthine (IBMX), theophylline, and caffeine. Collier et al. (1984) proposed that these drugs act by inhibiting cAMP phosphodiesterase (cAMP-PDE), which in turn raises the amount of cAMP available in the brain. A parallel of the aforementioned drugs to true opioids lies in the fact that opioid dependence is characterized by an increase in cAMP, that which is originally caused by an opioid’s tendency to inhibit adenylyl cyclase. Essentially, these non-opiate drugs elicit hyperalgesia akin to that seen after morphine treatment by causing the release of endogenous opioid (Crain & Shen, 2008).
Crain and Shen (2008) extended Collier et al.’s (1974, 1981) research by illustrating that a more specific cAMP-PDE inhibitor, rolipram, rapidly evokes thermal hyperalgesia in mice, mediated by excitatory opioid receptor signaling. However, extending their own research beyond their original findings with exogenous opioids (Crain and Shen, 1994; Shen and Crain, 1998; Crain and Shen, 2000), Crain and Shen (2008) found that this effect is blocked by cotreatment of low-dose naltrexone, and in fact a state of endogenous, bimodally acting (excitatory/inhibitory) opioid-mediated analgesia is exposed. Additionally, the same analgesic effect is found when naltrexone is replaced with the kappa-opioid receptor antagonist nor-binaltorphimine (nor-BNI) or the mu-opioid receptor antagonist β-funaltrexamine (β-FNA). The aforementioned research provides a base by which the endogenous opioid system could potentially be used in the management of pain when specific cAMP-PDE inhibitors and low-dose naltrexone, nor-BNI, or β-FNA are combined. However, this research was performed using paradigms formulated within the mouse spinal cord, and thus the degree of applicability of these effects to supraspinal centers as well as general extrapolation is unknown.

In a series of electrophysiological studies, Crain and Shen (1994) demonstrated that while extremely low doses of many mu-, kappa-, and delta-opioid receptor agonists can elicit excitatory effects on their respective receptors (resulting in hyperalgesia), higher doses evoke inhibition of these receptors (resulting in analgesia), indicating a need for higher doses of opioids in the treatment of pain (Shen and Crain, 1998). More recent studies have shown that these low, subanalgesic doses of morphine may actually be sufficient for the treatment of chronic pain, but only when accompanying low doses of opioid antagonists are present. Crain and Shen (2000)
found that although extremely low doses of morphine (ca. 0.1 mg/kg) elicited hyperalgesia in mice, this effect was blocked by concomitant treatment of equally low doses of naltrexone (ca. 1–100 pg/kg). Additionally, this accompanying treatment unmasked potent analgesia, and thus can be used to make lower doses of opioids effective, such that patients did not require quite as much drug to alleviate their chronic pain states. In turn, this can alleviate adverse affects (Crain & Shen, 2000). Shen and Crain (1998) reported clinical evidence from 60 post-hysterectomy patients with the ability to self-administer morphine, such that patients receiving morphine in conjunction with low doses of the opioid antagonist naloxone required less morphine to alleviate their post-operative pain when compared to patients only receiving morphine, indicative of the development of tolerance in the latter group. This evidence provides a possible solution to the manifestation of hyperalgesia in clinical settings, where there exists a delicate balance between the prescription of opioids in attempt to alleviate pain and hyperalgesia, and the consequent increase in this phenomenon due to the protocol currently used to avoid it (titration of the drug).

Shen and Crain (1998) found that GM1 ganglioside, a member of the cAMP-PKA-dependent glycolipid family shown to regulate the binding of growth factors and to manipulate second-messenger systems, has a role unique to the opioid system. Specifically, alterations in the concentration of GM1 ganglioside are likely responsible for the conversion of opioid receptors from inhibitory (G<sub>i</sub>/G<sub>o</sub> second messenger-coupled) to excitatory (G<sub>s</sub>-coupled), and vis-à-vis, such that intraperitoneal (i.p.) injections of this glycolipid rapidly weaken the analgesic effects of morphine. Additionally, where naloxone is known to induce hyperalgesia as a result of physical dependence in mice treated with chronic morphine, a similar state is evoked by injection of a low dose of naloxone in mice pretreated with GM1 ganglioside, and naltrexone or the kappa opioid
receptor antagonist nor-binaltorphimine blocks this naloxone-evoked hyperalgesia. In fact, treatment with naltrexone or nor-binaltorphimine in GM1 pre-treated mice produces potent analgesia, likely by some mechanism involving the release of endogenous opioid agonists via inhibitory kappa opioid receptor signaling. Such evidence suggests a possible role for this glycolipid in morphine-induced hyperalgesia (Crain & Shen, 2007). In accordance, the binding of GM1 by extremely low doses of the non-toxic B-subunit of cholera toxin (CTX-B) blocked the excitatory but not inhibitory effects of morphine, such that CTX-B blocks morphine-induced hyperalgesia (Shen and Crain, 2001). Such results were similar to those obtained that showed a reduction in the manifestation of morphine hyperalgesia and accompanying increases in morphine analgesia after concomitant treatment of low-dose naltrexone. Thus, the plasticity of GM1 ganglioside may be the cellular mechanism so highly sought after, by which opioids can produce both analgesic/inhibitory effects, as well as hyperalgesic/excitatory effects on opioid receptors.

3b. Cellular sensitization. Studies indicate that prolonged opioid treatment not only results in a loss of opioid analgesic efficacy, but also leads to activation of a pronociceptive system that results in a reduction of nociceptive thresholds, indicative of both negative system adaptation (desensitization) and positive system adaptation (sensitization), respectively (Mao, 2002). Whereas desensitization refers to the gradual weakening of a response to a repeated stimulus, or a reduction in neuronal signaling after repeated stimulation (Freedman & Lefkowitz, 1996), central sensitization refers to increased synaptic transmission/efficacy in somatosensory neurons of the dorsal horn as a result of peripheral noxious stimuli (Ji, Kohno, Moore, & Woolf, 2003). It is hypothesized that opioid-induced hyperalgesia may therefore result from sensitization of
primary afferent neurons, and thereby manifests as amplification in nociceptive responses. Additionally, this phenomenon may also result in enhanced production, release, and spread of excitatory amino acid neurotransmission and the suppressed reuptake of neurotransmitters, and/or from neuroplastic changes of various receptor systems in the peripheral and central nervous system that lead to sensitization of pain pathways (Vanderah et al., 2001; Li & Clark, 2002; Ji, Kohno, Moore, & Woolf, 2003).

An upregulation of G-protein coupled neurokinin-1 receptors (NK-1) has been implicated in opioid-induced hyperalgesia. Lamina I cells of the dorsal horn that express the NK-1 receptor have been shown to project to supraspinal areas that facilitate pain processing (Nichols et al., 1999). Substance P, an excitatory neurotransmitter synthesized by primary afferent nociceptors (McCarthy & Lawson, 1989), is the endogenous ligand for the NK-1 receptor. It is released in the spinal cord dorsal horn after nociceptive stimulation (Duggan, Morton, Zhao, & Hendry, 1987), and has been shown to have a role in central sensitization and hyperalgesia associated with inflammatory pain (King et al., 2005). In fact, it has been reported that in circumstances of pathological pain, there is increased substance P expression in primary afferents and increased release of substance P upon noxious stimulation, causing internalization of NK-1 receptors in both superficial regions and deep laminae of the spinal cord. Although this specific scenario is the neuronal substrate of inflammatory pain, this mechanism has been hypothesized to also play a potential role in neuronal plasticity associated with opioid-induced hyperalgesia (Takeda, Chou, Takeda, Sachais, & Krause, 1991). Studies have shown that substance P in mice either intermittently treated or chronically exposed to morphine evoked hyperalgesia on chemical, thermal and mechanical assays of pain. Additionally, these paradigms also demonstrated greater
c-Fos immunoreactivity in dorsal horn nuclei after morphine administration, presumably a result of substance P activity in these areas and indicative of neuronal sensitization. This is supported by studies indicating that both systemic and intrathecal morphine treatment elicits substance P activity, increases spinal NK-1 receptor expression, and results in opioid-induced hyperalgesia, suggesting a particular role for opioid receptors in the spinal cord (Vanderah et al., 2001; Li & Clark, 2002; King et al., 2005).

Descending facilitation of nociception from the RVM has been found to increase spinal levels of the endogenous opioid peptide dynorphin (Gardell et al., 2002; Wang et al., 2001; Laughlin et al., 1997). Although originally believed to possess antinociceptive properties, dynorphin has more recently been shown to be a paradoxical kappa-opioid receptor agonist that modulates synaptic transmission via non-opioid receptor mechanisms (Laughlin et al., 1997). That is, dynorphin potentiates naloxone-insensitive pronociception by increasing neuronal receptive field size and sensitizing NMDA receptors, thus increasing the release and binding of excitatory neurotransmitters (i.e. glutamate) and subsequent release of intracellular calcium (Laughlin et al., 1997; Lai, Ossipov, Vanderah, Malan, & Porreca, 2001). In fact, a single intrathecal injection of low dose dynorphin produces heightened sensitivity to mechanical von Frey filament stimulation for up to 70 days in mice. Additionally, acute intrathecally administered dynorphin induces long-lasting tactile, cold, and mechanical allodynia and mechanical hyperalgesia that is blocked by NMDA receptor antagonists MK-801 and LY235959, and is unaffected by opioid receptor antagonism. MK-801 administered following dynorphin transiently blocks allodynia, indicating that dynorphin-induced allodynia may not only be produced but also maintained by NMDA receptors. Additionally, intrathecal MK-801 or dynorphin antiserum obfuscates morphine-
induced hyperalgesia (Laughlin et al., 1997; Vanderah et al., 1996; Vanderah et al., 2000). These paradigms suggest a role for spinal endogenous opioids in the descending, facilitative control of opioid-induced hyperalgesia.

3c. Morphine metabolites. A major area of ongoing investigation into the substrates underlying morphine-induced hyperalgesia is that of morphine metabolites. Once administered to humans, morphine is transformed primarily in the liver by UDP-glucuronosyltransferase, and to a lesser extent in the brain and kidneys into two primary metabolites, morphine-3β-glucuronide (M3G) and morphine-6β-glucuronide (M6G) (Christup, 2008; Vaughan & Connor, 2003). By way of the UGT2B7 isoenzyme, morphine is broken down in the liver into ninety percent M3G, and ten percent M6G. Recently, it has been hypothesized from in vitro studies that the 9:1 ratio of M3G to M6G is due to the additional involvement of the UGT1A1 isoenzyme in the formation of M3G; however, in vivo studies do not support anything other than a role for UGT2B7 in the metabolism of morphine (De Gregori et al., 2012). In mice, a variant UGT isoform is involved in glucuronidation, allowing only for the formation of M3G and no M6G (Zelcer et al., 2005). Thus, M6G receives little attention with regard to a role in morphine-induced hyperalgesia, as mice to not produce this metabolite but nonetheless exhibit hyperalgesia.

Membrane transport systems limit morphine accumulation in the brain by way of limiting accretion of the morphine glucuronides after morphine is biotransformed. Studies indicate that multidrug resistant protein 3 (Mrp3) is the major transporter of the morphine glucuronides from hepatocytes into the bloodstream. Once in the bloodstream, the glucuronides exert their effects
via an unknown mechanism and are eventually excreted primarily through the urinary tract. In
the absence of Mrp3 in mice, M3G is transported from the hepatocytes into bile, from which it is
excreted through biliary routes. Two interesting observations are that absence of Mrp3 does not
affect morphine-induced analgesia, and that analgesia is still present in mice who lack the ability
to form the extremely potent analgesic-inducing morphine metabolite, M6G. With that said, lack
of Mrp3 is not ideal, as transport through bile and excretion via intestinal routes allows for
deglucuronidation, reabsorption, and intestinal toxicity (Zelcer et al., 2005).

M6G has been found to have an affinity at µ-opioid receptors that is comparable to morphine,
and is commonly associated with superior production of longer lasting analgesic properties
without causing many of the troublesome side effects that result from morphine treatment
(Christup, 1997; Rossi, et al., 1997; Dahan, van Dorp, Smith, & Yassen, 2007; De Gregori, et al.,
2012). This idea came from a series of receptor binding studies in which it became apparent that
morphine elicits its analgesic effects via action at µ₁ receptors, and that many of its side effects
(such as respiratory depression and gastrointestinal disturbances) are mediated via action at µ₂
receptors. M6G has little affinity for any receptor other than µ₁ (Chen, Irvine, Somogyi, &
Bochner, 1991; Pasternak, Bodnar, Clark, & Inturrisi, 1987). Thus, M6G is likely a significant
contributor to the analgesic effects of morphine via that receptor subtype (Francés, Gout,
Campstron, Panconi, & Cros, 1990). In fact, when administered systemically (subcutaneous
injection) or centrally (via intrathecal or intracerebroventricular routes), M6G has been found to
have an increased effect duration of anywhere from a 1.6- to 4-fold or 13- to 800-fold analgesic
potency, respectively, when compared to morphine (Christup, 1997). In addition, M6G has been
found to be the only metabolite with analgesic properties derived from morphine that has the
ability to cross the blood brain barrier (BBB) with relative ease (De Gregori et al., 2012). With that said, M6G was quickly becoming the choice analgesic over morphine in clinical settings for the treatment of pain. However, M6G does cross the BBB, it does so slower than morphine, thus resulting in a significantly slower onset of analgesic effect (Christup, 1997). In addition, more recent evidence has shown that both acute and continuous administration of M6G also results in a hyperalgesic state independent of opioid receptors, similar to that of morphine, reversed by an acute injection of the NMDA receptor antagonist MK-801 (van Dorp et al., 2009). Because of such recent findings, M6G is less and less often considered the golden alternative to morphine in the treatment of pain.

Morphine-3β-glucuronide is the primary result of morphine biotransformation in humans and the only morphine metabolite produced in rodents. It is speculated to have a significant role in morphine-induced hyperalgesia and the development of tolerance. Importantly, M3G has no appreciable affinity at any opioid receptor subtype (Labella et al., 1979) and therefore no analgesic potency, and the wide-spectrum opioid antagonist naloxone does not block its pronociceptive effects (Juni et al., 2006). In addition, M3G has been reported to antagonize the antinociceptive effects of morphine and M6G, and is postulated to contribute to the respiratory depression seen after morphine treatment (Christup, 1997). Not fully understood is the relationship between morphine-induced hyperalgesia and M3G, if any. Juni, Klein, & Kest (2006) report hyperalgesic cross-adaptation between morphine and M3G, after seven days of low dose morphine infusion followed by an acute injection of M3G. However, Swartjes et al. (2012) report a negative correlation between acute morphine-induced hyperalgesia and plasma levels of M3G, such that as hyperalgesia manifests, plasma concentrations of M3G decrease.
Additionally, there is no evidence to support M3G directly interacting with the MC1R system, and thus M3G is an unlikely candidate in the mediation of morphine hyperalgesia. Yet, there does appear to be a functional relationship between the other major morphine metabolite, M6G, and MC1Rs, as e/e mice and humans lacking functional MC1Rs both exhibit significantly more powerful analgesia after M6G administration (Mogil et al., 2005).

Injecting M3G in rodents via the intracerebroventricular (i.c.v.) (Labella et al., 1979), intrathecal (i.t.) (Woolf, 1981), or systemic/subcutaneous (s.c.) (Juni et al., 2006) route has been found to enhance nociception. Interestingly, i.t. injections of M3G reportedly induced hyperesthesia and allodynia (Yaksh, Harty, & Onofrio, 1986), while i.c.v. injections induced a variety of other excitatory behaviors such as excessive grooming, “wet dog” shakes (Bartlett, Cramond, & Smith, 1994), lethargy, and seizure-like behavior (Arout, Caldwell, McCloskey, & Kest, unpublished observations), suggesting the involvement of a variety of non-opioid receptor systems within the central nervous system. Furthermore, both naloxone (NLX) and naltrexone (NTX) exacerbate these excitatory behaviors, providing further evidence for non-opioid receptor involvement in the pharmacodynamics of M3G (Christup, 1997).

Notably, it appears that even after administration of the general opioid antagonist naltrexone, M3G is still significantly more active in the PAG when compared to morphine also preceded by NTX, via unknown mechanisms (Arout, Caldwell, McCloskey, & Kest, unpublished observations). Interestingly, the PAG is rich in μ-opioid receptors (as well as other receptors systems implicated in morphine-induced hyperalgesia) and is known for its role in pain modulation, whereas levels of δ-opioid and κ-opioid receptors are barely discernible in this
A role for M3G in morphine hyperalgesia has been proposed in humans and rats (Woolf, 1981). However, the activity of M3G is poorly characterized. There is a potential role for the GABA_\text{A} receptor complex in the mediation of M3G; Bartlett et al. (1994) have shown that midazolam (a GABA_\text{A} receptor agonist) attenuates the excitatory behaviors elicited by the morphine metabolite, although M3G has not been shown to have an affinity for this receptor complex. M3G is also thought to activate (though not bind) NMDA receptors, a finding consistent with the ability of MK-801 to reverse morphine hyperalgesia, as well as findings of the functional antagonism of the excitatory behavioral effects of M3G by the NMDA receptor antagonist LY274614 (Bartlett et al., 1994). It is therefore feasible that this metabolite is working indirectly with the NMDA receptor system; however, while the NMDA receptor antagonist MK-801 reverses morphine-induced hyperalgesia (Juni et al., 2006; Juni et al., 2008; Waxman et al., 2010), it does not appear to block M3G’s pronociceptive effects, further suggesting distinct
mechanisms underlying these hyperalgesic states (Arout, Caldwell, McCloskey, & Kest, unpublished observations). Therefore, it is likely that M3G elicits hyperalgesia via another unknown receptor system.

