SEX DIFFERENCES IN OPIOID ANTINOCICEPTION: MODULATION BY THE N-METHYL-D-ASPARTATE SYSTEM

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There is ample evidence that males and females differ markedly in their sensitivity to the antinociceptive effects of opioids that are active at µ and κ receptor. The following studies examined the role of the NMDA receptor system in modulating sexually dimorphic opioid antinociception using acute (hot-plate and warm water tail-withdrawal) and persistent (temporal summation and capsaicin) models of nociception.

Experiment 1 focused on the development of a behavioral procedure to induce temporal summation in rats and determined the sensitivity of this behavior temporal summation in rats to various parametric manipulations, sex, modulation by the NMDA receptor system, and sensitivity to reversal by opioids. Males displayed slightly higher levels of temporal summation than females. NMDA antagonists attenuated the level of temporal summation in both sexes. There were no sex differences in µ and κ opioids effectiveness in this procedure.

Experiment 2 evaluated sex differences in the antihyperalgesic actions of selected κ and mixed-action opioids in a persistent pain model and examined the role of the NMDA system to modulate these effects in a sexually-dimorphic manner in rats. In general, κ opioids were more potent in males when administered both peripherally and systemically. Sex differences were not observed with the mixed-action opioids. The NMDA antagonist
dextromethorphan attenuated κ opioid induced antihyperalgesia in both sexes. In contrast to the findings in acute pain models, antagonism at the NMDA receptor site did not modulate the effects of κ opioids in a sexually-dimorphic manner.

Experiment 3 examined sex differences in the influence of the NMDA antagonist dextromethorphan on μ opioid antihyperalgesia in the capsaicin persistent pain model and two acute nociceptive assays. Dextromethorphan enhanced the antihyperalgesic effect of morphine in males but not females in the capsaicin assay. Enhancement of antinociception was seen in both sexes in the acute pain models, with greater magnitude in males. These findings demonstrate a sexually dimorphic interaction between the NMDA antagonist dextromethorphan and morphine in a persistent pain model that can be quantitatively distinguished from those observed in acute pain models.

In summary these experiments demonstrate the importance of pain model in investigations of sex differences in opioid analgesia and its possible mechanisms.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>cm</td>
<td>centimeter</td>
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<td>Celsius</td>
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<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective dose required to produce a 50% effect</td>
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<td>GDX</td>
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CHAPTER 1
GENERAL INTRODUCTION

There is now an abundance of evidence indicating differences in the sensitivity of males and females to \( \mu \) and \( \kappa \) opioid analgesics, as well as sex differences in the effects of various mixed-action analgesics (Gear et al., 1996, 1999; Sarton et al, 1999). The root of research in sex differences in opioid analgesia lies in the finding of an ineffectiveness of clinically employed doses of morphine in almost a third of post-surgical pain patients (Lasagna and Beecher, 1954). This finding prompted investigation into the reasons behind this lack of opioid effectiveness in such a large group of patients. While there is an extensive list of factors that could account for individual differences in opioid effectiveness, such as the patients’ age, opioid history and willingness to report pain (Burns et al 1989; Mercandante et al 1997; Miaskowski and Levine 1999); a primary physiological factor appears to be the sex of the individual.

Clinically, males and females have been shown to differ in the amount of morphine required to control post-surgical pain, as well as differing in the level and duration of analgesia produced by opioids (Gear et al., 1996; Sarton et al., 2000; Mogil et al., 2003). As observed in humans, marked sex differences in opioid antinociception have been found in many different species (Cicero et al., 1996; Kest et al., 1999; Negus and Mello 1999; Craft and Bernal, 2000; Mogil et al., 2000; Craft, 2003). For example, the \( \mu \) opioid morphine has
been shown to be more potent in producing antinociception in male rodents than in their female counterparts, and this effect has been observed across a variety of nociceptive stimuli and rodent strains (Cicero et al., 1996, 1997; Kest et al., 1999; Terner et al., 2003). A number of mechanisms have been proposed to account for these sex differences; however, it is now known that sex differences in opioid antinociception are not a consequence of sex-specific opioid binding affinity, G-protein stimulation, or receptor density (Messing et al., 1980; Kepler et al., 1991; Candido et al., 1992; Selly et al., 2003). Pharmacokinetic factors have also been ruled out, as blood and brain concentrations of morphine are similar in males and females after systemic injection (Cicero et al., 1997).

Although the activational and organizational effects of gonadal hormones are key factors in modulating sex differences in opioid antinociception, it is also clear that other factors play critical roles (Cicero et al., 2002). Recent in vivo and in vitro studies, for example, suggest that the NMDA receptor system may be intricately involved in this interaction. Arguably, the strongest indication of a relationship between these two systems comes from morphological evidence indicating co-localization of NMDA and opioid receptors in the central nervous system. Specifically, NMDA and opioid receptors have been identified in the spinal cord dorsal horn and periaqueductal grey (Commons et al., 1999; Aicher et al., 2002). The periaqueductal gray (PAG), and its projections to the rostral ventromedial medulla (RVM) and spinal cord, makeup the primary circuit for opioid analgesia (Basbaum and Fields, 1984). In vivo studies support these observations, as NMDA antagonists enhance the antinociceptive effects of μ opioids in various acute pain models (Allen and Dykstra, 2001; Redwine and Trujillo, 2003; Nemanni et al., 2004), and minimize
the development of both µ opioid tolerance and dependence (Trujillo and Akil, 1991; Kolesnikov and Pasternak, 1999).

Recent studies also suggest that the interaction between the NMDA and opioid systems may be sexually dimorphic. For example, stress-induced antinociception has been shown to be attenuated by NMDA antagonists in male but not female mice. In females, this effect is mediated by the gonadal hormones, as NMDA antagonists reverse stress-induced antinociception in ovariectomized females (Mogil and Belknap, 1997). Perhaps more importantly, NMDA antagonists selectively attenuate the effect of κ opioid antinociception in males (Suacier and Kavaliers, 1994; Kavaliers and Choleris, 1997) and in ovariectomized females (Sternberg et al., 2004).

New findings indicate that µ opioid antinociception can be dramatically enhanced by NMDA antagonists, and this effect also appears to be sex-specific (Holtman et al., 2003; Nemmani et al., 2004). Such findings suggest further that there are fundamental differences between males and females in the extent to which the NMDA system can modulate opioid antinociception. This sexually dimorphic interaction is not only observed in behavioral measures of nociception; NMDA antagonists also reduce morphine induced c-Fos expression in a sexually dimorphic manner (D’Souza et al., 1999). It is likely the sex differences observed in opioid antinociception are due, in part, to a sexual differentiation in the NMDA system and how this system modulates the opioid system.