Both morphine and M3G have been reported to have significant activity at the toll-like receptor 4 (TLR4), whereas its analgesia-inducing morphine metabolite counterpart M6G showed no such activity (Hutchinson et al., 2010; Lewis et al., 2010). This receptor system is therefore indicated in the manifestation of neuroexcitatory effects of morphine and M3G (Due, Piekarz, Wilson, Fledman, Ripsch, et al., 2012). However, while NTX increases morphine glucuronidation and thus results in increased concentrations of M3G (Antonilli, Petecchia, Caprioli, Badiani, & Nencini, 2005), it has also been found to be a TLR4 antagonist (Li, 2012), so any paradigm employing NTX pretreatment should conceivably narrow the possibility of M3G (or morphine) working directly via TLR4 to mediate hyperalgesia. Indeed, recent research (Lewis et al., 2010) indicates a role for M3G working indirectly via this receptor system to modulate a microglial-originated state of TLR4 regulated hyperalgesia. That is, it is possible that M3G is producing hyperalgesia that is initiated via a glial cell mechanism, as both astrocytes and microglia have been found to express all three subtypes of opioid receptors and play a role in the development of opioid-induced side effects. As such, these glial cells in part cause the release of numerous neuroexcitatory transmitters, proinflammatory cytokines, and neuromodulators, which in turn produce increased excitability via direct and indirect connections to AMPA and NMDA receptor pathways, and downregulation of GABA receptors (Li, 2012). Such evidence supports the notion that opioid-induced hyperalgesia is a response elicited by the body to counteract analgesia and regain homeostasis (Simonnet & Rivat, 2003); in other words, hyperalgesia may be an
immune response in itself (Watkins, Hutchinson, Rice, & Maier, 2009). However, whether this immune response-elicited hyperalgesia is the same to that seen after morphine treatment is unknown. Seeing as M3G has no affinity for opioid receptors and has demonstrated activity at TLR4, it is reasonable to speculate that the hyperalgesia seen after M3G treatment is regulated by this system. However, it is again notable that NTX serves as a TLR4 antagonist, abolishing the possibility of a role for this receptor system in studies employing NTX pretreatment paradigms (Li, 2012). Indeed, it appears that NTX does not block the ability of M3G to increase cellular activity in the PAG (Arout, Caldwell, McCloskey, & Kest, unpublished observations) and therefore makes such speculation impractical. As such findings involving TLR4 are novel, additional corroborative and expansive studies are required. Additionally, behavioral pharmacological studies investigating a role for TLR4 in M3G-induced hyperalgesia appear nonexistent.

Although it has been suggested that M3G can be transformed back into morphine, it is entirely possible that morphine-induced hyperalgesia and the hyperalgesic state present after administration of M3G are two independent phenomena. It has been observed that morphine antagonized by NTX results in a marked decrease in the number of c-Fos active neurons in the PAG, while mice pelleted with NTX prior to M3G administration show no such decrease in active neurons in this same brain region. This suggests that M3G hyperalgesia is mediated by non-opioid mechanisms independent of transformation back into morphine (Arout, McCloskey, & Kest, unpublished data), or activity at hyperalgesia-mediating ORL1 opioid receptors (Moran & Smith, 2002; Rossi, Leventhal, Bolan, & Pasternak, 1997). Nonetheless, it is still unknown what precise mechanisms are recruited to cause M3G’s effects or where it is active in the central
nervous system (CNS), as all currently known opioid receptor subtypes have been excluded (Moran & Smith, 2002). Other brain regions relevant to opioid pain modulation such as the locus coeruleus, anterior cingulate cortex, ventral tegmental area, nucleus accumbens, and medulla may play a significant role in morphine-induced hyperalgesia involving morphine metabolites and therefore require further study.

Though there is an abundance of data supporting a possible role for the morphine metabolites in hyperalgesia, new research contradicts such speculations. It is becoming apparent that the morphine metabolites, though able to elicit hyperalgesia, do so through mechanisms independent of the hyperalgesia seen after morphine treatment. For example, Swartjes and colleagues (2012) demonstrated a negative correlation between hyperalgesia (evidenced by tail withdrawal latency) and blood plasma concentrations of M3G. In fact, it was found that as plasma levels of morphine increased, so did the magnitude of hyperalgesia. This evidence suggests that morphine itself may be the mediator in opioid-induced hyperalgesia. In addition, MRP3−/− mice lacking the ability to export M3G from the liver into systemic circulation also develop hyperalgesia after an acute morphine injection (Swartjes et al., 2012). Such evidence serves to eliminate the role of the morphine metabolites in opioid-induced hyperalgesia, leaving all roads likely pointing to the direct involvement of another receptor system: the NMDA receptor.

4) **Opioid-Induced Hyperalgesia and the N-Methyl-D-aspartate Receptor**

The NMDA receptor system has a well-studied and important role in neural and behavioral plasticity, long-term potentiation, learning, and memory. For these reasons, it is an excellent candidate receptor for the study of the mechanism underlying morphine-induced behaviors
(Trujillo & Akil, 1991). Specifically, Trujillo and Akil (1991) conducted studies indicating a likely role for this receptor system in the development of opioid tolerance and dependence, resulting from neural adaptations after repeated morphine exposure. Accordingly, as there is an apparent relationship between the NMDA receptor and morphine tolerance and dependence, investigators became interested in the possible role of this system in morphine-induced hyperalgesia. Also important is the parallel between the aforementioned dynorphin-induced allodynia and hyperalgesic states and morphine-induced hyperalgesia, in that both produce naloxone-insensitive pronociception that is blocked by NMDA receptors. This suggests that opioid-induced pronociceptive states are served by the NMDA receptor system (Laughlin et al., 1997).

4a. **NMDAR distribution.** NMDA receptor expression is widespread throughout both the central and peripheral nervous system, and is primarily responsible for fast excitatory synaptic transmission in the brain. Interestingly, it has recently been found that NMDA receptors are not necessarily static, and have the ability to travel from synaptic to extrasynaptic sites. This apparent mobile nature of NMDA receptors is increasingly being implicated in early cognitive dysfunction as well as a variety of neurodegenerative disorders (Gladding & Raymond, 2011). Though this receptor system’s most notable role to date is memory function and the control of synaptic plasticity, it is becoming more and more apparent that NMDA receptors also play a significant role in opioid-induced hyperalgesia. This speculation is supported by the fact that NMDA receptors are present in particularly dense populations in areas that modulate pain signaling, such as the dorsal root ganglion, superficial layers of the dorsal horn laminae, thalamus, and the hippocampus (Swartjes et al., 2012).
The postsynaptic density (PSD), which is a protein dense specialization attached to the postsynaptic membrane, is rich in glutamate receptors. This specialization ensures that receptors on the postsynaptic side are close enough to the presynaptic neurotransmitter release sites. Here, NMDA receptors form a large macromolecular complex that is comprised mainly of scaffolding, and adaptor and effector proteins that are involved in the activation of downstream signaling cascades and the overall regulation of NMDA receptor function, stability, and trafficking (Gladding & Raymond, 2011; Kennedy, 1997). NMDA receptors are also located in dense populations both peri- and extra-synaptically. Those receptors found perisynaptically likely serve as mobile receptors traveling to and from the PSD, while extrasynaptic NMDA receptors are those found on the soma, dendrites, and spines of neurons. Not only do these receptors redistribute between the PSD, peri- and extrasynaptic locations, but they also navigate between the plasma membrane and intracellular compartments. Synaptic and extrasynaptic NMDA receptors found in the hippocampus and the cortex are associated with the activation of neuroprotective and apoptotic signaling cascades, respectively (Gladding & Raymond, 2011). Since the activation of NMDA receptors commonly results in any number of second messenger and other downstream signaling pathways, further in-depth research is required as it is plausible that one of these second-order activations could be the connecting pathway between the NMDA system and the initiation of morphine-induced hyperalgesia.

There are seven known subunits of the ionotropic NMDA receptor; GluN1, GluN2A-GluN2D, and GluN3A-GluN3B, with several variants of each type of subunit. Most common are NMDA receptors that are tetrameric proteins made up of two GluN1 and two GluN2 subunits, and these
receptors are found both extrasynaptically as well as synaptically (Gladding & Raymond, 2011). During development, GluN1 expression is at its plateau in most areas of the brain, with GluN2B sharing a similar pattern of developmental expression. However, GluN2B expression declines in adulthood, and the emergence of GluN2A is much more apparent as the hippocampus and cortex fully mature (Gladding & Raymond, 2011). The synaptic target of each fully assembled NMDA receptor is determined by the NMDA receptor subunit composition, which is conceived during development. In order to activate the ionotropic NMDA receptor complex, the binding of glycine and glutamate to the GluN1 and GluN2 subunits, respectively, is required. However, to remove the Mg$^{2+}$ block and open the channel to allow a flux of ions additionally requires postsynaptic depolarization. A subsequent influx of Ca$^{2+}$ through the NMDA receptor is the neural substrate responsible for synaptic changes seen in long-term potentiation (LTP) and long-term depression (LTD). However, overstimulation of this receptor system can be detrimental in that Ca$^{2+}$ dependent proteases, lipases, and DNAases can be activated (Gladding & Raymond, 2011).

4b. NMDAR-mediated opioid hyperalgesia. Perhaps most intriguing is the finding that morphine-induced hyperalgesia occurs independent of opioid receptors. Several studies have indicated that morphine hyperalgesia is not a consequence of either prior or concurrent opioid receptor activity (Yoburn, Cohen, & Inturrisi, 1986; Juni et al., 2006; van Dorp et al., 2009; Juni, Cai, Stankova, Waxman, Arout, Klein, Dahan, Hruby, Mogil, & Kest, 2010). In fact, the blockade of NMDA receptor activity using MK-801 in naltrexone-pelleted mice results in a complete reversal of morphine and M6G-induced hyperalgesia in both acute and chronic
paradigms, providing further support that this reversal is independent of opioid receptor activity (Juni et al., 2006; van Dorp et al., 2009).

Juni et al. (2006) reported that in mice where hyperalgesia subsided after continuous infusion of a large dose of morphine, acute administration of a low dose of morphine immediately reinstated a hyperalgesic state. In addition, hyperalgesia is evident after continuous morphine infusion in opioid receptor triple knock out (TKO) mice lacking all three genes encoding the prominent opioid receptors µ, δ, and κ (Juni, Klein, & Kest, 2006; Juni, Klein, Pintar, & Kest, 2007). Currently, there is no reliable consensus as to the location of specific neural substrates that underlie the manifestation of this hyperalgesic state. However, there are several proposed mechanisms that seem to play a significant role, particularly involving the N-Methyl-D-aspartate receptor system.

The NMDA receptor system has long been speculated to modulate opioid-induced hyperalgesia. However, the precise mechanism by which this receptor system may be enabling such a state remains quite nebulous. It is clear from an abundance of research that NMDA receptors play a particular role in central sensitization and consequent inflammatory pain, although a knowledge base for the role it plays in peripheral sensitization is not yet established (Du, Zhou, Coggshall, and Carlton, 2003). It has been found that glutamate does indeed act on unmyelinated cutaneous sensory axons in rodents and humans, suggesting action at the same C- and Aδ-fiber neurons that play a primary role in hyperalgesia. In fact, intraplantar injections of glutamate induce both thermal and mechanical hyperalgesia, and subcutaneous activation of NMDA receptors results in
allodynia and hyperalgesia that is easily abolished following an injection of the antagonist MK-801 (Du et al., 2003).

As opioids do not have any binding affinity for the NMDA receptor, it is implausible that opioids directly interact with this receptor system. In addition, NMDA receptor antagonists do not affect acute opioid analgesia, further illustrating a lack of direct interaction between opioids and the NMDA receptor system (Mao et al., 2002). However, a role for the NMDA receptor in morphine-induced hyperalgesia is indicated by findings that this hyperalgesic state is reversed by the non-competitive antagonist MK-801 (Juni et al., 2006; Juni et al., 2008; Waxman et al., 2010). Additionally, Swartjes and colleagues (2012) attenuated hyperalgesia by administration of ketamine or the novel NR2B selective NMDA antagonist traxoprodil. Such evidence provides a promising illustration of a role for the NR2B subunit in the development of opioid-induced hyperalgesia; this subunit coincidentally is particularly dense in both spinal and supraspinal sites such as the dorsal root ganglia, superficial layers of the dorsal horn laminae, the thalamus, hippocampus, and cortex (Swartjes et al., 2012). Interestingly, physicians do not prescribe ketamine to counteract opioid induced hyperalgesia due to the manifestation of psychotropic side effects; however, traxoprodil appears to have no such effects, making it a plausible consideration for physicians in preventing hyperalgesia in patients undergoing opioid treatment.

Several studies have indicated that exposure to morphine through intracellular protein kinase C (PKC) may prime NMDA receptors and cause increased excitability (L. Chen & Huang, 1992; Mao, Price, & Mayer, 1994; Martin, Malmberg, & Basbaum, 2001; Sluka & Audette, 2006). The relationship between PKC and NMDA receptors in this context is not concrete; it is likely
that PKC somehow modulates NMDA receptors by removing the Mg$^{2+}$ block, thereby allowing an influx of Ca$^{2+}$ (Mao et al., 2002) which subsequently further stimulates calcium-sensitive protein kinase, particularly the gamma isoform (PKCγ). PKCγ catalyzes NMDA receptor phosphorylation, leading to augmentation of the NMDA mediated glutamate responses and to long-term potentiation of synaptic transmission (L. Chen & Huang, 1992; Sluka & Audette, 2006). Coincidentally, most of the isoforms of the PKC enzyme are found in the superficial laminae of the dorsal horn, indicating an anatomical overlap of opioid receptors, NMDA receptors, and PKC activity (Sluka & Audette, 2006). When activated in the spinal cord, PKC results in decreased heat and mechanical latency thresholds, increased release of glutamate, and sensitization of dorsal horn neurons as well as the spinothalamic tract (Sluka & Audette, 2006). It has been reported that development of morphine hyperalgesia is preventable by intrathecal administration GM1 ganglioside, an intracellular inhibitor of PKC translocation/activation. Such evidence illustrates a critical role for the activity of this protein that occurs in response to NMDA receptor activation in morphine-induced hyperalgesia (Mao et al., 1994).

**4c. Role of glutamate transporters in opioid-induced hyperalgesia.** The homeostasis of extracellular glutamate is regulated by the glutamate transporter (GT) system, which contains minimally five differentially expressed Na$^{+}$-dependent GT proteins. EAAC1 is a GT that is primarily expressed in neurons, while GLAST and GLT-1 are primarily found in glial cells. Evidence has shown that GTs play a crucial role in the prevention of glutamate neurotoxicity. Mao and colleagues (2002) used immunocytochemical methods, western blot analysis, and behavioral methods (tail-flick and paw-withdrawal tests) to illustrate that continuous morphine administration induces a downregulation of spinal glutamate transporters, suggesting a definitive
role for this system in morphine hyperalgesia. Indeed, additional studies in various brain regions have demonstrated changes in GLT-1 mRNAs subsequent to naloxone-precipitated morphine withdrawal, and morphine tolerance is decreased after subcutaneous injection of the glial GT activator MS-135 (Mao et al., 2002).

The most commonly proposed mechanism of morphine-induced hyperalgesia involves the colocalization of NMDA receptors and opioid receptors in the nervous system. Of particular interest is that fact that both NMDA receptors and µ-opioid receptors are both found pre- and postsynaptically, and are particularly abundant in lamina I-III of the dorsal horn (Mao et al., 2002; Gladding & Raymond, 2011). It is hypothesized that even though opioids are reliably inhibitory in nature, they may somehow activate NMDA receptors. Subsequently, continuous opioid administration may cause a reduction in GT function, thereby causing an increase in the amount of synaptic glutamate available for uptake, particularly in the spinal cord where opioids most commonly exert their effects. These physiological changes, in turn, may be responsible for the development of both tolerance and hyperalgesia following prolonged opioid treatment. Indeed, Mao et al. (2002) found that levels of EAAC1 and GLAST were significantly reduced in lamina I, II, III, IV, V, and VI (the lowest levels in laminas III-VI) of rats receiving seven days of intrathecal morphine in both acute injection and continuous infusion paradigms, when compared to baseline levels and saline groups. In addition, these reductions in GTs appear to be dose-dependent, as significant differences were seen after treatment with 10µg, and more so after treatment with 20µg of morphine. Coadministration of naloxone not only prevented the downregulation of both EAAC1 and GLAST, but it also prevented the development of morphine tolerance and thermal hyperalgesia. Interestingly, the downregulation of GTs is not apparent
until day six of morphine infusion and persists until minimally day eight, which corresponds well
with the timeline of the manifestation of morphine hyperalgesia (Mao et al., 2002). Furthermore,
hyperalgesia in rats treated with morphine is intensified by exogenous glutamate, evidenced by
reduced paw-withdrawal latencies and a prolonged state of hyperalgesia. Mao and colleagues
(2002) also found that this downregulation of GT after morphine administration is preventable if
NMDA receptors are inhibited. Finally, administration of GT regulators (i.e. riluzole) 30
minutes prior to exogenous glutamate prevents the development of tolerance and/or hyperalgesia,
but does not abolish an already-present state of tolerance and/or hyperalgesia. When
administered without glutamate, riluzole results in a 10-12% increase from baseline paw-
withdrawal latencies prior to onset of hyperalgesia (Mao et al., 2002). Such evidence suggests
that continuous morphine impacts glutamate homeostasis in the spinal cord, which in turn
somehow induces both morphine tolerance and hyperalgesia. Therefore, it is likely that spinal
changes in GT activity play a role in the development of opioid tolerance and possibly morphine-
induced hyperalgesia (Mao et al., 2002). However, the aspect of the aforementioned study
evidencing a temporal and therefore neuroanatomical correlation between morphine analgesic
tolerance and hyperalgesia no longer holds precedence, since more recent studies have shown
otherwise (Juni et al., 2006). A definitive answer as to how morphine causes hyperalgesia via
non-opioid mechanisms that likely involve NMDA receptors in the spinal cord remains
unknown.