Sex difference in opioid antinociception and its relationship to the NMDA system have been examined in a limited set of conditions and nociceptive models. Pain models in animals are an important means by which the mechanisms and maintenance of pain can be understood (Aloisi et al., 1995). Analyses of these models have revealed that the mechanisms
underlying nociception are dependent upon a number of factors, including the duration of the nociceptive stimulus (McLaughlin and Dewey, 1994). Behavioral models of pain can be classified by the duration of nociceptive stimulus as either phasic (acute) tests such as the tail flick or tail-withdrawal assay or tonic tests (more long lasting; persistent). These models vary in transmission of nociceptive information as well as nociceptive response (Le Bars et al, 2001). In acute tests there is typically the application of a brief noxious stimulus followed by a withdrawal behavior by the animal (Mogil et al., 2000). Persistent models, which seek to model more long term pain, often result in hyperalgesia, inflammation and/or tissue damage (Le Bars et al, 2001). Moreover, in acute pain models, opioids produce effects via spinal and supraspinal sites, alternatively in some persistent pain models opioids are effective when administered peripherally at the site of injury, which is not true in acute models (Yaksh, 1997). While there is limited research on sex differences in opioid antinociception in persistent models of pain, there is some indication that females have a greater nociceptive response in these assays. For example, in the formalin assay which is a model of persistent pain, injections of formalin produced greater hindpaw licking in females rats compared to males. Male rats are also less sensitive to the hyperalgesic effects of capsaicin compared to their female counterparts in the capsaicin model of persistent pain (Aloisi et al., 1994; Gaumond et al., 2002; Barrett et al., 2003). Recent studies indicate, at least with μ opioids, there may not be large sex differences in opioid antinociception in the capsaicin persistent model of pain (Barrett et al., 2003). The issue of chronicity of the pain model is critical in that these models vary on so many dimensions. Evaluation in both persistent and acute models of pain is necessary in order to have a full understanding of sex differences in opioid antinociception.
Central sensitization is a phenomenon thought to be an integral factor in the development of persistent pain (Coderre, 1993; Woolf, 1996; Li et al., 1999). Central sensitization is most commonly described as an increased excitability of central wide dynamic range neurons in the spinal cord (Eide, 2000). This event is a reaction to increased activity at nociceptors, such as from injury or inflammation (Woolf, 1996). This central activity may play a role in many chronic pain conditions and is specific to persistent pain; it is typically not a factor in acute pain. One proposed factor underlying central sensitization is wind-up, in which the repeated stimulation of the C primary nociceptive afferents induces hyperactivity of dorsal horn neurons (Mendell and Wall, 1965; Woolf, 1996). The behavioral correlate of this phenomenon, as seen in humans, is temporal summation of pain (Ren, 1994; Li et al., 1999), or the increase in pain following repeated presentation of a nociceptive stimulus. This short-lasting change in nociceptive sensitivity has been employed in humans to examine factors that influence the central processing of pain and the mechanisms underlying some chronic pain conditions. Interestingly the NMDA receptor system appears to play in a role in the development of central sensitization and wind-up as NMDA antagonists have been shown to inhibit wind-up in dorsal horn neurons (Dickenson and Sullivan, 1987). The purpose of Experiment 1 was to provide a detailed evaluation of sex differences in the sensitivity of opioids in a model of persistent pain by developing a model of temporal summation of pain in rats. Male and female rats were evaluated for nociceptive response in a novel behavioral model of temporal summation (i.e., decrease tail-withdrawal latency following repeated presentations of a nociceptive thermal stimulus). Sensitivity to various parametric manipulations, sex, modulation by the NMDA receptor system, and sensitivity to reversal by opioids were examined.
Sex differences in the potency of the antinociceptive effects of κ opioids have been reported in acute pain models and when opioid activity is centrally mediated. In acute models, the nociceptive stimulus activates large Aδ nociceptive fibers. In contrast, nociception in persistent pain models is associated with slower, C nociceptive fiber activation as well as tissue damage and inflammation. The distinction between acute and persistent pain models is critical to the study of sex differences in opioid analgesia as these models can be differentially modulated by the NMDA system. Sex differences in antinociception may be dependent upon the opioid system’s interaction with the type/duration of nociception elicited (Barrett et al., 2003; Lomas et al., 2007). Recent studies support this contention as the magnitude of sex differences in opioid antinociception has been demonstrated to be larger in models of acute than chronic pain in a study on μ opioids (e.g., Barrett et al., 2003). Given the possible importance of pain model in determining antinociceptive responses and role of the NMDA receptor system, both experiment 2 and 3 utilized the non-NMDA mediated capsaicin model of persistent pain. In experiment 2 a capsaicin model of persistent pain was used to compare κ opioid-induced antihyperalgesia in intact F344 male and female rats. Various κ opioids and mixed action opioids were tested in this 45°C tail-withdrawal assay measuring hyperalgesia. There is evidence that opioids can produce their effects not only by central receptor action, but also by peripheral receptors in models of inflammation, with select opioids having greater potency peripherally (Ko et al., 1998; Ko et al., 2000). The capsaicin model was used to identify potential sex differences in κ opioid effectiveness both peripherally and centrally. Additional tests were conducted to examine the effect of κ opioid/NMDA antagonist combinations on antihyperalgesia in this persistent model of pain.

There is a large body of evidence suggesting the NMDA system modulation of the
opioid effects. NMDA antagonists have been shown to inhibit the development of opioid
tolerance, dependence and sensitization (Mao, 1999; Popik, 2000; Trujillo, 2000). NMDA
antagonist enhancement of μ opioid antinociception has been observed in a number of
species (Allen and Dykstra, 2001; Caruso, 2000; Holtman et al, 2003; Redwine and Trujillo,
2003). To date, most evidence demonstrating that NMDA antagonists enhance opioid
antinociception, as well examination of sex differences in this effect, have been obtained in
models of acute pain. In Experiment 3, a capsaicin model of persistent pain was used to
examine sex differences in NMDA modulation of μ opioid-induced antihyperalgesia. At the
supraspinal level, NMDA receptors are found in the hippocampus, thalamus and brainstem
(Roth et al., 1996; Suzuki et al., 1996), whereas, at the spinal level, NMDA receptors have
been primarily identified in the dorsal horn (Petralia et al., 1994; Tolle et al., 1995). Opioid
receptor density is also relatively high at these sites. Therefore NMDA/μ opioid interactions
were also evaluated using acute nociceptive assays with both a nociceptive response that is
primarily supraspinally mediated (hot-plate: hind paw lift or escape) and one that is primarily
spinally mediated (warm water tail-withdrawal) (Le Bars, 2001).
CHAPTER 2

EXPERIMENT 1: BEHAVIORAL ASSESSMENT OF TEMPORAL SUMMATION
IN THE RAT: SENSITIVITY TO SEX, GONADAL HORMONE DEPLETION,
OPIOIDS AND MODULATION BY NMDA RECEPTOR ANTAGONISTS

Introduction

Several lines of evidence suggest that hyperalgesia, allodynia and some chronic pain conditions may be due, in part, to the enhanced excitability of central wide dynamic range neurons in the spinal cord. This excitability can be induced by inflammation, tissue damage (central sensitization) and artificially by repeated stimulation of dorsal horn neurons, a phenomenon known as wind-up (Mendell and Wall 1965; Coderre et al. 1993; Woolf 1996). Wind-up frequently occurs following repetitive heat pulses at intervals of 3 seconds or less and is associated with a gradual increase in pain perception mediated by activity in the central nervous system (Price 1977; Fillingim et al. 1998). A behavioral manifestation of this central excitability is temporal summation (Ren 1994; Li et al. 1999), which in humans is characterized by an increase in pain sensitivity following repeated presentations of a nociceptive stimulus. This increase in pain sensitivity typically outlasts exposure to the nociceptive stimulus, with painful after-sensations reported as long as 15 and 120 secs following the termination of the nociceptive stimulus (Staud et al. 2001, 2003).