Though the majority of evidence supports a role for spinal NMDA receptors in morphine
hyperalgesia, other studies have illustrated a critical role for supraspinal medullary NMDA
receptor activity as well. Urban and Gebhart (1999) found that inactivating the RVM attenuated
hyperalgesia. Additionally, it has been reported that bilateral lesions or lidocaine injections into the RVM not only reversed hyperalgesia, but also restored analgesia that had been previously obfuscated (Vanderah, Ossipov, et al., 2001; Vanderah, Suenaga, et al., 2001). A role for the on- and off-cell system in the RVM has been proposed after evidence that the application of mustard oil resulted in an increase of on-cell firing. When on-cell activation was blocked by infusing the NMDA receptor antagonist AP5 into the RVM, hyperalgesia was avoided. In addition, secondary thermal hyperalgesia after mustard oil application was also correlated with a decrease in off-cell firing that was unchanged by AP5 infusion. Thus, NMDA receptors appear to have two distinct roles in the RVM; they potentiate analgesia when they are recruited to activate off-cells, while producing hyperalgesia when contributing to activation of on-cells after an acute inflammatory stimulus (Xu et al., 2007). Therefore, it appears that NMDA receptor-mediated activation of on-cells is critical for the development of secondary thermal hyperalgesia in an acute context. Such evidence provides support beyond solely a spinal mechanism that employs the use of NMDA receptors in opioid-induced hyperalgesia, to include supraspinal mechanisms as well.

Administration of the noncompetitive NMDA receptor antagonist MK-801 has been shown in various studies to block morphine hyperalgesia when given in conjunction with GT inhibitors (Mao et al., 2002). Such studies have shown MK-801 to reverse and in some cases even enhance opioid analgesia in a sex- and dose-dependent manner (Bodnar & Kest, 2009; Juni et al., 2009; Waxman et al., 2009; Waxman et al., 2010). For example, hyperalgesia induced by low morphine doses in both male and female mice is reversed after acute injections of MK-801, while hyperalgesia induced by high doses of morphine infusion is reversed by MK-801 in males and ovariectomized females lacking circulating ovarian hormones. It is hypothesized that at high
doses of morphine, females recruit a melanocortin-1 receptor dependent system to process nociceptive information (Juni et al., 2009). In addition to sex differences with regard to hyperalgesic responses, male and female mice also show differences in analgesia. Studies have shown that the NMDA receptor antagonist MK-801 reverses stress-induced analgesia in males, but not females, further suggesting a role for a non-NMDAergic system in the processing of pain information in females (Mogil et al., 2003). However, the locus of action of these effects is unknown; whether these processes are mediated by spinal mechanisms, supraspinal mechanisms, or substrates working collaboratively in both regions remains unclear.

5) The Melanocortin-1 Receptor and Opioid-Induced Hyperalgesia

5a. Melanocortin receptor distribution. The melanocortin (MC) receptor system has become the main receptor system under investigation for its role in regulating female opioid-induced hyperalgesia. The receptor system is composed of five subtypes (MC1 through MC5), all of which are G protein-coupled receptors. Each subtype has vastly different functions, ranging from pigmentation, to energy balance, to the regulation of gland functioning within mammals. Of particular interest in the regulation of nociceptive processing is the MC1R, which is unusually polymorphic with more than 60 known naturally occurring variants (García-Borrón, Sánchez-Laorden, and Jiménez-Cervantes, 2005). The seven transmembrane MC1R is primarily expressed on the surface of melanocytes, or melanin-producing cells in the epidermis layer of the skin (Nakayama, et al., 2006). Past in situ hybridization and immunohistochemical studies have revealed a widespread distribution of this receptor system in the peripheral nervous system, yet a restricted distribution in the central nervous system, limited to the ventral periaqueductal gray
and in brain glial cells. Some reports have hypothesized the endogenous ligands for this receptor to be α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), which result in an increase in cAMP; this proopiомelanocortin gene product has been found to elicit inhibition of thermal pain, while contradictory studies have shown it to have an anti-opioid role (Xia, Wikberg, & Chhajlani, 1995; Mogil et al., 2003; Delaney et al., 2010).

Several variants of the MC1R have been identified and found to have a role in hair and skin pigmentation, a possible role in the susceptibility to skin cancer, and finally, a likely role in inflammatory and pain responses via the mitigation of proinflammatory cytokines (Mogil et al., 2003; Dessinioti, et al., 2011). The latter function has received remarkably little attention in the literature, as most studies on this receptor system focus on its role in hair and skin pigmentation (Rana, Hewett-Emmett, Jin, Chang, Samsuughin, et al., 1998; Sánchez-Más, 2004; Mogil et al., 2005; Nakayama, et al., 2006; Dessinioti et al., 2011; García-Borrón et al., 2005). The bit of research on this receptor system that has focused on its role in pain has studied differences in analgesic pain responses as a function of MC1R variants (Mogil et al., 2005).

5b. Sex differences in opioid-induced analgesia and hyperalgesia. Although research examining the NMDA receptor system reveals promising evidence for a solution to opioid induced hyperalgesia, this system cannot be entirely responsible for such a phenomenon. NMDA receptor antagonists do not reverse hyperalgesia across the board, as they leave hyperalgesia undisturbed in female CD-1 mice in some cases (Juni et al., 2008; Juni et al., 2010). It has been shown after continuous infusion of a low dose of morphine (1.6mg/kg/24h), hyperalgesia was present in males for six days but persisted in females for minimally 14 days.
At a high dose of morphine (40mg/kg/24h), there appears an analgesic period of two to three days, followed by hyperalgesia in both males and females that persists through day 11. Additionally, this hyperalgesic state occurs independently of prior or current opioid receptor activity, as all mice were concurrently treated with pellets containing the general opioid antagonist, naltrexone (Juni et al., 2008; Juni et al., 2010). As such, systemic administration of NDMAR antagonists attenuated hyperalgesia in both sexes at a low dose, while it reversed hyperalgesia exclusively in males at the high dose. After ovariectomy (OVX), females resorted to male-typical hyperalgesic patterns, suggesting that they do in fact possess the same systems but are diverted from using them by circulating ovarian hormones. This is further evidenced in that when ovariectomized females are subjected to estrogen replacement, their female-typical hyperalgesic patterns are restored. Therefore, it is believed that not only is there a role for NMDA receptor pathways in hyperalgesia, but also a hormonally regulated role for minimally a second receptor system (Juni et al., 2008; Juni et al. 2010).

The MC1R system’s role in skin pigmentation and anti-inflammatory processes is well studied and understood. What is not well characterized is this receptor system’s role in the inhibition and regulation of non-inflammatory pain, namely, analgesia and hyperalgesia. However, due to the MC1R’s presence in the PAG, this proposed function is feasible. Recent quantitative trait locus (QTL) mapping studies have directed attention to the MC1R system in terms of sex differences in opioid analgesia. Specifically, the MC1R system appears to mediate κ-opioid analgesia in females, but not males. Additionally, clinical use of opioids modulated by the κ-opioid receptor is more efficacious in females than in males. Mogil and colleagues (2003) performed a QTL study using the highly selective κ-opioid receptor agonist U50,488H in which
they found the MC1R gene on chromosome 8 to be the most likely candidate gene to mediate states of female-specific analgesia. Additionally, it was found that analgesia in females was completely obfuscated after administration of the ₾-selective opioid antagonist norbinaltorpimine, even though it was given 48 hours prior to U50,488H injections. This state of U50,488H analgesia is likely mediated centrally, as exclusively i.c.v. administration of MK-801 in males reverses this ₾-opioid’s analgesic state. Finally, whereas MK-801 had no effect in mutant B6 female mice, mutant C57BL/6J-Mc1r<sup>e/e</sup> (spontaneous mutants of the B6 background lacking functional MC1Rs) mice differentiated from B6 mice only by a lack of functional MC1Rs, demonstrated male-like, NMDA-mediated U50,488H analgesia. Therefore, Mogil et al. (2003) concluded that minimally B6 female mice are using the melanocortin system to mediate pain responses resulting from U50,488H administration, and QTL studies makes the contribution of any other protein to this process unlikely.

In this same series of studies, Mogil et al. (2003) explored the effects of blocking the MC1R system during U50,488H administration, using the antagonist Ac-Nle-Asp-Trp-DPhe- Nle-Trp-Lys-NH<sub>2</sub>. They hypothesized that such activities would cause the mice to “switch” systems, in that they would begin using the NMDA receptor system when the MC1R system was not available. Indeed, this is what occurred; U50,488H analgesia was potentiated in outbred, exclusively female mice after i.c.v. injection of the aforementioned antagonist that was reversed by injection of MK-801, demonstrating a shift in the system being used. Similar results were obtained using cyclopentylglycine, another MC1R antagonist (Mogil et al., 2003).

The finding that the MC1R has a role in opioid analgesia consequently led investigators to
question the role of MC1R in morphine-induced hyperalgesia. Recent studies have illustrated a sex difference, as female and male C57BL/6J (B6) naltrexone-pelleted mice showed hyperalgesia beginning on day 1 and 3, respectively, after continuous high dose morphine infusion. However, only naltrexone-pelleted B6 e/e male mice showed hyperalgesia from Day 2 to 7 (Juni, et al. 2010). Furthermore, MK-801 reversed hyperalgesia in B6 and CD-1 males but not in B6 or CD-1 females. A selective MC1R antagonist, MSG606, reversed hyperalgesia in B6 and CD-1 females but not in B6 or CD-1 males, all undergoing continuous infusion of the high dose of morphine. Conversely, continuous infusion of a low dose of morphine caused hyperalgesia in both e/e males and females (Juni, et al. 2010), thus further illustrating the involvement of a number of neural substrates in morphine-induced hyperalgesia.

Juni et al.’s (2010) findings reveal a striking counterpart to Mogil et al.’s (2003) rodent and clinical findings, in that these newer findings supported the idea that κ-opioid analgesia, and now morphine-induced hyperalgesia, is mediated by NMDA receptors in males and MC1Rs in females. Furthermore, it is likely that hyperalgesic mechanisms are located supraspinally as MC1Rs have been found to be expressed particularly in the midbrain PAG, a region critical to pain modulation (Juni et al., 2010). Noted, however, is the fact that these findings are restricted to a high dose of morphine and that similar findings are not yielded by low morphine infusion doses, thus suggesting minimally two distinct neural networks responsible for morphine hyperalgesia. This is further evidenced by aforementioned findings that although both male and female hyperalgesia are reversed by MK-801 after infusion of a low dose of morphine, female hyperalgesia begins later and lasts several infusion days, if not weeks, longer than males (Juni, et al. 2010, and unpublished observations).
Interestingly, it has been demonstrated that in the absence of functional µ-opioid receptors, morphine analgesia is regulated by κ-opioid receptors. This effect is minimal at low doses, but quite remarkable at high doses of morphine. Such evidence suggests minimally an interaction between µ- and κ-opioid receptors in morphine analgesia, which may indicate a role for this interaction in hyperalgesia as well (Yamada, et al., 2006). However, such studies were confounded by possible inadequate morphine doses and therefore are preliminary. Together, these studies indicate that NMDA receptors mediate hyperalgesia during a low morphine dose in male and female mice, whereas MC1Rs and NMDA receptors respectively play a role in female and male hyperalgesia during infusion of the high morphine dose. Thus, this demonstrates a critical role for the MC1R system in female opioid-induced hyperalgesia. However, beyond the aforementioned data, little is known as to the precise location and further mechanism of action of how MC1Rs mediate female opioid-induced hyperalgesia, as MSG606 was administered through a single systemic bolus injection and not directly into a specific site of the central nervous system in these studies (Juni et al., 2010).
RATIONALE

As demonstrated in the aforementioned literature review, the neural substrates underlying opioid-induced hyperalgesia remain unknown and require further investigation. Elucidating the precise mechanisms and corresponding locations underlying opioid-induced hyperalgesia is imperative, as morphine remains the most effective pharmacological intervention for the treatment of moderate to severe pain. This paradoxical state of sex- and dose-dependent hyperalgesia creates the most prominent and serious barrier to successful treatment. The next section will provide 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 Continuous Morphine Infusion

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 as the main opioid agonist for study in this dissertation. Although chronic infusion is the most clinically relevant paradigm for morphine administration, methods employing repeated injection/oral administration might allow for hyperalgesia resulting from withdrawal due to interruptions in treatment. Our paradigm uses continuous, uninterrupted delivery, which eliminates this confound. Obtaining an understanding of the hyperalgesic liabilities following continuous morphine exposure can provide a substantial and important scientific contribution to the field.

Rationale for the Use of Mice
My studies require continuous infusion of opioids into healthy subjects. Clearly, this is not possible in human populations. Furthermore, multiple genetic and environmental factors may confound the study of hyperalgesia in this population. These considerations are irrelevant in laboratory-obtained rodents. For this dissertation, mice were chosen as subjects because of the large body of literature documenting pain related characteristics in the mouse, the high degree of genetic similarity with human beings, and the ready availability.

**Rationale for Investigating Different Routes of Administration for Antagonists**

The morphine studies described in this dissertation utilized two central routes of antagonist drug administration. Systemic administration results in widespread distribution along the neuraxis and periphery, leading to activation at spinal, supraspinal, and peripheral levels, sites which are too diffuse to pinpoint a precise locus of action in the central nervous system. Therefore, more focal administration paradigms of intracerebroventricular and intrathecal administration were utilized in order to assess distinctly supraspinal and spinal loci contributions to morphine-induced hyperalgesia, respectively.

**Rationale for Selected Experimental Assay 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. As these measures assess pain as a result of vastly different evocation and presumably underlying physiological mechanisms, Mogil and colleagues (1999) conducted a multivariate analysis. In their studies, they analyzed the responses of 11 inbred mouse strains on
12 common measures of nociception and later identified three clusters of pain tests which appear to share common genetic substrates and presumably underlying physiology (Mogil et al., 1999a, 1999b). The extensive 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, peripheral nerve injury, and mechanical hypersensitivity). Mogil and colleague’s (1999a, 1999b) 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 relevance.

Studies of nociception are further limited by the specificity of the nociceptive process under investigation. For example, some of the most common techniques for studying pain sensitivity include measures of behavioral changes following exposure to high temperatures, nerve ligation, mechanical compression, or inflammation. Not only do these measures differ in modality, but they also show little commonality in their response to pharmacologic intervention (Lai, Ossipov, Vanderah, Malan, Jr., & Porreca, 2001) and may even be modulated by different genes (Mogil, 1999). Thus, an organism’s responses to one measure of thermal pain may have little bearing on its responses to noxious mechanical or inflammatory stimuli. This makes comparisons between studies especially difficult, as it demands congruence of the nociceptive modality. Additionally, such measures in themselves would likely serve as confounds for the proposed series of studies, as they would likely alter baseline pain latencies.
For the present studies, a measure of nociception is required that is valid, reliable, non-invasive, and could be easily repeated in a within-subjects design that includes repeated testing of the same animals carried out over a short period of time (less than two hours). To address these concerns, all of the proposed sets of studies described within this dissertation employed one experimental nociceptive assay, the tail withdrawal test.

The tail withdrawal assay: The tail withdrawal assay is a well-known measure of nociceptive sensitivity based on phasic, reflexive limb withdrawal from a noxious stimulus. Although first described using rats exposed to a focused light beam (D'Amour & Smith, 1941), various modifications of this procedure have been utilized over the years to objectively assess animal’s sensitivity to noxious stimulation. A modified version of this classic test was selected for the current proposed dissertation (Janssen, Niemegeers, & Dony, 1963), based on its minimally invasive nature and its stability and reliability 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, in seconds, between water immersion and reflexive withdrawal of the tail. For the experiments discussed in the current proposed dissertation, latency of tail-withdrawal from the hot-water bath was used as the dependent measure to reflect nociceptive sensitivity.

The underlying physiological 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). Those neurons within the dorsal root ganglion extend their connections to the dorsal horn of the spinal cord where they synapse onto local interneurons and on projection neurons. These signals are then sent via sensory afferents primarily to the brain stem, thalamus and hypothalamus (Hanai, 1998). Back near the site of initiation, local neurons in the dorsal horn process efferent regulatory nociceptive inputs, leading to activation of the autonomic nervous system and motor neurons mediating local withdrawal reflexes (Y. P. Chen, Chen, & Pan, 2005). Regulation of the phasic tail withdrawal can therefore occur via modulation of either peripheral or central mechanisms (McCormack, Prather, & Chapleo, 1998).