Temporal summation of pain has been shown to be sensitive to organismic factors, such as health status, sex and age. For example, in some chronic pain patients and the elderly
there is heightened level of temporal summation (Maxiner et al. 1998; Edwards and Fillingim 2001; Staud et al. 2001), whereas in healthy young subjects temporal summation of pain is inversely correlated with perceptions of general health (Edwards and Fillingim 2001). Moreover, females display greater levels of temporal summation of pain and unpleasantness ratings than males with these effects evident for both thermal and mechanical nociceptive stimuli (Fillingim et al. 1998; Sarlani and Greenspan 2002; Sarlani et al. 2004). That females also display lower pain thresholds and are more likely to develop certain chronic pain conditions (Unruh 1996), suggests that temporal summation of pain may represent a model to investigate the factors involved in the development of chronic pain.

There is a growing body of evidence indicating that activation of the N-methy-D-aspartate (NMDA) system is involved in some chronic pain conditions, thus it is not surprising that this system has also been linked to the development of temporal summation of pain. For example, NMDA receptor antagonists attenuate temporal summation of pain in humans, with the magnitude of this effect varying markedly across NMDA antagonists (Arendt-Nielsen et al. 1994; 1995; Price et al. 1994). Likewise, NMDA antagonists can be effective at reducing evoked pain in individuals with neuropathic pain (Eide et al. 1994; Mathison et al. 1995) and may hold promise as a treatment for certain chronic pain conditions (Eide 2000).

Opioids have been reported to be effective in reducing the development of temporal summation in humans (Price, 1985). Although sex differences in this effect have not been systematically examined in the temporal summation procedure (for an exception see Fillingim et al. 2004), sex differences in opioid sensitivity have been observed in the post-surgical setting (Gordon et al. 1995; Fillingim and Gear, 2004). This effect appears to be
least evident with high efficacy opioids (e.g., morphine) and most evident with less efficacious opioids (e.g., butorphanol). In non-humans, sex differences have been consistently observed with low efficacy opioids in procedures that induce acute (e.g., Cook et al. 2000; Craft and Bernal 2001) but not chronic nociception (Barrett et al. 2003). As in the development of temporal summation, the NMDA system appears to play a critical role in the development of chronic nociception (Przewlocki and Przewlocki 2001). Consequently, the use of the temporal summation procedure should provide important information concerning sex differences in opioid sensitivity under chronic pain conditions.

The purpose of this investigation was to determine if repeated presentations of a thermal nociceptive stimulus produces systematic increases in nociceptive sensitivity in rats. Such an effect would be functionally equivalent to temporal summation of pain in humans, and would thus allow for the assessment of the mechanisms underlying central sensitization and the factors that influence some chronic pain conditions. In this investigation, we focused on the sensitivity of temporal summation in rats to sex, gonadal hormone depletion, modulation by non-competitive NMDA receptor antagonists and reversal by opioids active at \( \mu \) and \( \kappa \) receptors.

**Methods**

**Animals**

Gonadally intact and gonadectomized (GDX) male and female F344 rats were obtained from Charles River Suppliers (Raleigh, N.C., USA). F344 rats were selected for study as this strain was shown to display marked differences in both nociceptive and opioid sensitivity (Cook et al. 2000; Barrett et al. 2003; Terner et al. 2003). All testing occurred when the rats were
between 3 and 6 months of age. Rats were housed individually in a colony maintained on a 12-h light/dark cycle and had unlimited access to food and water. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina, and the “Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research” (Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, National Research Council National Academy Press, 2003).

Temporal summation procedure

After a habituation procedure (see Terner et al. 2003), rats were placed in restraint tubes, the distal 7 cm of the tail immersed in warm water, and the time recorded to tail-withdrawal (baseline tail-withdrawal latency). For all baseline tests, a 15 sec cut-off time was imposed to prevent tissue damage. Approximately 2 mins later, the temporal summation testing procedure was initiated. This procedure consisted of repeatedly placing the rat’s tail in the warm water for 1.0-3.0 secs. After a set number of presentations of the nociceptive stimulus, with each presentation separated by a brief interval, tail-withdrawal latency was again recorded with the 15 sec cut-off still imposed.

Parametric analyses

Extensive parametric testing was required, as in humans temporal summation of pain is highly dependent on the inter-nociceptive stimulus intervals, nociceptive stimulus intensities and the frequency of nociceptive stimulus presentations (Price 1977; Fillingim et al. 1998; Maixner et al. 1998). Five parameters of the procedure used to assess temporal summation
magnitude were altered, including (1) repetitions of the nociceptive stimulus (number of times the tail was placed in warm water), (2) the duration of the nociceptive stimulus (length of time in which the tail was placed in warm water), (3) interval between nociceptive stimulus presentations (time between when the tail was removed from the warm water and then placed back in warm water), (4) intensity of the nociceptive stimulus (water temperature), and (5) test time (time between the final presentation of the nociceptive stimulus and when the test for temporal summation was conducted). Each group of animals (n=7-8 males/females) was generally used for 8-10 tests (one parametric manipulation), with each test separated by a minimum of 48 hrs.

Data from studies using human subjects indicated that the optimal time between nociceptive stimulus presentations is 3.0 secs (Price et al. 1977), therefore the majority of parametric tests were conducted using this time interval (inter-stimulus interval, ISI). Preliminary tests also indicated that after approximately eight presentations of the nociceptive stimulus, the rats failed to leave their tails in the water for a stimulus duration of 3.0 secs, thus limiting both the duration of exposure to the nociceptive stimulus and the frequency at which it could be presented in this model. Due to these constraints, much of the testing was conducting using eight presentations of the nociceptive stimulus, a 3.0-s nociceptive stimulus and a 3.0-s ISI. The specific parameters used and testing conditions are indicated in Table 1.

Drug testing: NMDA antagonists and Opioids

For drug testing, the stimulus presentation number was set at 8 with a 1.0-sec exposure to the warm water stimulus, a 3.0-sec ISI, and tests of temporal summation conducted 3-secs after
the final nociceptive stimulus presentation. All drug tests were conducted following a 30 min pretreatment interval with an i.p. administration of saline or selected doses of the following drugs: the NMDA antagonists ketamine (3.0-30 mg/kg), dizocilpine (0.03-0.1 mg/kg), and dextromethorphan (10-30 mg/kg), the µ opioids morphine (3.0-10 mg/kg) and buprenorphine (0.3-3.0 mg/kg), the mixed-action opioid butorphanol (3.0-30 mg/kg) and the κ opioid spiradoline (10-30 mg/kg). All drug doses were tested at 51ºC water, whereas the highest doses of the NMDA antagonists were also tested at 49ºC water. These parameters were selected as they produced both low (49ºC) and high (51ºC) levels of temporal summation. For all tests, each group of rats (n=6-8) was tested with a single drug, and at least 6 days separated each test.

Data analysis

Latencies to tail-withdrawal following the temporal summation procedure were converted to percent of the baseline tail-withdrawal latency using the following equation: % of Baseline = \(((\text{observed} - \text{baseline}) \times \text{baseline} \times 100) + 100\). For this analysis, 100% of baseline indicated that the tail-withdrawal latency following the temporal summation procedure was equal to baseline latency prior to initiation of the temporal summation procedure. Less than 100% of baseline represented a decrease in tail-withdrawal latency, or hyperalgesia. Mixed model analyses of variance (ANOVA) were conducted to determine the influence of the various parameter manipulations and sex. Baseline tail-withdrawal latencies were also analyzed using a repeated measures ANOVA with sex as the between groups factor and temperature as the repeated measures factor.
For determining the antinociceptive effect of drugs, baseline tail-withdrawal latencies following administration of drug were converted to a percentage of antinociception using the following equation: % antinociceptive effect = [(observed drug baseline – saline baseline)/(15s-saline baseline)] x 100. To determine the antihyperalgesic effective of drugs, tail-withdrawal latencies following administration of drug were converted to a percent antihyperalgesic effect using the following equation: % antihyperalgesia = [(temporal summation level with drug – saline temporal summation level) / (saline temporal summation baseline – saline temporal summation level)] x 100. For NMDA antagonists an ANOVA was conducted with sex, drug and temperature as factors to determine their influence on temporal summation. For all tests, the α level was set at 0.05. For opioids, the dose of each drug required to produce a 50% (ED\(_{50}\)) maximal antinociceptive effect, 50% (ED\(_{50}\)) antihyperalgesic effect (the dose of drug that increased temporal summation tail-withdrawal latency by 50%) and the 95% confidence limits were derived mathematically (least-squares method) using log linear interpolation with at least three doses on the ascending limb of the dose–effect curve. Relative potency estimates were calculated in a manner described by Tallarida and Murray (1987), in which the relative potency of each opioid tested in male rats was compared to their female counterparts. Differences between sexes in the relative potency of opioids were considered to be significant if the 95% confidence interval did not overlap 1.0.

**Drugs**

The following drugs were examined: ketamine HCl (Abbott Laboratories, North Chicago, IL), dizocilpine hydrogen maleate, dextromethorphan hydrobromide monohydrate,
spiradoline mesylate, butorphanol tartrate (all purchased from Sigma-Aldrich, St Louis MO),
morphine sulfate, and buprenorphine HCl (both provided by the National Institute on Drug
Abuse, Bethesda, MD). All drugs were dissolved in distilled water and administered i.p. in an
injection volume of 0.5–1.0 ml/kg. Doses of drugs are expressed in terms of salts.

**Results**

*Baseline latencies*

Prior to testing in the temporal summation procedure, baseline tail-withdrawal latencies were recorded across a range of nociceptive stimulus intensities (water temperature) in the warm water tail-withdrawal procedure. In both male and female rats baseline tail-withdrawal latencies decreased with increases in the nociceptive stimulus intensity (Table 1). At almost all intensities tested, tail-withdrawal latencies were longer in males than their female counterparts. Analyses confirmed a main effect for both nociceptive stimulus intensity ($F_{5,60}=34.8$, $P<0.05$) and sex ($F_{1,12}=8.96$, $P<0.05$).

*Temporal Summation: Nociceptive stimulus intensity*

Figure 1 shows the level of temporal summation in males and females at nociceptive stimulus intensities ranging from 45 to 52°C. Data illustrated in this and other figures reflect a representative subset of the data: in almost all instances, parametric tests were conducted using various nociceptive stimulus presentations, ISIs, nociceptive stimulus durations and nociceptive stimulus intensities (A complete list of all testing conditions are shown in Table 1.) Data portrayed in this and other figures are generally consistent with the findings obtained across the parameters tested. Unless otherwise noted (see specifics of each panel
and each figure), data displayed in these panels, as well as for other figures, are for tests conducted using 51°C water, 8 nociceptive stimulus presentations, 3.0 sec ISI, 3.0 sec duration of the nociceptive stimulus, with tests for temporal summation conducted 3.0 secs following the final presentation of the nociceptive stimulus. In this figure, increases in the level of temporal summation are represented by a decrease in the percentage of baseline latency. In both males and females, the level of temporal summation (i.e., hyperalgesia) increased with increases in the nociceptive stimulus intensity. In general, the magnitude of this effect was larger in males than females. At the lowest nociceptive stimulus intensity tested (45°C), there was little evidence of temporal summation, whereas the largest effects were obtained at the higher nociceptive stimulus intensities (49-52°C). Analyses of these data confirmed a main effect for nociceptive stimulus intensity ($F_{5,60}=41.3$, $P<0.05$), a main effect for sex ($F_{1,12}=11.1$, $P<0.05$) and a nociceptive stimulus intensity x sex interaction ($F_{5,60}=2.84$, $P<0.05$).

Comparisons were also made on the basis of absolute tail-withdrawal latencies in males and females (data not shown). Although this analysis identified a main effect for nociceptive stimulus intensity ($F_{5,60}=89.3$, $P<0.05$), there was no main effect for sex or a nociceptive stimulus intensity x sex interaction. Thus, sex differences in temporal summation were apparent when the data were expressed as a percent of baseline but not as absolute latencies. Such finding suggested that sex differences in baseline latencies may contribute to the differential development of temporal summation and thus the interpretation of all parametric manipulations and drug tests (see below).

Temporal Summation: Nociceptive presentation number, inter-stimulus interval (ISI) and nociceptive stimulus duration The level of temporal summation varied as a function of
the number of presentations of the nociceptive stimulus with temporal summation evident at each presentation number (Fig. 2A). The smallest effect was observed when the nociceptive stimulus was presented a single time and the largest at presentations of 8 and 12. Similar findings were obtained at the other nociceptive stimulus intensities tested as well as at the other testing parameters. Across the different presentation numbers and nociceptive stimulus intensities tested, the magnitude of temporal summation was generally greater in males than females. Analyses across all tests confirmed a main effect for number of stimulus presentations (F_{3,55}=44.8, P<0.05), sex (F_{1,55}=30.1, P<0.05) and nociceptive stimulus intensity (F_{3,165}=4.0, P<0.05), whereas there was no stimulus presentation x sex interaction.

In both males and females, temporal summation was observed at each of the ISIs, with the 3.0 sec interval producing the largest effect and the 12 and 24 sec intervals the smallest effect (Fig. 2B). The magnitude of temporal summation was generally greater in males than in females, but this sex difference was not significant. Analyses of all tests conducted with the ISIs confirmed a main effect for ISI (F_{3,52}=44.34, P<0.05), but not for sex or ISI x sex interaction.

Temporal summation was apparent at each of the nociceptive stimulus durations tested with the magnitude of this effect being slightly larger in males than females (Fig. 2C). The level of temporal summation did not systematically increase or decrease with increases in the nociceptive stimulus duration, but rather was characterized by a U-shaped function. Analyses confirmed a main effect for duration of the nociceptive stimulus (F_{2,40}=19.3, P<0.05), sex (F_{1,40}=24.9, P<0.05), nociceptive stimulus intensity (F_{3,120}=5.37, P<.05) as well as a duration of the nociceptive stimulus x sex interaction (F_{2,40}=4.0, P<0.05).
**Temporal summation: Duration**

The duration of temporal summation under conditions in which tests were conducted at intervals ranging from 3.0 to 60 secs after the final presentation of the nociceptive stimulus was tested. The level of temporal summation systematically declined with increases in duration of the test interval (Fig.3). Temporal summation was evident at the 3.0 sec test time, only slightly below baseline at the 15 sec test time, and back to pre-temporal summation baseline levels at the 30, or 60 sec test times. Analyses of these data confirmed a main effect for sex ($F_{1,56}=5.09$, $P<0.05$) and test time ($F_{3,56}=21.13$, $P<0.05$), but no ISI x time of temporal summation test interaction.