**Rationale for Specific Aims of the Present Dissertation**

Morphine hyperalgesia is reversed in a sex- and dose-dependent manner in male and female mice (Juni et al., 2010). Whereas the NMDA receptor antagonist MK-801 reverses hyperalgesia in males, and females receiving a low dose of morphine, the melanocortin-1 receptor antagonist MSG606 conversely reverses morphine hyperalgesia exclusively in females receiving a high dose of morphine. In Juni et al. (2010), these drugs were injected systemically, thus the location of the neural mechanisms contributing to morphine hyperalgesia, whether they are spinal or supraspinal, is unknown. The goal of this dissertation is to further investigate the mechanisms underlying morphine-induced hyperalgesia, specifically regarding the potential central sites of action of this phenomenon. Accordingly, the aforementioned findings are subjected to replication with additional exploration of possible spinal and supraspinal involvement in the regulation of morphine hyperalgesia.
The goal of Specific Aim 1 was to evaluate whether or not supraspinal sites contribute to morphine hyperalgesia. We aim to investigate the role of the NMDA and melanocortin-1 receptor systems in morphine hyperalgesia, using antagonists of these substrates injected directly into the lateral ventricles (intracerebroventricular injections; i.c.v.). As previous research has been limited to systemic administration (Juni et al. 2010), performing such proposed studies will allow us to further examine the specific supraspinal regions in the central nervous system relevant to this hyperalgesic state. We hypothesize that hyperalgesia evoked by low doses of continuous morphine infusion will be reversed by i.c.v. injections of the NMDAR antagonist, MK-801, in both males and females. Conversely, we believe that following high doses of continuous morphine infusion, while males will continue hyperalgesic mediation by the NMDAR system, female-specific hyperalgesia will be reversed exclusively by i.c.v. injections of the MC1R antagonist MSG606. Assuming that the findings of these studies support our hypotheses, a second series of studies will entail investigation of these same proposals at a spinal level.

The goal of Specific Aim 2 was to evaluate whether or not spinal mechanisms contribute to morphine hyperalgesia. We aim to assess if either, neither, or both aforementioned antagonists work via the spinal cord (intrathecal injections; i.t.) to reverse morphine hyperalgesia. When combined with the findings from Specific Aim 1, such studies will help us determine if the neural substrates for morphine hyperalgesia are present in both the brain and spinal cord. We propose that at low doses of continuous morphine-induced hyperalgesia, this state will be reversed exclusively by spinal injection of the NMDAR antagonist, MK-801, in males and females. At high doses of continuous morphine infusion, we believe that males will continue to
use the NMDAR system to modulate morphine-induced hyperalgesia. However, we are particularly interested in investigating the spinal pathways associated with the melanocortin-1 receptor, as the literature on this receptor and its general neuronal network is scarce, suggesting minimal presence in spinal loci. The anatomy of this receptor system in the spinal cord as it relates to MSG606 is of particular interest, as we hypothesize that hyperalgesia elicited by high doses of morphine infusion in females may not be reversed by this antagonist. These findings, combined with the findings from Specific Aim 1, will lead to our third investigation. Specifically, as there are distinct sex differences in continuous morphine-induced hyperalgesia, we will further investigate the contribution of female hormones to this state.

The goal of Specific Aim 3 was to assess hormonal contributions to sex differences evident in morphine hyperalgesia. We have previously shown that morphine hyperalgesia is sex- and dose-dependent; it is reversed in males by NMDA receptor antagonists, while this same effect is purportedly obfuscated in females because their ovarian hormone production (Juni et al. 2008, 2010). However, the reversal of hyperalgesia in females mirrors the male model after administration of melanocortin-1 receptor antagonists, suggesting a role for this receptor system in morphine hyperalgesia as well (Juni et al. 2010). The present dissertation further investigated such claims with the use of ovariectomized females who were later subjected to hormone replacement using acute progesterone. We hypothesize that intact males will continue using the NMDAR system to modulate continuous morphine-induced hyperalgesia, and that removing circulating ovarian hormones in females will cause them to “switch” systems to mirror male patterns. However, following acute subcutaneous progesterone replacement, we propose that female-typical patterns of hyperalgesia will be restored. Finally, as it is believed that sex
differences in morphine-induced hyperalgesia are a result of the presence of female hormones rather than a lack of male hormones, it is quite possible that progesterone administration in intact males may cause them to mimic female-typical patterns of hyperalgesia.
CHAPTER 2.

1. 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 unless otherwise noted in the methods, and for all groups, \( n \geq 8 \).

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

3. Surgical Procedures:
Where indicated, female mice were subjected to ovariectomy (OVX) surgery through a single ventral midline incision. The fallopian tubes were then exposed and ligated with surgical silk proximal to the ovaries before removing the distal ends (including the ovaries and surrounding ovarian fat). Incisions were closed using surgical silk sutures (3-0) and mice were allowed a 20-day recovery period before undergoing any testing manipulations. This surgical procedure is a quite common and demonstrated to be an effective way to disrupt estrus cycles and effectively
prevent estrogen expression and subsequent circulation (Cohen & Milligan, 1993).

4. Drugs:

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

**Naltrexone** – In order to block opioid receptors and to control for any confound related to opioid analgesia and/or corresponding withdrawal, the opioid receptor antagonist naltrexone was co-administered for all studies in this dissertation. Pellets containing 30 mg of drug were obtained from the National Institute of Drug Abuse (Rockville, MD) and surgically implanted subcutaneously (see below).

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

**MSG606** – A selective MC1R antagonist, MSG606 ([Cyclo-((CH$_2$)$_3$CO-Gly-His-DPhe-Arg-D-Trp-Cys(S-))-Asp-Arg-Phe-Gly-NH$_2$]), is a potent and novel cyclic thioether peptide. It was synthesized in the laboratory of Victor J. Hruby, Ph.D. (Regents Professor, Department of Chemistry, University of Arizona, Tucson, Arizona) and provided as a gift. It was dissolved in a saline and 10% dimethyl sulfoxide vehicle. For a complete explanation of the synthesis of this compound, please see Juni et al., 2010.

**Progesterone** – Belonging to the progestogen class of hormones, progesterone is a steroid
hormone involved in the female menstrual cycle, pregnancy, and embryogenesis of humans and mice, along with other species. It was chosen because it has been shown that the hormone replacement-regulated reversal seen in past studies (Juni et al., 2008) is likely attributable to concentrations of progesterone produced by estrogen replacement therapy. Specifically, this compound has been shown to be sufficient to acutely reinstate female-typical patterns of morphine-related pain responses (Waxman et al., 2010). It was obtained from Sigma-Aldrich (St. Louis, MO) and delivered in a commercial sesame oil vehicle.

5. Drug Delivery Mechanisms:

**Pellet implantation** – NTX pellets containing 30mg of drug were wrapped in a sterile nylon mesh and subcutaneously implanted into the nape of the neck under oxygen/isofluorane inhalant anesthesia, through a small incision and closed with stainless steel surgical staples.

**Continuous administration paradigms** – Throughout this dissertation, morphine administration paradigms involved continuous infusion. Osmotic pumps (Alzet Model 2001, Alza, MountainView, CA) were implanted subcutaneously via the same dorsal midline incision created for pellet implantation. Pumps, which dispense 1.0 microliter/hour for up to seven days and thus control for withdrawal commonly seen with the use of acute injection paradigms, were filled with either 2mg/ml or 50mg/ml of morphine (1.6mg/kg/24h or 40mg/kg/24h, respectively), and implanted 24 hours after naltrexone pellets. The incision was closed with a small stainless steel surgical clip. Muscle, tissue, or bone was not disturbed.

**Acute injection paradigms** - Throughout this dissertation, injection paradigms for NMDAR and
MC1R antagonists and their corresponding control groups involved intracerebroventricular (i.c.v.) or intrathecal (i.t.) injections. The NMDAR antagonist MK-801 was dissolved in 0.9% saline solution, while the MC1R antagonist MSG606 was dissolved in a 10% dimethyl sulfoxide (DMSO) vehicle. As a vehicle control condition for the MK-801-injected groups, a solution of 0.9% saline was used. Serving as a vehicle control condition for the MSG606-injected groups, a solution of 10% DMSO/90% saline was used. Intracerebroventricular 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. Intrathecal injections (2 µl volume) were performed under light oxygen/isoflurane inhalant anesthesia using a 10-µl Hamilton micro-syringe fitted with a 27-gauge needle and administered by lumbar puncture (adapted from Hylden and Wilcox, as cited in Li & Clark, 2002). Progesterone was dissolved in a sesame oil vehicle and administered via a subcutaneous bolus injection. The volume of drug for subcutaneous injections was administered based on the animal’s weight in kilograms, according to formula of 10ml drug solution per kg of mouse weight.

6. Nociceptive Assay:

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 10 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. 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 47.5°C, since in pilot studies baseline latencies of 9–10 seconds were consistently obtained, thus minimizing the possibility of floor effects during hyperalgesia. Nociception was 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 their likely effect upon peripheral pain processing in the tail. Likewise, animals that showed obvious signs of disease, motor impairment, or failed to exhibit the tail withdrawal response during baseline behavioral assessments (withdrawal latencies exceeding 30s), were not included for further analysis. Finally, animals that demonstrated motor impairments following i.c.v. or i.t. injections were not included in final data analyses.

Animals 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°C ± 0.2°C by an immersion circulator pump (Fisher Isotemp Model 71). Latency between water immersion and reflexive
withdrawal of the tail was measured twice to the nearest hundredth of a second, with each
determination separated by at least 30 seconds to ensure adequate recovery time between
assessments. The 2 measures were then averaged. A 30s cutoff latency was employed to
prevent possible tissue damage. Ambient room temperature for all assessments trials was at 22-
23 °C, as it has been documented that changes in tail skin temperature can affect tail withdrawal
latencies (Tjolsen & Hole, 1993).

7. Data analysis

Chronic opioid infusion hyperalgesia was expressed as raw withdrawal latencies. Withdrawal
latencies were analyzed using three-way analyses of variance (sex * antagonist drug * time)
followed by a Fisher’s LSD (protected t-test) for post-hoc comparisons. P-values < 0.05 were
considered significant. Data from low- and high-dose morphine studies were analyzed
separately, as were data from studies employing intracerebroventricular versus intrathecal
antagonist injection paradigms.
CHAPTER 3.

SPECIFIC AIM ONE:

*The contribution of supraspinal receptor sites to morphine-induced hyperalgesia*

1. INTRODUCTION

At the forefront of clinical treatment for moderate to severe pain lies morphine; however, unwavering successful treatment with this opioid is complicated by the manifestation of hyperalgesia (Woolf, 1981; Crain & Shen, 2001; Vaughan & Connor, 2003; Chieng, Hallberg, Nyberg, & Christie, 2005; Galeotti, Stefano, Guarna, Biaehni, & Ghelardini, 2006; van Dorp, et al., 2009). While clinicians are concerned solely with relieving hyperalgesia in their patients, scientific investigators are focusing on uncovering the mechanism by which hyperalgesia manifests, such that more efficacious treatment options can be studied. Delineating the receptor system by which hyperalgesia is regulated is at the forefront of importance in terms of pain treatment with morphine. Earlier studies have insisted direct mediation by the opioid receptor system and have linked hyperalgesia to tolerance and withdrawal (Vanderah et al., 2001a; Ossipov et al., 2003); however, more recent evidence suggests oppositional results (Juni et al, 2006). Specifically, Juni et al. (2006) demonstrated that this paradoxical state occurs independently of opioid receptors, and is distinct from both withdrawal and tolerance. A finding that further complicates elucidating morphine-induced hyperalgesia (MIH) is the fact that there exists a dose- and sex-dependent interaction. That is, lower infusion doses of morphine render mice immediately hyperalgesic, a state which is reversed by an acute bolus subcutaneous injection of the NMDAR antagonist MK-801. Higher infusion doses of morphine elicit analgesia (which is blocked by NTX), followed by a period of hyperalgesia that is reversed exclusively in males by an acute injection of MK-801. In contrast, females undergoing a high dose of
morphine infusion exhibit hyperalgesia that is reversed exclusively by the MC1R antagonist MSG606 (Juni et al., 2008, Juni et al., 2010). Such findings suggest several distinct mechanisms involved in the initiation and maintenance of MIH.

The wide variance of experimental procedures utilized in studies of MIH makes determining the exact mechanism and loci by which this state is mediated quite difficult. Along with various dosing and administration paradigms and the investigation of several species models, a great deal of the literature studies hyperalgesia as a result of nerve injury or neuropathic pain. Thus, the resulting hyperalgesia may or may not be neuroanatomically, neurophysiologically, or neurochemically the same as hyperalgesia evoked by opioid treatment. Finally, it is often assumed that the hyperalgesia seen in concomitance with opioid withdrawal is one in the same with all states of MIH. While our lab has previously shown that this is not the case (Juni et al., 2006), former studies investigated the involvement of receptor systems manipulated by antagonists that were injected systemically using subcutaneously bolus injections (Juni et al., 2008; Juni et al., 2010), allowing for general distribution throughout both the peripheral and central nervous system. Additional studies that investigated the role of these non-opioid receptor systems employed acute paradigms (Waxman et al., 2010; Waxman et al., 2012). Such methodologies allowed us to determine the receptor systems that mediate MIH, but do not permit us to adequately extrapolate information regarding the precise location that mediates this troubling phenomenon. Thus, whether the location of the neural mechanisms contributing to morphine hyperalgesia is supraspinal or spinal remains unknown.
There is substantial evidence in the literature to support a significant contribution of supraspinal loci to the development and maintenance of hyperalgesia. Supraspinal sites that are rich in, and activated by opioid receptors yield many descending inhibitory projections. Generally, studies have shown significant levels of opioid receptor mRNA in cortical, diencephalic, and brainstem regions (Quirion, 1984). While opioid receptors can be found in numerous supraspinal areas including the frontal cortex, hippocampus, thalamus, and hypothalamus, the PAG and RVM are most commonly associated with MIH and share reciprocal connections in terms of their role in nociceptive processing (Quirion et al., 1983; Quirion, 1984; Ossipov, 2004). Specifically, it is hypothesized that hyperalgesia likely begins in the PAG, where excitation of opioid receptors in turn excites neurons in the RVM. In addition to dense reciprocal connections with the RVM, the PAG is also heavily entwined with the hypothalamus and limbic forebrain structures including the amygdala, anterior cingulate cortex, and medial prefrontal cortex (Heinricher, Tavares, Leith, & Lumb, 2009).

The PAG-RVM system has been shown to play a large role in inhibitory control and functions to suppress nociceptive inputs, thus propitiating pain (Morgan, Whittier, Hegarty, & Aicher, 2008). While opioids initially activate neurons in the PAG, these projections directly communicate with the RVM, resulting in excitation (Basbaum, Clanton, & Fields, 1978; Basbaum & Fields, 1978, 1984). The RVM also receives input from several other supraspinal areas, and thus is a critical region to bidirectional nociceptive modulation. Additionally, specialized “on”, “off”, and “neutral” cells in the RVM are considered to be the neural basis for the bidirectional control of this site in nociception (Fields & Heinricher, 1985; Fields, Heinricher, & Mason, 1991; Heinricher & Morgan, 1992; Heinricher et al., 2009; Neubert, Kincaid, & Heinricher, 2004;
With a documented role in antinociception, it has more recently been proposed that in addition to suppressing pain, this system may also facilitate nociception by way of on-cells mediating the majority of descending facilitative information (Heinricher et al., 2009; Neubert et al., 2004).

Aforementioned increases in the activation of on-cells in the RVM, the activation of NK-1 receptors, and the increased release of excitatory neuropeptides (including cholecystokinin; CCK) bring into play a role for NMDA receptors at the supraspinal level. At the supraspinal level, the importance of NMDA receptors in areas such as the RVM and PAG in acute morphine-induced hyperalgesia has been confirmed by past studies in our lab (Waxman, 2012); however, their role in MIH remains unknown. It is possible that opioids elicit hyperalgesia through RVM activity, inducing neuroplastic changes (Vanderah, Suenaga, et al., 2001). Evidence demonstrates that the NMDA receptors may have both direct and indirect involvement in this descending information, and additionally in these neuroplastic changes. Specifically, inhibition of medullary NMDA receptors or of nitric oxide synthase weakens somatic and visceral hyperalgesia (Coutinho et al., 2001; Urban & Gebhart, 1999). Changes in the excitability of the RVM have been observed during inflammatory hyperalgesia, and are likely a result of changes in NMDA receptor activation (Guan, Terayama, Dubner, & Ren, 2002; Terayama, Dubner, & Ren, 2002).

Additionally, activation of NK-1 receptors in the RVM augments excitability of on-cells, evoked by NMDARs (Budai et al., 2007). Furthermore, NK-1 receptors have been found to coexist with NMDA receptors on a subset of neurons in the RVM. These findings suggest that substance P acting on RVM NK-1 receptors can facilitate excitatory amino acid transmission either by
enhancing their presynaptic response, or by enhancing responses of on-cells to these excitatory amino acids (Budai et al., 2007). Thus, activation of NK-1 and NMDA receptors localized to the RVM, paired with the subsequent sensitization of on-cells may contribute to the development of central sensitization hypothesized in MIH. Additionally, efferent influences resulting from the release of excitatory peptide neurotransmitters such as CCK may also underlie supraspinal involvement in MIH, by way of indirectly activating NMDA receptors. Specifically, both acute and chronic morphine treatment has been found to cause a dose-dependent increase of CCK activity in the RVM and the PAG (Ding & Bayer, 1993; Rosen & Brodin, 1989). Collectively, the finding that NMDAR blockade at the supraspinal level completely abolishes acute morphine-induced hyperalgesia, it is likely that the activities of the RVM and PAG that directly and indirectly involve supraspinal NMDA receptors may have a greater contribution to MIH than NMDA receptors in the spinal cord.