**Temporal Summation: Allodynia**

A comparison of the level of temporal summation in males and females using a non-noxious (45ºC) and a noxious (51ºC) intensity was made to determine if heightened sensitivity to a non-noxious stimulus would develop. Following 8 presentations of the 51ºC water, high levels of temporal summation were observed in the 51ºC test, but temporal summation was not evident in the test with non-noxious, 45ºC, stimulus (Fig. 4). Analysis indicated a main effect of nociceptive only for stimulus intensity ($F_{1,14}=211.3$, $P<0.05$), sex ($F_{1,14}=13.9$, $P<0.05$) and a sex x temperature interaction ($F_{1,14}=13.9$, $P<0.05$). Thus, repeated presentations of a noxious stimulus induced temporal summation of nociception but not allodynia.

**Temporal Summation: Gonadal hormone depletion**

For intact and gonadectomized females, temporal summation levels increased with increasing nociceptive stimulus intensities ($F_{5,60}=56.3$, $P<0.05$) (Fig. 5). The level of
temporal summation was greater in gonadectomized females than their intact counterparts \((F_{1.12}=19.0, \ P<0.05)\) and there was an interaction of treatment condition with nociceptive stimulus intensity \((F_{5.60}=6.1, \ P<0.05)\). For both intact and gonadectomized males, temporal summation levels increased with increasing nociceptive stimulus intensities \((F_{5.65}=93.4, \ P<0.05)\), but there was no treatment condition x temperature interaction \((F_{5.65}=0.92, \ P>0.05)\). The level of temporal summation was higher in gonadectomized males than their intact counterparts \((F_{1.13}=8.9, \ P<0.05)\), although the magnitude of this effect was extremely small and not observed at all the nociceptive stimulus intensities.

**NMDA Antagonists**

To determine if the NMDA antagonists ketamine, dizocilpine and dextromethorphan produced antinociceptive effects, these drugs were tested in the warm water tail-withdrawal procedure (baseline conditions for the temporal summation procedure) using \(51^\circ\)C water. Across the doses examined, these NMDA antagonists failed to produce greater than a 9\% antinociceptive effect (data not shown) in either males or females.

Low doses of these compounds produced only small changes in the level of antihyperalgesia (data not shown), whereas the higher doses (Fig. 6) reduced the level of temporal summation with the magnitude of this effect being consistently larger at the 49 then \(51^\circ\)C water and slightly greater in females. At no dose or water temperature tested was temporal summation eliminated in either the males or females. Analyses confirmed a main effect of the drugs on the level of temporal summation \((F_{3.105}=903.2, \ P<0.05)\), a main effect of nociceptive stimulus intensity \((F_{1.105}=35.3, \ P<0.05)\) and a main effect of sex \((F_{1.105}=6.0, \ P<0.05)\). Note that at the \(49^\circ\)C water, baseline tail-withdrawal latencies approached the cut-
off (15 sec) imposed in this procedure, and thus tests of antinociceptive effects of the various NMDA antagonists could not be evaluated.

**Opioids**

Morphine, buprenorphine, butorphanol and spiradoline produced dose-dependent increases in antinociception in the warm water tail-withdrawal (51°C) procedure, with the highest doses tested producing maximal or near maximal effects (Fig. 7). The ED$_{50}$ values (95% confidence limits) for these opioids in the warm water tail-withdrawal procedure were generally larger in males than females, with an analysis of potency ratios indicating the less efficacious opioids buprenorphine and butorphanol were more potent (P<0.05) in males than females (Table 3).

Similarly, in the temporal summation procedure these opioids produced a dose-dependent antihyperalgesic effect, with the highest dose tested generally producing tail-withdrawal latencies comparable to baseline levels (Fig. 8). That is, these opioids completely reversed the hyperalgesia produced by the temporal summation procedure. These opioids were equally efficacious in males and females, and there were no sex differences (P<0.05) in opioid potency in the temporal summation procedure (Table 3).

Comparison between males and females generally indicated sex differences in opioid sensitivity that was dependent upon the nociceptive procedure and type of opioid tested. In males, morphine, buprenorphine and butorphanol were more potent in producing antinociceptive effects than antihyperalgesic effects. The opposite effect was obtained in females, as these opioids were more potent in producing antihyperalgesic effects. In both males and females, the κ opioid spiradoline was more potent the antinociception procedure.
Discussion

As observed in humans, temporal summation in rats was rapidly established and demonstrated to be sensitive to changes in the frequency, intensity, ISI, and time of testing following the final presentation of the nociceptive stimulus. Across the range of parameters tested, the magnitude of the temporal summation was slightly greater in males than females. Moreover, temporal summation appeared to be modulated, in part, by the NMDA system, as evidenced by the ability of NMDA receptor antagonists to attenuate its development. Various opioids reduced the development of temporal summation, but there was no sex difference in either their potency or effectiveness.

A critical variable in the development of temporal summation in humans is the ISI, with temporal summation being most evident when the nociceptive stimulus is presented with intervals of less than 3.0 secs (Price 1977; Vierck et al. 1997; Staud et al. 2001; Sarlini and Greenspan 2002). The intervals most and least effective in producing temporal summation have been shown to correspond closely with those observed in assays of wind-up (Herrero et al. 2000), lending support to a relationship between temporal summation and wind-up. Consistent with these observations is the present finding that an ISI interval of 3.0 secs produced the highest level of temporal summation in rats. Although temporal summation was still apparent at an ISI of 24 secs, the magnitude of this effect was extremely small.

Temporal summation in the rat was also sensitive to the number of presentations of the nociceptive stimulus, with the smallest effect obtained following a single presentation of the nociceptive stimulus and the largest following 4-12 presentations. Similarly, enhanced perception of pain with progressive presentations of the nociceptive stimulus is also a
characteristic of temporal summation in humans with maximal levels of temporal summation typically reached after 6-30 repetitions (Vierck et al. 1997; Fillingim et al. 1998; Edwards and Fillingim 2001).

The intensity of the nociceptive stimulus also had a significant effect on the development of temporal summation, with increases in water temperature producing increases in the magnitude of the temporal summation. At the lowest temperature tested, 45°C, temporal summation was not observed, a finding consistent with this temperature being a non-noxious stimulus (Falcon et al. 1996) and studies reporting the absence of temporal summation following presentations of non-noxious stimuli (Vierck et al. 1997; Edwards and Fillingim 2001). There are numerous studies indicating that in humans the level of temporal summation increases across thermal nociceptive stimulus intensities ranging from 45-53°C (Vierck et al. 1997; Edwards and Fillingim 2001).

Previous investigations indicate that temporal summation established using a thermal stimulus can produce after-sensations, such as a burning or hot feeling, that can persist for approximately 15 secs in healthy controls and for 2 mins in patients with fibromyalgia (Staud et al. 2001). Pain ratings are also elevated in patients with fibromyalgia 60 secs after the establishment of temporal summation (Staud et al. 2003). Although subjective assessments of pain cannot be made in rats, tests of nociceptive sensitivity were conducted at varying intervals following the final presentation of the nociceptive stimulus. As observed in humans, the level of temporal summation in rats decayed after 3.0 secs with a return to baseline by 30 secs. Collectively, these findings demonstrate a strong similarity between humans and non-humans in the factors that modulate temporal summation, including ISI, nociceptive stimulus...
intensity, number of presentations of the nociceptive stimulus and duration of nociceptive sensitivity.

Across various parametric manipulations, male rats exhibited a slightly greater level of temporal summation than females, at least when analyses took into account sex differences in baseline latencies. These findings are somewhat surprising considering that human females exhibit higher levels of temporal summation than males (Fillingim et al. 1998; Sarlini and Greenspan 2002). It has been suggested that these higher levels in females reflect enhanced central hyperexcitability of nociceptive processing which, in turn, may predispose females to the development of certain chronic pain conditions. Indeed, the prevalence of fibromyalgia, migraines and other chronic pain conditions are higher in females (Unruh 1996). Female rodents are also more likely to develop certain persistent pain conditions, such as neuropathic pain following partial sciatic nerve ligation and autoimmune diseases (Coyle et al. 1995; Verthelyi et al. 2001). Although there are a number of procedural differences in the temporal summation procedure used in humans and rats, one critical difference is that humans are required to give a verbal pain rating in response to the nociceptive stimulus (Arendt-Neilsen et al. 1995; Graven-Nielsen et al. 2000; Staud et al. 2001; Price et al. 2002) and it is well established that pain has a prominent, sex-dependent emotional component (Riley et al. 2000). In fact, Robinson et al. (2004) recently reported that sex differences in temporal summation in humans are influenced by sex differences in anxiety levels and gender role stereotypes. Importantly, when these factors are taken into account, sex does not function as a predictor of levels of temporal summation.

In contrast to studies conducted in humans, in the present study sensitivity to the nociceptive stimulus in rats was inferred from a decrease in the tail-withdrawal latency from
warm water. Moreover, testing was initiated only after an extensive habituation procedure, thus minimizing potential sex differences in stress or emotional responsiveness (Dhabhar et al. 1997). It is important to note that while temporal summation in humans is considered to be a correlate of wind-up, at this time there has been no exploration of sex differences in wind-up therefore comparison of sex difference in this model and wind-up are not possible.

Receptors for sex hormones are widely distributed throughout the CNS and are known to have an effect on nociceptive pathways. For example, gonadal hormones can affect nociceptive sensitivity in models of both acute and chronic nociception using chemical, thermal and mechanical stimuli (Fry et al. 1993; Gaumond et al. 2002; Aloisi 2003; Barrett et al. 2003; Terner et al. 2003; Vinogradova et al. 2003). Although this issue has not been specifically addressed in humans, there is evidence that the elderly display a greater level of temporal summation than younger individuals, thus there is the possibility that depletion of gonadal hormones associated with aging may contribute to this phenomenon (Edwards and Fillingim 2001; Staud et al. 2001). In the present investigation, the level of temporal summation in gonadectomized females was greater than those in intact females. In contrast, gonadectomy had minimal effects on temporal summation in males, a finding suggesting that male gonadal hormones may not be directly involved in the development of temporal summation. The reasons for these differential effects of hormones remain unclear.

Wind-up and temporal summation are both models used to investigate the central mechanisms thought to underlie the pathophysiology of chronic pain. Attenuation of wind-up and temporal summation of pain in humans by the administration of NMDA antagonists has been used to suggest the contribution of the NMDA system and its central involvement (Dickenson and Sullivan 1987; Price et al. 1994; Arendt-Nielsen et al. 1995; Graven-Neilson
et al. 2000; Guirimand et al. 2000). In the present investigation, administration of various non-competitive NMDA antagonists decreased the level of temporal summation in a generally dose-dependent manner. This effect was observed in both males and females at doses of the NMDA antagonists that failed to produce an antinociceptive effect. The magnitude of this effect appears comparable in both humans and rats, with complete blockade of temporal summation not obtained under any of the conditions tested. Although the adverse side effects of NMDA antagonists typically limit the testing of high doses in humans, even the relatively high doses tested in rats failed to completely block the development of temporal summation. Whereas these findings provide evidence that the NMDA system plays a modulatory role in the development of temporal summation, they also suggest that other systems are involved in this process.

In humans, the opioids morphine, codeine and pentazocine have been reported to be effective in reducing the development of temporal summation, with pentazocine being equally potent in both males and females (Price et al. 1985; Enggaard et al. 2001; Fillingim et al. 2004). Similarly, in the present investigation the µ opioids morphine and buprenorphine, the mixed-action opioid butorphanol and the κ opioid spiradoline were effective in reducing temporal summation. Moreover, each of these opioids were equally potent in males and females. Temporal summation is thought to reflect the development of central sensitization and thus is considered a model of some types of chronic pain (e.g., Ren 1994; Li et al. 1999). As such, the present findings extend a recent report indicating the absence of sex differences in opioid sensitivity in model of chronic pain. Indeed, in rats morphine, buprenorphine and dezocine were found to be equally potent and effective at reducing the hyperalgesia induced by the administration of capsaicin (Barrett et al. 2003). Presently, it is unclear as to whether
the temporal summation procedure and the capsaicin-induced hyperalgesia can be considered models of long-term pain, as in rats the duration of the hyperalgesic effect is relatively short-lived.

In contrast to the findings obtained in the temporal summation procedure, in the warm water tail-withdrawal procedure, a model of acute pain, both buprenorphine and butorphanol were more potent in producing antinociception in males. Such effects were not consistently observed with the µ opioid morphine or the κ opioid spiradoline. These findings extend a large body of evidence indicating that in non-humans, males are generally more sensitive than their female counterparts to the antinociceptive effects of opioids in models of acute pain and that these sex differences are most evident with less efficacious opioids (Negus and Mello 1999; Cook et al. 2000; Craft and Bernal 2001; Terner et al. 2003). Such findings provide strong support for the idea that different mechanisms underlie sex differences in opioid sensitivity in acute and chronic models of pain.

The present investigation describes a simple method of establishing temporal summation in rats. In addition to demonstrating the generality of temporal summation across species, the similarities of temporal summation in humans and rats allow for number of potential applications of this model. Of particular importance is that this model can be adapted to screen novel pharmacological treatments for chronic pain and to identify the factors that modulate the effectiveness of different treatment approaches. For example, the present findings identified a potential role for sex in determining the potency of opioids in the treatment of chronic pain, as analyses indicated that in males the µ opioids tested were generally more potent in producing antihyperalgesia than antinociception, whereas in females the µ opioids were generally more potent in producing antinociception.
This model can also be used to explore the mechanisms underlying the development of some chronic pain conditions as well as the factors that can modulate these conditions (e.g., age, sex, health status). For example, in the present study the effectiveness of NMDA antagonists was dependent upon nociceptive stimulus intensity, with greater levels of attenuation observed at a relatively low nociceptive stimulus intensity. Such findings may help explain the varying levels attenuation produced by NMDA antagonists observed in studies of human temporal summation, and suggest that high nociceptive stimulus intensities may require activation of NMDA and non-NMDA systems. Additionally, whereas NMDA antagonists have played a key role in establishing the importance of the NMDA system in modulating chronic pain models, these compounds are relatively non-selective and thus this model of temporal summation may allow identification of the critical NMDA receptor subtypes (e.g., see Kovacs et al. 2004).
Table 1. Baseline tail withdrawal latencies (standard error) expressed in seconds for male and female rats (N = 7-8) in the warm water tail-withdrawal procedure.