The current study uses morphine infusion pumps in mice pretreated with NTX pellets. This protocol allows for investigation of the mechanisms involved in morphine-induced hyperalgesia not confounded by initial states of analgesia, and additionally eliminates the possibility of opioid receptor involvement. Furthermore, although morphine-induced hyperalgesia has been demonstrated in chronic paradigms that employ repeated injection (Ammon-Treiber & Hollt, 2005; Suzuki, Porreca, & Dickenson, 2006), it is believed that in the context of discontinuous morphine delivery (i.e. multiple injections) hyperalgesia may be intensifsied by “mini withdrawal” episodes (Gutstein, 1996; Angst et al., 2003; Ossipov et al., 2004). As opioid withdrawal-induced hyperalgesia has been shown to be mechanistically distinct from the opioid-induced hyperalgesia currently under investigation (Harris et al., 2004; Dunbar et al., 2006), the
use of osmotic pumps for morphine infusion eliminates the possible occurrence of withdrawal by providing uninterrupted, constant morphine exposure over several days. Indeed, past research using continuous infusion protocols (Vanderah et al., 2001a; Xie et al., 2005; Juni et al., 2006) also uncovered hyperalgesia that was hypothesized to be unassociated with withdrawal.

The goal of the present study is to further investigate the potential supraspinal mechanisms underlying morphine-induced hyperalgesia. Accordingly, aforementioned findings (Juni et al., 2008; Juni et al., 2010) are subjected to replication with additional exploration of supraspinal involvement in the regulation of morphine hyperalgesia. We aim to investigate the role of the NMDARs and MC1Rs in morphine hyperalgesia using antagonists of these receptor systems administered directly into the lateral ventricles via i.c.v. injection. As previous research has been limited to systemic administration (Juni et al. 2010), performing these studies will allow us to further examine the specific neural networks specific to supraspinal loci relevant to this hyperalgesic state. We hypothesize that hyperalgesia evoked by low doses of continuous morphine infusion will be reversed by i.c.v. injections of the NMDAR antagonist, MK-801, in both males and females. Conversely, we believe that following high doses of continuous morphine infusion, while males will continue hyperalgesic mediation by the NMDAR system, female-specific hyperalgesia will be reversed exclusively by i.c.v. injections of the MC1R antagonist MSG606.

2. METHODS

Subjects
Adult male and female CD-1 mice (Charles Rivers, Kingston, NY), six to ten weeks of age, were housed in cages of four with same-sex littermates. All mice were maintained on a 12:12-hour light/dark cycle in a climate-controlled room (22 °C ± 2°C) with ad lib access to food and filtered tap water. Each subject was used once. For all conditions, \( n \geq 8 \).

**Drug Delivery**

Twenty-four hours prior to initiation of morphine administration (labeled “day -1”), naltrexone pellets were subcutaneously implanted. One day later (labeled “day 0”), continuous morphine treatment of two distinct dosing paradigms was introduced via subcutaneous osmotic pump implantation. One group received a low dose of morphine (2mg/ml, yielding a cumulative dose of 1.6mg/kg/24h), whereas a second group received a high dose of morphine (50mg/ml, yielding a cumulative dose of 40mg/kg/24h). When mice were rendered hyperalgesic (day 4), intracerebroventricular injections of MSG606 (1.5 mg/ml), MK-801 (.05 mg/kg), the MSG606 control, or the MK-801 control were performed under oxygen/isoflurane inhalant anesthesia. Injections (5µl volume) were made directly through the skull at a point 2mm rostral and lateral to lambda at a depth of 3mm using a 10µl Hamilton micro-syringe fitted with a 27-gauge needle. A stainless steel wound clip was used to close the incision after each injection.

**Nociceptive Assay**

The warm-water tail-withdrawal test, as described in detail above in the General Methods section, was chosen to assess nociception. This measure has repeatedly been shown to be stable and reliable in the context of recurrent testing (Elliott et al., 1995; Kest et al., 2002; Nemmani et al., 2004). Each subject was tested prior to NTX pellet implantation as a baseline measure, and before pump implantation to confirm no effect of NTX. All subjects were tested each day.
following pump implantation to assess hyperalgesia. Finally, after intracerebroventricular injection of each group’s respective treatment, each mouse was tested at 15, 30, 45, and 60 minutes to assess a time course of possible hyperalgesia reversal.

**Data Analysis**

Opioid-induced hyperalgesia was expressed as raw withdrawal latencies. Withdrawal latencies were analyzed using three-way analyses of variance (Sex * Drug * Time) followed by a Fisher’s LSD (protected t-test) for post-hoc comparisons. Data from low- and high-dose morphine studies were analyzed separately. *P*-values < 0.05 were considered significant. Values reported are mean ± SEM.

**3. RESULTS**

**Study 1: Hyperalgesia in males and females during continuous low-dose morphine infusion**

All subjects were rendered significantly hyperalgesic by day 4 of continuous low-dose morphine administration (1.6 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both males and females were injected via the i.c.v. route with an acute dose of MSG606, MK-801, a DMSO + saline vehicle control for MSG606, or a saline-only vehicle control for MK-801 and tested every 15 minutes post-injection for one hour.

Significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was already evident in both sexes at 15 minutes post-injection, and persisted throughout the entire one-hour testing period (Figure 1A-B). However, no such effect was observed in males or females following injection of MSG606. Additionally, no reversal of
hyperalgesia was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on low-dose morphine-induced hyperalgesia.
1A. Females

| Minutes After Antagonist Injection on Day 4 |

1B. Males

| Minutes After Antagonist Injection on Day 4 |
**Figure 1A-B.** Time course of hyperalgesia in CD-1 females (1A) and males (1B) receiving continuous low-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a low dose of morphine (1.6 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.c.v. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MK-801 control, or the MSG606 control. After which, all mice were assayed for nociception every 15 minutes for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on day 4 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
Study 2: Hyperalgesia in males and females during continuous high-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous high-dose morphine administration (40 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both males and females were injected via the i.c.v. route with an acute dose of either MSG606, MK-801, MSG606 vehicle control, or MK-801 vehicle control, and tested every 15 minutes for one hour.

Significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was already evident in exclusively males at 15 minutes post-injection, and persisted throughout the entire one-hour testing period (Figure 2B). In solely females, however, significant equal magnitude reversal of morphine-induced hyperalgesia after injection of the MC1R antagonist MSG606, and not MK-801, was observed at 15 minutes post-injection (Figure 2A). This female-exclusive reversal resolved within the one-hour testing period. No reversal of hyperalgesia was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on high-dose morphine-induced hyperalgesia.
Figure 2A-B.  Time course of hyperalgesia in CD-1 females (2A) and males (2B) receiving continuous high-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a high dose of morphine (40 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.c.v. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MK-801 control, or the MSG606 control. After which, all mice were assayed for nociception every 15 minutes for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on day 4 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
4. DISCUSSION

The major findings of Study 1 and Study 2 confirmed all hypotheses, and were as follows: 1) hyperalgesia in both male and female mice undergoing continuous low-dose morphine infusion is reversed exclusively by i.c.v injection of the NMDA receptor antagonist MK-801, but not the MC1R antagonist MSG606; 2) hyperalgesia resulting from continuous high-dose morphine infusion is reversed exclusively in males by the NMDA receptor antagonist MK-801, but reversed exclusively in females by i.c.v. injection of the MC1R antagonist MSG606. These two outcomes suggest that there are minimally two dose- and sex- dependent supraspinal mechanisms contributing to morphine-induced hyperalgesia. These findings are discussed in detail below.

**Morphine-induced hyperalgesia is reversed by dose- and sex-dependent mechanisms specific to supraspinal loci.** Systemically administered opioids produce profound antinociceptive effects peripherally, supraspinally, and spinally, but the precise mechanism of action of the ensuing hyperalgesia is not as well understood (Jensen, 1997; Juni et al., 2008; Juni et al., 2010; Waxman et al., 2010). In order to better classify the location of mechanisms modulating continuous morphine-induced hyperalgesia, the current study employed an intracerebroventricular receptor antagonist injection paradigm, intended to provide blockade of systems at exclusively supraspinal loci. Our previous findings have shown there to be a systemically initiated sex- and dose-dependent mediation of morphine hyperalgesia, such that acute bolus injections of MK-801 or MSG606 reversed this state differentially in male and female mice (Juni et al., 2008; Juni et al., 2010). For these reasons, the current studies also investigated the NMDA receptor antagonist MK-801, and the melanocortin-1 receptor antagonist
MSG606. However, as our previous studies utilized systemic antagonist administration that allowed for widespread distribution throughout the peripheral and central nervous systems, it was unknown if the resulting reversal of hyperalgesia was modulated exclusively by supraspinal, spinal, or some combination of both central areas. The results of the current study suggest that these hormone-dependent mechanisms are in fact regulated by supraspinal loci that function independently of ascending nociceptive input.

While research examining the NMDA receptor system is at the forefront of study as a modulator of opioid induced hyperalgesia, the present data provide further evidence that this system is not entirely responsible for supraspinally-mediated MIH. The NMDA receptor antagonist MK-801 does not reverse hyperalgesia across the board, leaving hyperalgesia undisturbed in female CD-1 mice receiving a high dose of morphine, corroborating our past studies (Juni et al., 2008; Juni et al., 2010). The female-counterpart in the modulation of nociception is the melanocortin-1 receptor, after it was discovered that the MC1R system appears to mediate κ-opioid analgesia exclusively in females. This is supported by the fact that κ-opioid receptor agonists are more efficacious in females than in males in clinical applications (Mogil et al., 2003; Mogil et al., 2005). Thus, the finding of a role for the MC1R in opioid analgesia led researchers to subsequently investigate a possible role of this receptor system in morphine-induced hyperalgesia. As discussed in the General Introduction, Mogil and colleagues (2003) found the MC1R gene on chromosome 8 to be the most likely candidate gene to mediate states of female-exclusive analgesia. This analgesia (induced by the κ-opioid agonist U50,488H) is likely mediated centrally, as exclusively i.c.v. administration of MK-801 in males reverses this κ-
opioid’s analgesic state. Thus, it is reasonable to postulate a supraspinally-mediated hyperalgesic mechanism involving this receptor system.

Our recent findings (Juni et al., 2010) extend Mogil et al.’s (2003) rodent and clinical findings beyond κ-opioid analgesia to include morphine-induced hyperalgesia, such that both states are mediated by NMDA receptors in males and MC1Rs in females. Taken together with the current finding that a sex- and dose-dependent state of morphine-induced hyperalgesia is differentially reversed specifically by i.c.v. injection of NMDAR and MC1R antagonists, it is likely that these hyperalgesic mechanisms are located supraspinally. NMDARs and MC1Rs are densely expressed throughout the brain and midbrain PAG, respectively (Renno, 1998; Juni et al., 2010; Gladding & Raymond, 2011; Swartjes et al., 2012; Xia, Wikberg, & Chhajlani, 1995; Mogil et al., 2005). Noted, however, is the fact that both past and current findings are limited to high doses of morphine and that similar findings are not yielded by low morphine infusion doses, suggesting minimally two distinct dose-dependent supraspinal neural networks in the modulation of morphine-induced hyperalgesia.

**Morphine-induced hyperalgesia is, at least in part, regulated by supraspinal mechanisms.** The current data suggest that opioid-induced hyperalgesia is regulated in a way that is similar to opioid analgesia, such that this sex- and dose-dependent phenomenon is mediated via supraspinal NMDA and MC1 receptor mechanisms. However, the current study is limited to ventricular administration of the respective antagonists, which allows for distribution throughout the cortex; thus, the precise brain locus of action remains unknown. More studies are needed to delineate the exact supraspinal location and further mechanism of action; these methods were not
employed in the current study as stereotactic administration into precise cortical and subcortical areas are typically performed in rats, and our paradigms are limited to mice. Likely candidates for more specific study are the PAG-RVM system, and the locus coeruleus (Heinricher, Tavares, Leith, & Lumb, 2009). Specifically, more studies are needed to assess the role of PAG MC1Rs in morphine-induced hyperalgesia.

The present study does not address the possibility that morphine-induced hyperalgesia may have independent supraspinal and spinal mechanisms, such that spinal loci could also mediate this nociceptive state on its own; however, we can now deduce that both areas are not collaboratively required to induce/maintain opioid-induced hyperalgesia. The second study of this dissertation will assess if spinal mechanisms are independently capable of mediating sex- and dose-dependent nociception.
SPECIFIC AIM TWO:

_The contribution of spinal receptor sites to morphine-induced hyperalgesia_

1. INTRODUCTION

While supraspinal sites such as the PAG and RVM are well-known associative areas involved in descending modulation of pain, the spinal cord is also thought to play a large role in this process. In fact, the RVM receives inputs from the dorsal horn of the spinal cord and additional rostral sites, and projects diffusely to both superficial and deep laminae of the dorsal horn (Zhuo & Gebhart, 1997). Thus, the spinal cord is likely a critical site for the mediation of the antinociceptive effects of morphine (Akil et al., 1984; Basbaum & Fields, 1984; Yaksh, 1981). Specifically, opioid receptors densely populate laminae I, II, and III of the dorsal horn (the marginal zone, the substantia gelatinosa, and the nucleus proprius, respectively), as well as dorsal root ganglia in the spinal cord (Quirion, 1984; Gouardères et al., 1985). These outer laminae are the principal spinal sites of action of morphine’s nociceptive signaling (Besse et al., 1990).

Giving further credence for a large role of the spinal cord in opioid processing, a study by Advokat and Burton (1987) demonstrated that in rats with transected spinal cords, the potency of systemically administered morphine was weakened in response to thermal nociceptive stimuli, while the potency of spinally administered morphine remained intact. This suggests that opioids act directly on spinal sites to modulate nociceptive inputs, and that the loss of this spinal mechanism renders morphine nearly ineffective (Advokat & Burton, 1987). Additionally, studies involving the blockade of spinal opioid receptors demonstrate to a marked reduction in the analgesic potency of opioids (Hara et al., 1999). In chronic paradigms, rats receiving daily
bolus spinal injections of morphine for eight days developed thermal hyperalgesia, though associated with antinociceptive tolerance (Mao, et al., 1994). In rats, continuous spinal delivery (via osmotic pumps) of DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin), a synthetic opioid peptide with high opioid receptor specificity/affinity also produced thermal hyperalgesia and tactile allodynia of the hind paws, accompanied by a decrease in analgesic potency and efficacy of spinal DAMGO (Vanderah, et al., 2000).

Past literature documents dose-dependent effects of opioids in the spinal cord. Specifically, both acute and chronic administration of high-dose opioids directly into the spinal cord produce a hyperalgesic response in both animals and humans (Sakurada, Komatsu, & Sakurada, 2005). Specifically, acute studies illustrate that high-dose morphine administration into the spinal subarachnoid space decreased tail-withdrawal latency, or induced hyperalgesia (Woolf, 1981). In rats implanted with continuous i.t. catheters, high-dose morphine yielded a series of pain behaviors such as biting and scratching, specifically at the dermatomes innervated by the spinal cord near the catheter tip (Yaksh & Harty, 1988; Yaksh, et al., 1986). Furthermore, pretreatment with NTX did not reduce these behaviors in studies employing similar paradigms (Sakurada, et al., 1996). Serving as a striking counterpart to the Yaksh and colleagues (1986) study, a case report was described by Ali (1986) in which a patient with terminal cancer receiving high-dose spinal morphine demonstrated hyperalgesia in her lower extremities. Subsequently, reports accumulated documenting 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; Sjögren, Jonsson, Jensen, Drenck, & Jensen, 1993; Werz & MacDonald, 1982).
In contrast to high-dose morphine literature, there is conflicting evidence on low morphine doses administered via the spinal route. Whereas some studies document that low-dose acute administration of the (-) morphine enantiomer produced significant analgesia, the (+) enantiomer, which is inactive at opioid receptor binding sites, produced hyperalgesia (Woolf, 1981). Other studies demonstrated that low-dose acute morphine produced brief excitatory effects in the flexor reflex, which is typically marked by the analgesic effect of morphine (Wiesenfeld-Hallin, et al., 1991).

As previously mentioned, the most commonly proposed mechanism of MIH involves the colocalization of NMDA receptors and opioid receptors in the nervous system. Of particular interest is that fact that both NMDA receptors and µ-opioid receptors are particularly abundant in lamina I-III of the dorsal horn (Mao et al., 2002; Gladding & Raymond, 2011). It is hypothesized that opioids may somehow activate NMDA receptors. Specifically, Mao et al. (2002) report a downregulation of glutamate transporters in the spinal cord after several days of either acute injection or chronic infusion paradigms of morphine. Interestingly, this reduction of GTs is not apparent until day six of morphine infusion and persists until minimally day eight, which partially corresponds with the manifestation of morphine hyperalgesia (Mao et al., 2002). Additional studies in mice and rats suggest that morphine-induced hyperalgesia is mediated by increased glutamate from primary afferent terminals in the dorsal horn of the spinal horn, and subsequent activation of NMDA receptors. Moreover, several studies have suggested that an NMDA-NO cascade in the spinal cord may mediate this phenomenon (Watanabe, Okuda, et al., 2003; Watanabe, Sakurada, et al., 2003). Specifically, high-dose spinally administered morphine has been shown to produce a significant increased rate of NO release in the spinal cord; in turn,
this increased generation of NO leads to increased activation of NMDA receptors in the spinal cord (Malmberg & Yaksh, 1993; Sorkin, 1993).