<table>
<thead>
<tr>
<th>Warm Water Temperature</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>45°C</td>
<td>15.00 (0.0)</td>
<td>14.47 (0.53)</td>
</tr>
<tr>
<td>47°C</td>
<td>13.56 (0.56)</td>
<td>11.87 (1.03)</td>
</tr>
<tr>
<td>49°C</td>
<td>13.24 (0.87)</td>
<td>10.85 (0.91)</td>
</tr>
<tr>
<td>50°C</td>
<td>10.67 (1.1)</td>
<td>8.50 (0.54)</td>
</tr>
<tr>
<td>51°C</td>
<td>10.00 (0.83)</td>
<td>6.72 (0.57)</td>
</tr>
<tr>
<td>52°C</td>
<td>8.00 (0.87)</td>
<td>6.34 (0.55)</td>
</tr>
</tbody>
</table>
Table 2. Testing conditions for the temporal summation procedure

<table>
<thead>
<tr>
<th>Testing Condition</th>
<th>Number of Stimulus Presentations&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inter-Stimulus Interval&lt;sup&gt;c&lt;/sup&gt; (sec)</th>
<th>Stimulus Duration&lt;sup&gt;d&lt;/sup&gt; (sec)</th>
<th>Test Interval&lt;sup&gt;e&lt;/sup&gt; (sec)</th>
<th>Stimulus Intensity&lt;sup&gt;a&lt;/sup&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulus Intensity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>45,47,49,50,51,52</td>
</tr>
<tr>
<td>Number of Stimulus Presentations&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1,4,8,12</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>49,50,51,52</td>
</tr>
<tr>
<td>Inter-Stimulus Interval&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
<td>3,6,12,24</td>
<td>3</td>
<td>3</td>
<td>49,50,51,52</td>
</tr>
<tr>
<td>Stimulus Duration&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8</td>
<td>3</td>
<td>0.5,1,3</td>
<td>3</td>
<td>49,50,51,52</td>
</tr>
<tr>
<td>Test Interval&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>3,15,30,60</td>
<td>51</td>
</tr>
<tr>
<td>NMDA Antagonist</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>49,51</td>
</tr>
<tr>
<td>Opioids</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>51</td>
</tr>
</tbody>
</table>

<sup>a</sup> Water temperature
<sup>b</sup> Number of times the tail was placed in warm water
<sup>c</sup> Time between when the tail was removed from the warm water and placed back in warm water
<sup>d</sup> Length of time in which the tail was placed in warm water
<sup>e</sup> Time between the final presentation of the nociceptive stimulus and when the test for temporal summation was conducted
Table 3. ED<sub>50</sub> (mg/kg) values (95% confidence limits) for male and female rats (n=6-8) in tests of antinociception (warm water tail-withdrawal) and antihyperalgesia (temporal summation procedure) conducted with morphine, buprenorphine, butorphanol and spiradoline.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antinociception</th>
<th>Antihyperalgesia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4.58 (3.43-6.12)</td>
<td>5.19 (4.10-6.58)</td>
</tr>
<tr>
<td>Female</td>
<td>5.90 (5.06-6.90)</td>
<td>5.50 (4.43-6.85)</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.01 (0.000012-15.19)*</td>
<td>0.22 (0.04-1.23)</td>
</tr>
<tr>
<td>Male</td>
<td>0.6 (0.36-0.99)</td>
<td>0.55 (0.35-0.87)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butorphanol</td>
<td>5.84 (1.92-17.75)*</td>
<td>9.91 (4.69-20.92)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiradoline</td>
<td>24.07 (17.91-32.35)</td>
<td>14.47 (10.13-20.69)</td>
</tr>
<tr>
<td>Male</td>
<td>31.82 (14.31-70.72)</td>
<td>18.31 (14.17-23.66)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference from females at the 0.05 level as determined by potency ratios
Figure 2.1 Tail-withdrawal latencies for male and female rats (7-8 animals per group) in the temporal summation procedure following repeated presentations of the warm water stimulus at temperatures ranging from 45 to 52°C. For all tests, the warm water stimulus was presented 8 times with each presentation lasting 3.0 secs and an inter-stimulus interval of 3.0 secs. The temporal summation test was conducted 3.0 secs after the final presentation of the warm water stimulus. All data are expressed as percent of baseline tail withdrawal latencies assessed prior to the start of the temporal summation procedure. A value of 100% indicates that the tail-withdrawal latency in the temporal summation procedure was comparable to that obtained prior to the repeated presentation of the warm water stimulus. Values of less than 100% indicate that repeated presentations of the warm water stimulus produced a decrease in the tail withdrawal latency indicative of hyperalgesia. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.2 Effects of various parametric manipulations on the magnitude of temporal summation in male and female rats (7-8 animals per group). All data are expressed as percent of baseline tail-withdrawal latencies assessed prior to the start of the temporal summation procedure. Unless indicated (see below), in all tests the warm water stimulus was presented 8 times with each presentation lasting 3.0 secs and an inter-stimulus interval of 3.0 secs. The temporal summation test was conducted 3.0 secs after the final presentation of the warm water stimulus. For all panels, vertical bars represent the standard error; where not indicated, the standard error fell with the data point. **Panel A:** Tail-withdrawal latency for the temporal summation procedure in which the number of presentations of the warm water ranged from 1 to 12. **Panel B:** Tail-withdrawal latency for the temporal summation procedure in which the interval between warm water stimulus presentations (inter-stimulus interval) ranged from 3.0 to 24 secs. **Panel C:** Tail-withdrawal latency for the temporal summation procedure in which the duration of exposure to the warm water stimulus ranged from 0.5 to 3.0 secs.
Figure 2.3 Tail-withdrawal latency in the temporal summation procedure in male and female rats (7-8 animals per group). Tests were conducted at intervals ranging from 3.0 to 60 secs after the final presentation of the warm water stimulus (51°C). For all tests, the warm water stimulus was presented 8 times with each presentation lasting 3.0 secs and an inter-stimulus interval of 3.0 secs. All data are expressed as percent of baseline tail withdrawal latencies assessed prior to the start of the temporal summation procedure. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.4 Effects of repeated presentation of a noxious stimulus (51°C water) on the development of temporal summation and allodynia in males and females rats (7-8 per group). For all tests, the 51°C warm water stimulus was presented 8 times with each presentation lasting 3.0 secs, and an inter-stimulus interval of 3.0 secs. The final presentation of the warm water stimulus was followed by the tail withdrawal latency test using either 45 (non-noxious) or 51°C warm water. For both males and females, a test temperature of 45°C produced tail-withdrawal latencies equal to that of baseline tail-withdrawal latencies. All data are expressed as percent of baseline tail-withdrawal latencies assessed prior to the temporal summation procedure. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.5 Effect of a sham operation (intact) and gonadectomy on the level of temporal summation in male and female rats (7-8 per group) using water temperatures ranging from 45-52°C. All data are expressed as percent of baseline tail-withdrawal latencies assessed prior to the temporal summation procedure for females (left panel) and males (right panel). For all tests, the warm water stimulus was presented 8 times with each presentation lasting 3.0 secs, and an inter-stimulus interval of 3.0 secs. The temporal summation tail-withdrawal latency test was conducted 3.0 secs after final presentation of the warm water stimulus. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.6 The effects of saline and the NMDA antagonists ketamine (30 mg/kg), dizocilpine (0.1 mg/kg) and dextromethorphan (30 mg/kg) on the level of temporal summation in male and female rats (6-8 per group). The temporal summation procedure for these tests was performed 30 mins following injection and consisted of 8 repetitions of the warm water stimulus (49 and 51°C), an inter-stimulus interval of 3.0 secs, using a 1.0 sec warm water stimulus duration, with the tail-withdrawal latency test conducted 3.0 secs following the final presentation of warm water stimulus. Saline data are expressed as percent of baseline tail-withdrawal latency taken 30 mins following the administration. For ease of comparison, drug data are expressed as percent of saline baseline tail-withdrawal latencies. Only data for the highest dose of ketamine, dizocilpine and dextromethorphan are illustrated in the figure: these drugs had no effect on baseline tail-withdrawal latencies. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.7 The effects of morphine, buprenorphine, butorphanol and spiradoline in male and females rats (6-8 per group) tested with a warm water, tail-withdrawal procedure at 51°C. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 2.8 The effects of morphine, buprenorphine, butorphanol and spiradoline in male and females rats on the level of temporal summation in male and female rats (6-8 per group). The temporal summation procedure for these tests was performed 30 mins following injection and consisted of 8 repetitions of the warm water stimulus (51°C), an inter-stimulus interval of 3.0 secs, using a 1.0 sec warm water stimulus duration, with the tail-withdrawal latency test conducted 3.0 secs following the final presentation of warm water stimulus. Data are expressed as percent antihyperalgesic effect, 100% antihyperalgesic effect reflects a temporal summation tail-withdrawal latency with drug equal to that of saline baseline tail-withdrawal latency. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
CHAPTER 3