Following our previous studies (Juni et al., 2008; Juni et al., 2010) combined with the results from Specific Aim 1, we also aimed to investigate the possible role of spinal MC1Rs. While a supraspinal MC1R system is feasible due to this receptor’s distribution, particularly in the PAG, this receptor does not appear to have a widespread spinal presence (Xia, Wikberg, & Chhajlani, 1995; Mogil et al., 2003). Interestingly, a recent study reported the involvement of MC4R, a subtype diffusely distributed both supraspinally and spinally, in neuropathic pain. Specifically, Delaney, Keighren, Fleetwood-Walker, and Jackson’s (2010) data suggest the involvement of more than one differentially distributed melanocortin receptor subtype in the modulation of female-specific pain.

In addition to roles for NMDARs and MC1Rs, an upregulation of G-protein coupled NK-1 receptors has been implicated in opioid-induced hyperalgesia. Specifically, lamina I cells of the dorsal horn that express the NK-1 receptor have been shown to project to supraspinal areas that facilitate pain processing (Nichols et al., 1999). Additionally, paradigms involving substance P administration in mice either intermittently treated or continuously exposed to morphine also demonstrated greater c-Fos immunoreactivity in dorsal horn nuclei after morphine administration, presumably a result of substance P activity in these areas and indicative of neuronal sensitization. This is supported by studies indicating that intrathecal morphine elicits substance P activity, increases spinal NK-1 receptor expression, and results in opioid-induced hyperalgesia, suggesting a role for spinal cord receptor systems (Vanderah et al., 2001; Li &
Clark, 2002; King et al., 2005). Furthermore, the presence and activation of NK-1 receptors are crucial for the generation of activity-dependent LTP that requires a substance P-induced rise in Ca$^{2+}$, likely involving Ca$^{2+}$ release from intracellular stores, and a substance P-facilitated Ca$^{2+}$ influx through NMDA receptors. This synaptic plasticity in spinal lamina I neurons has been shown to modulate hyperalgesia and may be augmented by the presence of opioids (Ikeda, et al., 2003). Finally, dynorphin (an endogenous peptide with significant non-opioid and NMDAR activity) evokes an array of self-injurious behaviors including persistent allodynia, and scratching, licking, and biting, conveniently mediated by NMDA receptors (Vanderah, et al., 2000; Vanderah, et al., 1996). It is suggested that opioids upregulate spinal dynorphin, which causes an increase of excitatory neurotransmitter release from primary afferent fibers. This, in turn, promotes exaggerated pain through an NMDA-dependent mechanism (Gardell, et al., 2002; Vanderah, Suenaga, et al., 2001). All of these aforementioned findings suggest a non-opioid receptor-mediated role of spinal NMDA receptors in opioid-induced hyperalgesia.

Other studies have indicated that exposure to morphine mediated by intracellular protein kinase C (PKC) may prime NMDA receptors and cause increased excitability (Chen & Huang, 1992; Mao, Price, & Mayer, 1994; Martin, Malmberg, & Basbaum, 2001; Sluka & Audette, 2006). Interesting, most isoforms of the PKC enzyme are located in the outer layers of the dorsal horn, demonstrating an anatomical overlap of opioid receptors, NMDA receptors, and PKC activity (Sluka & Audette, 2006). When activated in the spinal cord, PKC results in hyperalgesia on heat and mechanical assays, increased glutamate release, and sensitization of both dorsal horn neurons and the spinothalamic tract (Sluka & Audette, 2006). Additionally, the development of morphine hyperalgesia is preventable by intrathecal administration GM1 ganglioside, an
inhibitor of PKC activity. This illustrates a critical role for spinal activity of this protein that occurs in response to NMDA receptor activation in morphine-induced hyperalgesia (Mao et al., 1994).

The current study also uses morphine infusion pumps in mice pretreated with NTX pellets. This protocol allows for investigation of the mechanisms involved in MIH not confounded by initial states of analgesia, and thus eliminates the possibility of opioid receptor involvement. Additionally, although morphine-induced hyperalgesia has been demonstrated in chronic paradigms that employ repeated injection (Ammon-Treiber & Hollt, 2005; Suzuki, Porreca, & Dickenson, 2006), as well as those using continuous infusion (Vanderah et al., 2001a; Xie et al., 2005) protocols, it is believed that in the former context of discontinuous morphine delivery (i.e. multiple injections) hyperalgesia may be intensified by “mini withdrawal” episodes (Gutstein, 1996; Angst et al., 2003; Ossipov et al., 2004). As opioid withdrawal-induced hyperalgesia has been shown to be mechanistically distinct from the opioid-induced hyperalgesia currently under investigation (Harris et al., 2004; Dunbar et al., 2006), the use of osmotic pumps for morphine infusion eliminates the possible occurrence of withdrawal by providing uninterrupted, constant morphine exposure over several days.

The goal of the present study is to further investigate spinal mechanisms underlying morphine-induced hyperalgesia. Accordingly, aforementioned findings (Juni et al., 2008; Juni et al., 2010) are subjected to replication with additional exploration of spinal involvement in the regulation of morphine hyperalgesia. While there is documented research on the role of spinal NMDA receptors, we aim to investigate the role of both NMDA and the poorly characterized role of
spinal MC1 receptors in sex- and dose-dependent morphine hyperalgesia using antagonists of these receptor systems administered directly into the spinal cord via intrathecal injections. As previous research has been limited to systemic administration (Jun et al. 2010), and the first series of studies in the current dissertation investigated supraspinal involvement, performing the current series of studies will allow us to further examine the neural networks specific to spinal loci that may be relevant to this hyperalgesic state. We propose that at low doses of continuous morphine-induced hyperalgesia, this state will be reversed exclusively by spinal injection of the NMDAR antagonist, MK-801, in males and females. At high doses of continuous morphine infusion, we believe that males will continue to use the NMDAR system to modulate morphine-induced hyperalgesia. However, we are particularly interested in investigating the spinal pathways associated with the melanocortin-1 receptor, as the aforementioned literature on this receptor and its general neuronal network is scarce, suggesting minimal presence in spinal loci. The anatomy of this receptor system in the spinal cord as it relates to MSG606 is of particular interest, as we hypothesize that hyperalgesia elicited by high doses of morphine infusion in females may not be reversed by this antagonist. Findings yielded from the current studies combined with the findings from Specific Aim 1 will lead to our third series of investigations. Specifically, as there are distinct sex differences in continuous morphine-induced hyperalgesia, we will further investigate the contribution of female hormones to this state.

2. METHODS

Subjects

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

**Nociceptive Assay**

The warm-water tail-withdrawal test, as described in detail above in the General Methods section, was chosen to assess nociception. This measure has repeatedly been shown to be stable and reliable in the context of recurrent testing (Elliott et al., 1995; Kest et al., 2002; Nemmani et al., 2004).

**Drug Delivery**

Twenty-four hours prior to initiation of morphine administration (labeled “day -1”), naltrexone (NTX) pellets were subcutaneously implanted. One day later (labeled “day 0”), continuous morphine treatment of two distinct dosing paradigms was introduced via subcutaneous osmotic pump implantation. One group received a low dose of morphine (2mg/ml, yielding a cumulative dose of 1.6mg/kg/24h), whereas a second group received a high dose of morphine (50mg/ml, yielding a cumulative dose of 40mg/kg/24h). When mice were rendered hyperalgesic (day 4), intrathecal injections of MSG606 (1.5 mg/ml), MK-801 (.05 mg/kg), the MSG606 control, or the MK-801 control were performed under oxygen/isoflurane inhalant anesthesia. Intrathecal injections (2 µl volume) were performed under light oxygen/isoflurane inhalant anesthesia and administered by lumbar puncture.

**Data Analysis**
Opioid-induced hyperalgesia was expressed as raw withdrawal latencies. Withdrawal latencies were analyzed using three-way analyses of variance (Sex * Antagonist Drug * Time) followed by a Fisher’s LSD (protected t test) for post-hoc comparisons. \( P \)-values < 0.05 were considered significant. Data from low- and high-dose morphine studies were analyzed separately.

3. RESULTS

Study 3: Hyperalgesia in males and females during continuous low-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous low-dose morphine administration (1.6 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both males and females were injected via the i.t. route with an acute dose of either MSG606, MK-801, the MSG606 control, or the MK-801 control and tested every 15 minutes for one hour.

Significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was already evident in both sexes at 15 minutes post-injection, and persisted throughout the entire one-hour testing period (Figure 3A-B). However, no such effect was observed in males or females following injection of MSG606. Additionally, no reversal of hyperalgesia was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on low-dose morphine-induced hyperalgesia.
3A. Females

3B. Males
Figure 3A-B. Time course of hyperalgesia in CD-1 females (3A) and males (3B) receiving continuous low-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a low dose of morphine (1.6 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.t. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MK-801 or MSG606 control. After which, all mice were assayed for nociception every 15 minutes for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on day 4 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
**Study 4: Hyperalgesia in males and females during continuous high-dose morphine infusion**

All subjects were rendered significantly hyperalgesic on day 4 of continuous high-dose morphine administration (40 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both males and females were injected via the i.t. route with an acute dose of MSG606, MK-801, or the respective controls, and tested every 15 minutes for one hour.

Significant reversal of morphine-induced hyperalgesia after i.t. injection of the NMDA receptor antagonist MK-801 was already evident in exclusively males at 15 minutes post-injection, and persisted throughout the entire one-hour testing period (Figure 4B). In solely females, however, MSG606 did significantly reverse hyperalgesia (4A). Finally, no reversal of hyperalgesia was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on high-dose morphine-induced hyperalgesia.
4A. Females

Latency (sec)

Time Interval

Day -1 0 15 30 45 60

Minutes After Antagonist Injection on Day 4

Antagonist

4B. Males

Latency (sec)

Time Interval

Day -1 0 15 30 45 60

Minutes After Antagonist Injection on Day 4

Antagonist
**Figure 4A-B.** Time course of hyperalgesia in CD-1 females (4A) and males (4B) receiving continuous high-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a high dose of morphine (40 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.t. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MK-801 control, or the MSG606 control. After which, all mice were assayed for nociception every 15 minutes for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on day 4 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (−).
4. DISCUSSION

Findings from Study 3 and Study 4 partially confirmed our hypotheses. The major findings were as follows: 1) hyperalgesia in both male and female mice undergoing continuous low-dose morphine infusion is reversed exclusively by i.t. injection of the NMDA receptor antagonist MK-801 but not the MC1R antagonist MSG606; 2) hyperalgesia resulting from continuous high-dose morphine infusion is reversed exclusively in males by the NMDA receptor antagonist MK-801; 3) significant reversal of high-dose morphine-induced hyperalgesia is seen in exclusively females following i.t. administration of MSG606. This third finding was surprising, in that spinal administration of MSG606 significantly reversed female hyperalgesia elicited by continuous infusion of high dose morphine. These findings are discussed in detail below.

**Low dose morphine-induced hyperalgesia is reversed by the NMDAR system both supraspinally and spinally.** While systemically administered opioids produce profound antinociceptive effects peripherally, supraspinally, and spinally, the precise mechanism of action of the ensuing hyperalgesia is not as well understood (Jensen, 1997; Juni et al., 2008; Juni et al., 2010; Waxman et al., 2010). The studies of specific aim one provided support for the regulation of hyperalgesia by supraspinal mechanisms; however, the role of possible spinal mechanisms has remained inconclusive. In order to better classify the location of the entirety of mechanisms modulating continuous morphine-induced hyperalgesia, the current study employed an intrathecal receptor antagonist injection paradigm. Our previous findings have shown there to be a systemically and centrally initiated sex- and dose-dependent mediation of continuous morphine-induced hyperalgesia, such that both systemic injections (Juni et al., 2008; Juni et al., 2010) and i.c.v. injections (the current dissertation, specific aim one) of MK-801 or MSG606
reversed this state differentially in male and female mice. The current study therefore investigated the NMDA receptor antagonist MK-801 and the melanocortin-1 receptor antagonist MSG606, administered intrathecally. As our previous studies utilized systemic antagonist administration that allowed for widespread distribution throughout the peripheral and central nervous systems, it was unknown if the resulting reversal of hyperalgesia was modulated exclusively by supraspinal, spinal, or some combination of both central areas. According the results of the current dissertation, it appears that in males, and females at exclusively low doses of morphine, both supraspinal and spinal NMDARs play a role in morphine-induced hyperalgesia.

**High dose morphine-induced hyperalgesia is reversed by dose-, sex-, but not location-dependent mechanisms.** The results of the current study suggest that the hormone-dependent mechanisms of morphine-induced hyperalgesia are regulated independently in the brain and spinal cord. That is, at low doses of continuous morphine administration, NMDAR antagonism at the spinal level reverses hyperalgesia in both male and female mice. However, continuous high doses of morphine yield different results. Specifically, it appears that at high morphine doses, spinally regulated NMDAR antagonism remains sufficient for male mice in the reversal of this hyperalgesic state. Conversely, female mice undergo significant hyperalgesic reversal by spinally administered MSG606 following continuous administration of high morphine doses. Although not typical of quickly-absorbed lipophilic compounds such as morphine, it is possible for compounds to spread rostrally after spinal administration, making their way to the brain via cerebrospinal fluid. However, the dispersal of the 2 µl-injection volume used in these studies has been observed to travel approximately 0.5 to 1cm within 30 minutes. Thus, it would take quite
some time for an intrathecally-injected compound to exert an effect via action in the brain. Additionally, by the time rostral spread to the brain occurs, several half-lives of the drug would have passed, making for a much weaker or nonexistent drug concentration (Rossi, unpublished data and observations). Thus, we report that in addition to a hyperalgesic neural network in the brain, there exists a spinal locus that independently regulates morphine-induced hyperalgesia. That is, while the NMDAR mechanism that regulates hyperalgesia appears to be present and undoubtedly functional in the spinal cord, the current evidence suggests that the female-typical MC1R system also appears to be present in functionally significant concentrations beyond supraspinal loci. This provides contradictory evidence for reports that MC1Rs are found in dense concentrations in the PAG, and are not widespread in the spinal cord (Xia, Wikberg, and Chhajlani, 1995; Delaney et al., 2010). Indeed, it appears that MC1Rs are present in functionally significant concentrations in the spinal cord as well as the brain. However, much more research is needed to corroborate these findings and speculations.

In attempt to further define these sex-dependent mechanisms underlying morphine-induced hyperalgesia, the third series of studies in this dissertation replicates the first two series, with the addition of acute progesterone replacement following ovariectomy in females, as well as intact males.
SPECIFIC AIM THREE:

*Hormonal contributions to the sex differences in hyperalgesia evident during continuous morphine infusion*

1. INTRODUCTION

While the NMDA receptor system was the first to demonstrate promise in terms of regulating morphine induced hyperalgesia, it is now known that this system is not entirely responsible for this phenomenon. Previous literature documents that systemic administration of NMDA receptor antagonists reverses hyperalgesia in both males and females at a low dose, while it abolishes hyperalgesia exclusively in males at the high dose (Juni et al., 2008; Juni et al., 2010). After ovariectomy (OVX), females resort to male-typical hyperalgesic patterns, suggesting that they possess the same systems but are diverted from using them only by circulating ovarian hormones. This is further evidenced in that when ovariectomized females receive hormones via continuous estrogen replacement, their female-typical hyperalgesic patterns are restored. Therefore, it is believed that not only is there a role for NMDA receptor pathways in hyperalgesia, but also a hormonally regulated role for minimally a second receptor system in the MC1R system (Juni et al., 2008).

While the MC1R system’s role in skin pigmentation and anti-inflammatory processes is well documented, this receptor system’s role in the inhibition and regulation of pain is not satisfactorily characterized. In particular, this receptor system likely plays a role in hyperalgesia that is not clear. This proposed function is conceivable, as MC1 receptors are found in the PAG. Coincidentally, it was a series of quantitative trait locus (QTL) mapping studies by Mogil et al. (2003), elucidating sex differences in opioid analgesia that directed attention to the MC1R
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system. Specifically, the MC1R system appears to mediate κ-opioid analgesia in females, but not males. Mogil and colleagues (2003) used the highly selective κ-opioid receptor agonist U50,488H, in a study in which they found the MC1R gene on chromosome 8 to be the most likely gene to mediate female-specific analgesia. Interestingly, clinical use of κ-opioid agonists is more efficacious in females when compared to males. Additionally, it was reported that female analgesia was completely abolished after administration of the κ-opioid receptor antagonist nor-binaltorpimine, even though it was given two days prior to U50,488H injections. Finally, whereas MK-801 had no effect in mutant B6 female mice, mutant C57BL/6J-Mc1r<sup>e/e</sup> mice (differentiated from B6 mice only by a lack of functional MC1Rs) demonstrated male-like, NMDA-mediated U50,488H analgesia. Therefore, Mogil et al. (2003) concluded that B6 female mice are using the melanocortin system to mediate pain responses resulting from U50,488H administration. In an interesting addition to this same series of studies, Mogil et al. (2003) blocked the MC1R system during U50,488H administration, using the antagonist Ac-Nle-Asp-Trp-DPhe- Nle-Trp-Lys-NH₂. It was hypothesized that taking the MC1R system out of play would cause the mice to physiologically change the way they process pain, in that they would resort to using the NMDA receptor system when presented with a nonfunctional MC1R system. Indeed, this is precisely what occurred; U50,488H analgesia was potentiated in exclusively female mice following i.c.v. injection of the aforementioned antagonist, only to be reversed by injection of MK-801 (Mogil et al., 2003).

With these aforementioned findings in mind, it is likely that if this system regulates sex-differentiated analgesia, it also plays a role in sex-dependent hyperalgesia as well. Recent findings by Juni et al. (2010) provide an interesting accompaniment to Mogil et al.’s (2003)
findings, to now include morphine-induced hyperalgesia mediation by NMDA receptors in males and females following administration of low dose morphine, and exclusively MC1Rs in females proceeding high dose morphine treatment. Specifically, these studies illustrated a sex difference in hyperalgesia, as female and male C57BL/6J (B6) naltrexone-pelleted mice showed hyperalgesia beginning on day 1 and 3, respectively, after continuous high dose morphine infusion. However, only naltrexone-pelleted B6e/e male mice receiving a continuous infusion of high dose morphine were rendered hyperalgesia from Day 2 to 7 (Juni, et al. 2010). Furthermore, in mice undergoing continuous infusion of the high dose of morphine, MK-801 reversed hyperalgesia in B6 and CD-1 males but not in B6 or CD-1 females, while the selective MC1R antagonist MSG606 reversed hyperalgesia in exclusively B6 and CD-1 females. Conversely, continuous infusion of a low dose of morphine caused hyperalgesia in both e/e males and females (Juni, et al. 2010), thus further suggesting the involvement of more than one neural substrate in sex-dependent morphine-induced hyperalgesia; specifically, both the NMDAR and MC1R systems. This is further evidenced by aforementioned findings that although both male and female hyperalgesia is reversed by MK-801 after infusion of a low dose of morphine, female hyperalgesia begins later and lasts several infusion days, if not weeks, longer than males (Juni, et al. 2010, and unpublished observations).

Together, these studies indicate that NMDA receptors mediate hyperalgesia during administration of low doses of morphine in male and female mice, whereas MC1Rs and NMDA receptors, respectively, play a role in female and male hyperalgesia during infusion of the high morphine dose. Thus, this validates a critical role for the MC1R system in female MIH at some doses. However, beyond the aforementioned data, little is known as to how MC1Rs mediate
female opioid-induced hyperalgesia (Juni et al., 2010). Additionally, the results of the first two series of studies in this dissertation not only replicate sex- and dose-dependent differences, but also further these findings by showing that these differences in morphine-induced hyperalgesia are regulated independently at supraspinal and spinal levels of the nervous system. As such, further studies examining the role of ovarian hormones in this phenomenon are warranted. Our past studies employed a paradigm in which ovariectomized mice were subjected to continuous estrogen replacement. However, extended estradiol treatment can induce progesterone synthesis; thus, it is possible that our previous finding in which female-typical hyperalgesia was reinstated following continuous estrogen replacement is attributable to increased synthesis and presence of progesterone (Waxman et al., 2010). Thus, in the current studies, ovariectomized females and intact males were subjected to acute progesterone replacement.

In these studies, ovariectomized females and intact males were first subjected to identical treatment protocols as the first two series of studies. The purpose was to first assess if ovariectomized females would resort to male-typical patterns of supraspinally and spinally regulated hyperalgesia during continuous morphine infusion. An additional aim was to investigate these same ovariectomized females and intact males subjected to acute progesterone replacement in order to assess if female-typical patterns at both the supraspinal and spinal level could be reinstated or recruited, respectively. We hypothesize that intact males will continue using the NMDAR system to modulate continuous morphine-induced hyperalgesia, and that removing circulating ovarian hormones in females will cause them to “switch” systems to mirror male patterns. However, following acute subcutaneous progesterone replacement, we propose that female-typical patterns of hyperalgesia will be restored. Finally, as it is believed that sex
differences in morphine-induced hyperalgesia are a result of the presence of female hormones rather than a lack of male hormones, it is quite possible that progesterone administration in intact males may cause them to mimic female-typical patterns of hyperalgesia.

2. METHODS

Subjects

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

Ovariectomy Surgical Procedures

During surgery, female mice were subject to ovariectomy protocols, described in detail in the above General Methods section. All mice were allowed to recover for 20 days before undergoing experimental protocols.

Nociceptive Assay

The warm-water tail-withdrawal test, as described in detail above in the General Methods section, was chosen to assess nociception. This measure has repeatedly been shown to be stable and reliable in the context of recurrent testing (Elliott et al., 1995; Kest et al., 2002; Nemmani et al., 2004).

Drug Delivery
Continuous morphine of two distinct dosing paradigms was delivered via subcutaneous osmotic pump implantation. One group received a low dose of morphine (2mg/ml, yielding 1.6mg/kg/24h), whereas a second group received a high dose of morphine (50mg/ml, yielding 40mg/kg/24h). Naltrexone (NTX) pellets were subcutaneously implanted 24 h prior to pump implantation. Intracerebroventricular injections of MSG606, MK-801, DMSO + saline (MSG606 vehicle control), or saline (MK-801) vehicle control were performed under oxygen/isoflurane inhalant anesthesia. MK-801 was dissolved in saline, while MSG606 was dissolved in a 10% DMSO vehicle. Intrathecal injections of MSG606, MK-801, or their vehicles controls were performed under light oxygen/isoflurane inhalant anesthesia and administered by lumbar puncture. Acute bolus injections of progesterone (0.0016mg/kg) were administered according to the formula of 10 ml for every kg of body weight.

Testing Procedures

In accordance with the first two series of studies in the current dissertation, both ovariectomized females and intact males receiving continuous low or high morphine infusion doses were subjected to either i.c.v. or i.t. antagonist injections on Day 4, and nociception was assessed for one hour post-antagonist injection. On Day 6, all animals were subjected to identical procedures, with the addition of an acute subcutaneous injection of progesterone 30 minutes before antagonist injection.

Data Analysis

Opioid-induced hyperalgesia was expressed as raw withdrawal latencies. Withdrawal latencies were analyzed using three-way analyses of variance (Sex * Antagonist Drug * Time) followed by a Fisher’s LSD (protected t-test) for post-hoc comparisons. P-values < 0.05 were considered
significant. Data from low- and high-dose morphine studies were analyzed separately, as were i.c.v and i.t. studies.

3. RESULTS

Study 5: Hyperalgesia in intact males and ovariectomized females during continuous low-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous low-dose morphine administration (1.6 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both intact males and OVX females were injected via the i.c.v. route with an acute dose of either MSG606, MK-801, the MSG606 control, or MK-801 control, and tested every 15 minutes for one hour. Hyperalgesia was reinstated by Day 6 in all subjects; following a baseline measure on this day, both male and female mice received an acute s.c. injection of progesterone (0.0016mg/kg). Thirty minutes later, all subjects received an acute i.c.v. injection of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour.

Following continuous infusion of low morphine doses, intact females typically utilize the NMDAR system to mediate hyperalgesia; thus, OVX with subsequent progesterone replacement should not and did not cause a change in this pattern. On day 4, significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was already evident in both sexes at 15 minutes post-injection, and resolved within the one-hour testing period (Figure 5A-B). On Day 6, hyperalgesia in intact males and OVX females was reversed exclusively by the NMDAR system, even after progesterone replacement. No effect was
observed in males or females following injection of MSG606 on either testing day. Additionally, no effect was observed in either sex following injection of either control, indicating no influence of vehicle injections on low-dose morphine-induced hyperalgesia.
5A. Females

5B. Males
**Figure 5A-B.** Time course of hyperalgesia in CD-1 females (5A) and males (5B) undergoing continuous low-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a low dose of morphine (1.6 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.c.v. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MSG606 vehicle control, or MK-801 vehicle control. After which, all mice were assayed for nociception every 15 minutes for one hour. By Day 6, hyperalgesia was reinstated in all subjects. Following a baseline measure on this day (0), all subjects received an acute s.c. injection of progesterone (0.0016mg/kg), followed by an acute i.c.v. injection 30 minutes later of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on Day 4 and Day 6 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
Study 6: Hyperalgesia in intact males and ovariectomized females during continuous high-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous high-dose morphine infusion (40mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both intact males and OVX females were injected via the i.c.v. route with an acute dose of either MSG606, MK-801, or the MSG606 or MK-801 control, and tested every 15 minutes for one hour. On Day 6, hyperalgesia was reinstated in all subjects. Following a baseline measure on this day, both male and female mice received an acute s.c. injection of progesterone (0.0016mg/kg). Thirty minutes later, all subjects received an acute i.c.v. injection of MSG606, MK-801, and either the MSG606 or MK-801 vehicle control. All mice were tested every 15 minutes post-antagonist injection for one hour.

Significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was already evident in males and OVX females at 15 minutes post-injection on day 4, and resolved completely within the one-hour testing period. Following continuous infusion of a high morphine dose, intact females typically utilize the MC1R system to mediate hyperalgesia; thus, removal of circulating ovarian hormones via OVX should and did cause females to “switch” systems on day 4, using the NMDAR system. However, subsequent progesterone replacement on day 6 caused females to resort back to their female-typical patterns, using the MC1R system to mediate their hyperalgesia. However, females did appear to maintain use of the NMDAR system as well, although on a weaker scale than the MC1R system. Interestingly, on day 6, an acute progesterone injection caused the additional recruitment of a
female-typical system in males as well. That is, intact males were able to use either a supraspinal NMDAR or MC1R system to mediate their high-dose morphine induced hyperalgesia. However, reversal by the NMDAR system was significantly stronger than reversal by the MC1R system in males. It is possible that a higher dose of progesterone may have had a more significant effect in both sexes lacking ovarian hormones. No effect was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on high-dose morphine-induced hyperalgesia. (Figure 6A-B).
Figure 6A-B. Time course of hyperalgesia in CD-1 females (6A) and males (6B) receiving continuous high-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were
implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a high dose of morphine (40 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.c.v. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), the MSG606 vehicle control, or MK-801 vehicle control. After which, all mice were assayed for nociception every 15 minutes for one hour. By Day 6, hyperalgesia was reinstated in all subjects. Following a baseline measure on this day (0), all subjects received an acute s.c. injection of progesterone (0.0016mg/kg), followed by an acute i.c.v. injection 30 minutes later of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on Day 4 and Day 6 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
Study 7: Hyperalgesia in intact males and ovariectomized females during continuous low-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous low-dose morphine infusion (1.6 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both intact males and OVX females were injected via the i.t. route with an acute dose of MSG606, MK-801, or a MSG606 or MK-801 vehicle control and tested every 15 minutes for one hour. Hyperalgesia was reinstated by Day 6 in all subjects; following a baseline measure on this day, both male and female mice received an acute s.c. injection of progesterone (0.0016mg/kg). Thirty minutes later, all subjects received an acute i.t. injection of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour.

Significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was evident in both sexes at 15 minutes post-injection on day 4. Following continuous infusion of a low morphine doses, intact females typically utilize the NMDAR system to mediate hyperalgesia; thus, OVX with subsequent progesterone replacement on day 6 should not and did not cause a change in this pattern, as female hyperalgesia was mediated exclusively by the NMDAR system. Additionally, progesterone on day 6 had no effect on males, as they also continued to use the NMDAR system to mediate hyperalgesia. No effect was observed in males or females following injection of MSG606. Additionally, no effect was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on low-dose morphine-induced hyperalgesia.
Figure 7A-B. Time course of hyperalgesia in CD-1 females (7A) and males (7B) receiving continuous low-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were
implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a low dose of morphine (1.6 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.t. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), a MSG606 vehicle control, or a MK-801 vehicle control. After which, all mice were assayed for nociception every 15 minutes for one hour. By Day 6, hyperalgesia was reinstated in all subjects. Following a baseline measure on this day (0), all subjects received an acute s.c. injection of progesterone (0.0016mg/kg), followed by an acute i.t. injection 30 minutes later of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on Day 4 and Day 6 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
Study 8: Hyperalgesia in intact males and ovariectomized females during continuous high-dose morphine infusion

All subjects were rendered significantly hyperalgesic on day 4 of continuous high-dose morphine administration (40 mg/kg/24h) after concomitant treatment with NTX, as assessed using the warm water tail-flick withdrawal assay. Following a baseline measure on day 4, both intact males and OVX females were injected via the i.t. route with an acute dose of either MSG606, MK-801, a MSG606 vehicle control, or a MK-801 vehicle control, and tested every 15 minutes for one hour. Hyperalgesia was reinstated by Day 6 in all subjects; following a baseline measure on this day, both male and female mice received an acute s.c. injection of progesterone (0.0016mg/kg). Thirty minutes later, all subjects received an acute i.t. injection of MSG606, MK-801, or their respective controls. All mice were tested every 15 minutes post-antagonist injection for one hour.

On day 4, significant reversal of morphine-induced hyperalgesia after injection of the NMDA receptor antagonist MK-801 was evident in both sexes at 15 minutes post-injection. However, progesterone replacement on day 6 in exclusively OVX female mice allowed the recruitment of both the NMDAR and the MC1R system, while males continued to recruit exclusively the NMDAR system. No effect was observed in either sex following injection of either control, indicating no effect of the MSG606 or MK-801 vehicle on high-dose morphine-induced hyperalgesia.
**Figure 8A-B.** Time course of hyperalgesia in CD-1 females (8A) and males (8B) receiving continuous high-dose morphine infusion, as assessed by the tail-withdrawal test. Mice were implanted with NTX pellets (30 mg) on day -1, and implanted with morphine pumps containing a high dose of morphine (40 mg/kg/24h) on Day 0. When hyperalgesic (day 4), mice were administered acute i.t. injections of one of the following: the NMDA receptor antagonist MK-801 (.05mg/kg), the MC1R antagonist MSG606 (1.5 mg/ml), a DMSO + saline vehicle control, or a saline vehicle control. After which, all mice were assayed for nociception every 15 minutes for one hour. By Day 6, hyperalgesia was reinstated in all subjects. Following a baseline measure on this day (0), all subjects received an acute s.c. injection of progesterone (0.0016mg/kg), and an acute i.t. injection 30 minutes later of either MSG606, MK-801, a MSG606 vehicle control, or a MK-801 vehicle control. All mice were tested every 15 minutes post-antagonist injection for one hour. Values represent mean tail-withdrawal latency ± S.E.M.; following antagonist injections at “0”, significant increases in withdrawal latencies relative to baseline values obtained on Day 4 and Day 6 are indicated (+), denoting significant reversal of hyperalgesia assessed at 15, 30, 45, and 60 minutes post-injection. Where apparent, significant hyperalgesia is also indicated (--).
4. DISCUSSION

Studies 5, 6, 7, and 8, while almost entirely confirming our hypotheses, yielded interesting findings. The major findings of these studies were as follows: 1) at low doses of continuous morphine infusion, supraspinal NMDA, but not MC1, receptor blockade reverses hyperalgesia in male and OVX female mice, and this sensitivity to NMDA receptor blockade is unchanged by progesterone administration in either sex; 2) at high doses of continuous morphine infusion, supraspinal NMDA receptor, but not MC1R, blockade reverses hyperalgesia in both males and OVX females; 3) following progesterone administration in these same mice, OVX females and intact males are able to recruit the use of both MC1R and NMDAR systems; however, the effect of the MC1R- or NMDAR-regulated reversal is more robust in OVX females or intact males, respectively; 4) at low doses of continuous morphine infusion, male and OVX female mice recruit a spinal NMDAR system to modulate hyperalgesia, and progesterone replacement in these same animals leaves these patterns undisturbed; 5) at high doses of continuous morphine infusion, potent reversal by exclusively a spinal NMDAR system is evident in both males and OVX females; 6) following progesterone administration in these same mice, exclusively OVX females are able to recruit the use of both NMDAR and MC1R systems to mediate hyperalgesic reversal of similar magnitude. Unexpected was the finding that progesterone administration in males elicited female-typical patterns following the specific treatment paradigm of high dose morphine treatment paired with i.c.v. antagonist injections, but not i.t. antagonist injections. Additionally, while not surprising following speculation, we had not considered that progesterone-exposed OVX females would retain the use of both NMDAR and MC1R systems following high dose morphine treatment. Specifically, we hypothesized that intact female-typical patterns would be restored, rather than recruited as an additional mechanism. These
outcomes suggest that female ovarian hormones play a major role in the modulation of morphine-induced hyperalgesia. These findings are discussed in detail below.

**Female hormones play a large role in mediated morphine-induced hyperalgesia.** Our previous findings have shown there to be a systemically and centrally initiated sex- and dose-dependent mediation of continuous morphine-induced hyperalgesia, such that both systemic injections (Juni et al., 2008; Juni et al., 2010) and central injections (the current dissertation) of MK-801 or MSG606 reversed this state differentially in male and female mice. The previous studies of this dissertation illustrated that both supraspinal and spinal mechanisms are involved in morphine-induced hyperalgesia. The current study therefore investigated the role of female hormones in recruiting supraspinal and spinal NMDAR and MC1R systems in the mediation of morphine-induced hyperalgesia. The results of the current series of studies provide support for the notion that, not only are circulating ovarian hormones responsible for the switch between the NMDAR and MC1R system, but that these mechanisms work independently in both supraspinal and spinal loci. Interestingly, it is often reported that females demonstrate lower pain thresholds and largely represent those suffering from unremitting pain (Mogil, 2012); such clinical findings in conjunction with the current findings suggest that females are predisposed to the use of a dose-dependent hyperalgesic mechanism that is influenced by ovarian hormones. While what precisely cause females to have lower pain thresholds remains unknown, further investigation into the role of hormones in clinical studies is warranted.

Previous findings on the relationship between gonadal hormones and endogenous pain control show that the two are interrelated. Specifically, gonadal steroid receptors (in particular, α and β
estrogen receptors) and opioid receptors are co-localized on peripheral sensory neurons and neurons in the central nervous system. Research has shown that the endogenous opioid system is modulated in part by estrogen and testosterone; that estradiol causes μ-receptor internalization (Wiesenfeld-Hallin, 2005; Aloisi, Della Seta, Rendo, Ceccarelli, Scaramuzzino, et al., 2002). Interestingly, it is hypothesized that the sex differences seen in pain perception are in part due to increased activity of the endogenous opioid receptor system in males when compared to females. Investigations used mice lacking a subunit of the G-protein-coupled inwardly rectifying potassium receptor (GIRK); this particular channel has an important role in a neuron’s response to analgesics acting through all three opioid-GPCRs. Specifically, Mitrovic, Margeta-Mitrovic, Bader, Stoffel, Jan, et al. (2003) found that male mice are less sensitive to thermal nociception than are females. However, in GIRK2 knock-out mice this sex difference is no longer apparent, as male thresholds were reduced to that of females. Additionally, whereas male mice demonstrate stronger morphine-induced analgesia than females, this sex difference was also abolished in mice lacking GIRK2; however, the overall analgesic effect of morphine was also reduced. However, a major problem with the extrapolation of these findings lies in that humans tend to exhibit reverse patterns; that is, females typically exhibit stronger μ- and κ- receptor mediated analgesia than males in clinical populations. In fact, in studies on postoperative opioid consumption, women tend to consume less than half the amount of opioids that men do, suggesting a superior opioid analgesic effect in women. A second issue lies in the fact that women demonstrate varying pain thresholds depending of the follicular phase of their menstrual cycle (Wiesenfeld-Hallin, 2005). Nonetheless, it is not entirely known how sex differences in opioid-induced hyperalgesia are mediated, and these data suggest that hormones may a major role in the molecular and cellular underpinnings of hyperalgesia.
Administration of female hormones allows males to recruit supraspinal female-typical hyperalgesic mechanisms. The current data suggest that the female-typical mechanism of hyperalgesia is located supraspinally and spinally. It is interesting that in those current studies that investigated exclusively supraspinal mechanisms in intact male and OVX female mice, findings that supported specific aim 1 and 2 were discovered. That is, both male and OVX female mice recruited powerful supraspinal-level NMDAR systems during infusion of both doses of morphine. However, progesterone caused both cohorts to recruit powerful NMDAR and MC1R supraspinal systems to mediate hyperalgesia. At low doses of morphine infusion, males and OVX females continue to use a powerful spinal NMDAR-modulated hyperalgesic mechanism across the board. At high doses of continuous morphine, males and OVX females use the NMDAR system; however, following progesterone replacement, spinally administered MSG606 has no effect on males, yet evokes a significant effect in females. This supports the findings from specific aim 2; that there does appear to be a functional hyperalgesic mechanism in the spinal cord that involves the MC1R system. However, why males only recruit a supraspinal MC1R system after progesterone injection (Study 6) and not a spinal mechanism as well (Study 8) is unknown.
The overall aim of the current dissertation was to elucidate the precise location(s) of action of the regulatory mechanisms that underlie morphine-induced hyperalgesia. The current studies demonstrate several findings that expand upon what is currently known about this phenomenon:

1) Continuous low dose morphine-induced hyperalgesia is reversed by i.c.v. administration of the NMDAR antagonist MK-801 in both males and females;

2) Continuous high dose morphine-induced hyperalgesia is reversed by i.c.v. administration of MK-801 in exclusively males;

3) This same continuous high dose morphine-induced hyperalgesia is reversed exclusively by i.c.v. administration of the MC1R antagonist MSG606 in females only;

4) Continuous low dose morphine-induced hyperalgesia is reversed by i.t. administration of the NMDAR antagonist MK-801 in both males and females;

5) Continuous high dose morphine-induced hyperalgesia is reversed by i.t. administration of MK-801 in exclusively males;

6) This same continuous high dose morphine-induced hyperalgesia is significantly reduced by i.t. administration of the MC1R antagonist MSG606 in females only;

7) Following ovariectomy, females exhibit male-typical patterns of hyperalgesia at both high and low doses of continuous morphine administration;

8) After an acute injection of systemic progesterone, female typical patterns of hyperalgesia are restored following i.c.v. antagonist administration;

9) Following an acute injection of systemic progesterone, there appears a spinal MC1R mechanism that modulates female-typical high dose morphine-induced hyperalgesia;

10) Following progesterone replacement, males are capable of recruiting supraspinal female-typical hyperalgesic mechanisms.
There are several limitations to the studies detailed within this dissertation. All assessments were conducted using morphine, a substance that preferentially binds to the $\mu$ opioid receptor, a characteristic common to virtually all opioids reported to cause hyperalgesia in humans and rodents (Xu et al., 2003; Ossipov et al., 2004). While the current studies provide evidence for how these opioids cause hyperalgesia independently of opioid receptor activity, these findings are novel and thus we cannot say with utmost certainty if these assumptions can be extrapolated to include delta and kappa receptor opioids, or even other $\mu$-preferring opioids administered under other paradigms (Mogil, 2012). Further studies that assess the hyperalgesic tendencies of different opioids are required before such comparisons can be made. Furthermore, since the dependent nociceptive measure in all studies described in this dissertation, the tail withdrawal test, is a measure of thermal reflexive pain, it is possible that different results would be obtained on other nociceptive measures such as mechanical or chemical pain (Mogil et al., 1999a, 1999b, Mogil, 2012). Another issue is the possibility that spinally administered antagonists exerted their effects following rostral spread; in other words, it is possible that these substances travelled to the brain to exert their effects. While morphine is absorbed into the bloodstream of the spinal cord quickly and likely doesn’t spread beyond the spinal cord, it is unknown if MK-801 or MSG606 undergo rostral spread. However, the dispersal of the 2 $\mu$l-injection volume used in these studies has been observed to travel approximately 0.5 to 1cm within 30 minutes. Thus, it would take quite some time for an intrathecally-injected compound to exert a supraspinal effect. Additionally, by the time rostral spread to the brain occurs, it is likely that several half-lives of the drug would have passed, making for a much weaker or nonexistent drug concentration (Rossi, unpublished data and observations). Finally, only outbred CD-1 mice were used as subjects; different results may be found in mice of altered genetic background (Mogil et al.,
1999; Waxman, 2012). Thus, applicability beyond the narrow conditions described above should not be assumed.

In attempt to further characterize this dose- and sex- dependent paradoxical state, the current dissertation employed several paradigms to demonstrate that hyperalgesia may be mediated in a location-dependent manner as well. Previous continuous morphine infusion studies using receptor blockade to investigate the mechanisms involved in hyperalgesia employed systemic injections, which allows for widespread distribution throughout the peripheral and central nervous system and thus does not specify the precise central locus of action (Juni et al, 2008; Juni et al., 2010). The current dissertation employed a central paradigm of antagonist administration. Specifically, the role of supraspinal and spinal NMDAR and MC1R systems were investigated. It appears that at the supraspinal level, there exists a powerful NMDAR mechanism that modulates both male and female hyperalgesia as low doses of morphine infusion. However, while males continue to employ the NMDAR system at high doses of morphine infusion, females recruit a powerful supraspinal MC1R system.

In terms of spinal loci, it appears that there is a prevalent NMDAR mechanism that can independently regulate morphine hyperalgesia in males, and females at exclusively low doses of morphine infusion. Additionally, hyperalgesia following high dose morphine infusion in females is significantly regulated by a spinal MC1R mechanism, such that blockade of this receptor system resulted in hyperalgesic reversal. Thus, we hypothesize that while females possess a spinal male-typical NMDAR system, they also employ the use of a MC1R system to regulate spinally mediated hyperalgesia. While previous research debated the existence of MC1Rs in the
spinal cord, the current data suggest otherwise. Molecular and biochemical studies detailing MC1R distribution in the spinal cord are needed to corroborate these findings.

The current studies are intriguing when compared to previous studies reporting equivalent qualitative sex differences in opioid analgesia. Specifically, NMDA receptor antagonists have been shown to reduce kappa opioid analgesia in male but not female mice (Kavaliers & Choleris, 1997). In male mice, spinally regulated analgesia has been shown to require the activation of exclusively the $\mu$-opioid receptor, while females require the use of both mu- and $\kappa$-opioid receptors and the subsequent production of spinal dynorphin in order to experience the same magnitude of analgesia. Thus, it is apparent that sex differences are mediated by the presence of ovarian hormones in females (Liu, von, & Gintzler, 2007). Likewise, numerous studies have reported that $\kappa$-opioid receptor compounds utilize different physiological circuitry in males and females (Sternberg, Ritchie, & Mogil, 2004; Holtman, Jr. & Wala, 2006; Lomas, Barrett, Terner, Lysle, & Picker, 2007; Mogil, 2012), leading $\kappa$-agonists to be significantly more effective analgesic agents in both rodent and human females (Miller & Ernst, 2004; Mogil et al., 2005). This study extends the current findings to analgesia; that MK-801 effectively augments analgesia in OVX females, while OVX followed by estrogen injection reinstates the MK-801 insensitivity characteristic of intact females. Based on these findings, the authors concluded that estrogen diverts pain modulation in females towards a system that functions independently of the NMDAR system characteristic of males.

While a comprehensive understanding of the mechanisms underlying morphine-induced hyperalgesia still remains at large, the current series of studies have contributed several
important findings in terms of sex differences in morphine-induced hyperalgesia. As suggested by its susceptibility to NMDA receptor antagonism, typical morphine-induced hyperalgesia in males (but not intact females) is under the influence of exclusively NMDA receptors. Thus, it is practical that others report greater increases in morphine analgesia in male mice following MK-801 administration relative to females (Lipa & Kavaliers, 1990). Moreover, the failure of MK-801 to reverse hyperalgesia in females across the board, and the ability for i.c.v. MSG606 to reverse male hyperalgesia following progesterone administration provides additional support favoring the existence of supraspinal sex-specific hyperalgesic mechanisms. Interestingly, the current studies demonstrate that if males are given female hormones, they have the ability to recruit an exclusively supraspinal female-typical hyperalgesic mechanism. Conversely, males do not appear to recruit a spinal MC1R system after progesterone administration. It appears as though in such a case, the spinal NMDAR is superior.

While the current studies further define morphine hyperalgesia during continuous infusion, the mechanisms underlying this paradoxical state still remain unclear. In particular, the current dissertation suggests a more complicated sex-dependent mechanism, in that males may also recruit female-typical systems while still producing their own gonadal hormones. Interestingly, both male castration and the administration of gonadal hormones in OVX females decrease the potency of morphine-induced analgesia, suggesting that male hormones increase the efficacy of opioid analgesics (Bodnar and Kest, 2009). Thus, one could speculate that the lack of male hormones in females leaves them more susceptible to morphine-induced hyperalgesia. There are many studies that document sex differences in the clinical perception of pain, but while these differences have long been thought to be purely societal, it is possible that women may have a
hormonally disadvantageous pain processing system.

While a supraspinal MC1R system is feasible due to this receptor’s distribution in the PAG and in brain-glial cells (which more recently are speculated to play a role in hyperalgesia), this receptor is not reported to have widespread spinal distribution (Xia, Wikberg, & Chhajlani, 1995; Mogil et al., 2003). Unfortunately, most of the research concerning this receptor system investigates its role in hair and skin pigmentation, and other skin-related variables. Most of the research that exists on the role of the MC1R in pain relates to its action in antinociception, as detailed above. Thus, not much is known beyond the expanded findings of this dissertation in terms of how the MC1R regulates female-typical hyperalgesia. Interestingly, a recent study reported the involvement of MC4R, a subtype diffusely distributed both supraspinally and spinally, in neuropathic pain. Specifically, Delaney et al. (2010) found an upregulation of the fourth melanocortin subtype specifically in the spinal cord in response to peripheral nerve injury. While this type of pain sensitivity is not identical to morphine-induced hyperalgesia, these findings suggest the involvement of more than one differentially distributed melanocortin receptor subtype in the modulation of female-specific pain. Future research must investigate differential processes of all five of the MC receptor subtypes, particularly the relationship between the MC1R and MC4R. It is possible that spinal MC4Rs may serve as a counterpart to supraspinal MC1Rs, mediating neuropathic pain and hyperalgesia, respectively. Finally, more studies that detail the distribution of the MC1R, particularly in the spinal cord, are imperative. As suggested by the current studies, there appears to be both a supraspinal and spinal MC1R system that modulates pain processing. Specifically, as MC1Rs, opioid receptors, and estrogen receptors are all present in the PAG, this area serves as an idyllic locus for interactions between
opioids, their proposed hyperalgesic mechanisms, and the gonadal steroid hormones that determine these effects. While this site is already known to play a role in pain inhibition, corroborative studies detailing its role in pain facilitation are needed (Bodnar and Kest, 2009). As no such spinal locus is immediately evident, related future studies could detail the existence of a spinal female MC1R pain processing system.

Research on general sex differences in pain, both preclinically and clinically, a consensus is not immediately evident. While there is a vast amount of animal studies that investigate pain, most paradigms use exclusively males in their studies. Ironically, females are often excluded due to the very issue that causes their sex-differentiated pain: fluctuations in ovarian hormones. Because of their exclusion, this leaves the important area of female-typical pain unaddressed. For instance, the phase of the estrous cycle appears to play a role in animal models of female-mediated analgesia; specifically, systemically initiated analgesia is most potent during the metestrus and proestrus phases, and least effective during the estrous phase (Mogil, 2012; Terner, Lomas, and Picker, 2005; Bodnar and Kest, 2009). In humans, females tend to have higher pain tolerance during the follicular phase (Mogil, 2012). Additionally, preclinical literature suggesting superior analgesic effect in women following treatment with κ-agonists extrapolates to the clinical population. Thus, pharmacokinetic and pharmacodynamic sex differences are likely also at play, and it is likely that these hormone fluctuations play a role in hyperalgesia.

While many of the sex differences seen in pain research are detailed above as quantitative variables, there are likely also qualitative issues; for instance, women may be more likely to seek
treatment for unremitting pain. Secondly, women could be more susceptible to these chronic pain syndromes (likely due to hormonal cyclicity). Finally, it is possible that women demonstrate lower pain thresholds than men, due to variations in descending and ascending pain transmission pathways, or genetic variations. While all of these hypotheses present valid arguments, the only trend that unanimously holds up to the rigors of methodical scientific research is that women show reduced pain thresholds and increased pain sensitivity when compared to men (Mogil, 2012). Thus, the current dissertation suggests a hormonal mechanism by which women may demonstrate inferior pain processing. While the current studies by no means advocate for a reduction in the use of opioids in clinical settings, our findings shed a brighter light on sex differences in pain processing. Although there is an overwhelming amount of research on sex differences in pain, the clinical impact remains inadequate. As chronic pain is an increasingly growing issue that overwhelmingly encompasses the woman patient, it is imperative that these sex differences are comprehensively understood in order to provide adequate care.
GLOSSARY OF ABBREVIATED TERMS

α-MSH: α-melanocyte-stimulating hormone
AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ACTH: adrenocorticotropicin
β-FNA: β-funaltrexamine
BBB: blood brain barrier
BL: baseline
cAMP-PDE: cyclic adenosine monophosphate phosphodiesterase
C: degrees Celsius
CCK: cholecystokinin
cm: centimeters
CTX-B: cholera toxin B-subunit
DMSO: dimethyl sulfoxide
DRG: dorsal root ganglia
GABA: γ-aminobutyric acid
GIRK2: second subunit of the G-protein-coupled inwardly rectifying potassium receptor
GT: glutamate transporter
IBMX: 3-isobutyl-1-methylxanthine
i.c.v.: intracerebroventricular
i.m.: intramuscular
i.t.: intrathecal
i.v.: intravenous
kg: kilogram
µL: microliter
mg: milligram
mg/kg/24h: cumulative 24 hour dose
ml: milliliter
M3G: morphine-3β-glucuronide
M6G: morphine-6β-glucuronide
MC: melanocortin
MC1R: melanocortin-1 receptor subunit
MC4R: melanocortin-4 receptor subunit
MIH: morphine-induced hyperalgesia
Mrp3: multidrug resistance protein 3
NMDA: N-methyl-D-aspartate
NMDAR: N-methyl-D-aspartate receptor
NK: neurokinin
NTX: naltrexone
NLX: naloxone
NO: Nitric oxide
Nor-BNI: norbinaltorphimine
OIH: opioid-induced hyperalgesia
ORL1: opioid receptor-like type receptor
OVX: ovariectomy
PAG: periaqueductal gray
PKC: protein kinase C
PSD: post-synaptic density
QMWS: quasi-morphine withdrawal syndrome
QTL: quantitative trait loci
RVM: rostral ventromedial medulla
s: seconds
s.c.: subcutaneous
TLR4: toll-like receptor subtype 4
TKO: triple knock out
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