EXPERIMENT 2: SEX DIFFERENCES IN THE POTENCY OF κ OPIOIDS AND MIXED-ACTION OPIOIDS ADMINISTERED SYSTENICALLY AND AT THE SITE OF INFLAMMATION IN A CAPSAICIN-INDUCED MODEL OF HYPERALGESIA

Introduction

There is now evidence that male and female rodents differ in their sensitivity to the antinociceptive effects of κ opioids (e.g., Craft and Bernal 2001; Holtman and Wala 2006). In a recent review and analysis, Craft (2003) reported that when collapsed across drugs, studies and species, 39% of the reports indicated that κ opioids were more potent in males, 50% equally potent in males and females, and 11% more potent in females. Both the direction and magnitude of these sex differences appear to be influenced by the dose, type of κ opioid, genotype, and type of pain model (for reviews see Craft 2003; Barrett 2006). In rats, for example, reports of κ opioids being more potent in males have appeared exclusively in studies utilizing acute pain models and are more likely to occur when complete dose-effect functions are determined. Although there are only two reports in which κ opioids were examined in persistent pain models, in these studies the κ opioids tested were either more potent in females or equally potent in males and females (Binder et al. 2000; Clemente et al. 2004).

Presently, it is unclear if these conflicting findings are a consequence of a sexually-dimorphic interaction of opioids with the distinct mechanisms underlying acute versus
persistent nociception. For example, persistent nociception requires activation of unmylenated C-fibers and acute nociception activation of large A\(\delta\) nociceptive fibers (e.g., LeBars et al. 2001). Acute and persistent nociception also differ in terms of their duration, neurochemical substrates, and sensitivity to pharmacological manipulation (LeBars et al. 2001; Richardson and Vasko 2002; Mogil et al. 2003). Thus, the primary goal of the present investigation was to evaluate sex differences in the effects of \(\kappa\) opioids and selected mixed-action opioids with low efficacy at the \(\kappa\) receptor in a persistent pain model. To this end, we conducted tests using the capsaicin-induced hyperalgesia procedure, which is a persistent pain model adapted to the study of sex differences in \(\mu\) opioid sensitivity (Barrett et al. 2003). One limitation of some persistent pain models is that it is often difficult to control the level of nociception, and baseline levels of nociception are known to be a determinant of sex differences in opioid sensitivity (Cook et al. 2000; Barrett et al. 2002). By altering the dose of capsaicin, however, it is possible to produce comparable levels of nociception in males and females (Barrett et al. 2003). Moreover, inflammation is localized to the tail in the capsaicin model and thus it is possible to examine sex differences in the effects of relatively small doses of \(\kappa\) opioids when their actions are directed at \(\kappa\) opioid receptors located at the site of inflammation. From a clinical perspective, a result of administering small doses locally is that it minimizes potential adverse effects, which is especially critical for \(\kappa\) opioids as adverse central effects limit their clinical utility (Kumor et al. 1986; Rimoy et al. 1991).

Although the mechanisms that mediate the sexual dimorphism in \(\kappa\) opioid sensitivity are unclear, several studies have implicated the NMDA receptor system. Indeed, NMDA antagonists attenuate \(\kappa\) opioid antinociception in male mice and rats but not intact females (Kavaliers and Choleris 1997; Mogil et al. 2003; Holtman and Wala 2006). Ovariectomized
female mice (Sternberg et al. 2004) display a similar sensitivity to NMDA antagonists as males, suggesting further that males and females have similar opioid/nociceptive circuitry, but in females the NMDA component of this circuitry is actively suppressed by the gonadal hormones.

To date, studies of NMDA involvement in sex differences of κ opioid antinociception have been conducted exclusively in models of acute pain and thus its contribution to persistent pain remains to be determined. Experiments of this nature can be complicated by the fact that NMDA antagonists have a direct effect on the development of persistent nociception induced by injections of formalin and Freund’s adjuvant, as well as in post-surgical nociception (Eisenberg et al. 1993; Przewlocki and Przewlocki 2001; Nishimura et al. 2004). Such effects are not observed following the administration of capsaicin (Sakurada et al. 1998), and thus this persistent pain model may provide an opportunity to evaluate the contribution of the NMDA system to sex differences with κ opioids. For comparison, tests were conducted in a 50°C warm water tail-withdrawal procedure as in this procedure NMDA antagonists antagonize the effects of κ opioids in a sexually dimorphic manner (Mogil et al. 2003; Holtman and Wala 2006).

Methods

Animals

Gonadally intact male and female F344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). F344 rats were chosen as previous studies in our lab utilizing this strain have shown large sex differences in opioid antinociception. All testing occurred
between 3 and 6 months of age, and rats were individually housed in a colony on a 12-h/12-h light/dark cycle. All rats had unlimited access to food and water.

**Apparatus and testing**

A warm-water tail withdrawal procedure was used to assess hyperalgesia. Before testing, rats were habituated to handling on one occasion. During testing, each rat was lightly restrained, with the distal 7 cm of the tail immersed in water maintained at 45°C. This temperature was selected as it approximates threshold nociceptive values in adult rats (Falcon et al. 1996). Baseline tail withdrawal latencies were determined in each rat before administration of any compounds, and rats that failed to maintain their tails in the water for a full 15 secs were excluded from testing. Subsequently, capsaicin was injected 3.5 cm from the tip of the tail. All injections of capsaicin were made under light halothane anesthesia, with rats recovering from this procedure within 2-3 mins. Following administration of capsaicin, tail-withdrawal latencies decreased from 15 sec to approximately 3.5 - 4.5 secs with this effect being consistent in both males and females. Rats were tested once per week with no more than 5 tests per animal. Previous studies conducted in our laboratory indicated that this frequency of testing and the number of tests produced reliable and consistent tail-withdrawal latencies (Barrett et al. 2003).

**Antihyperalgesic effects of opioids**

For tests examining the antihyperalgesic effects of opioids, a 3.0 and 1.0 µg dose of capsaicin was chosen for males and females, respectively, as these doses produce a comparable magnitude and duration of hyperalgesia (Barrett et al. 2003). Before initiating
testing of opioids, a capsaicin baseline was assessed in each animal. To determine the antihyperalgesic effects of opioids and to determine potency differences in local versus systemic administration, capsaicin was administered in the tail, and one dose of each opioid was injected systemically (s.c.) or locally in the tail. Tail-withdrawal latencies were determined 15 min after the capsaicin/opioid injection, which is the time point corresponding to the peak effect of capsaicin. Additional tests were conducted in which the non-competitive NMDA antagonist dextromethorphan was administered (s.c. or tail) 15 mins prior to capsaicin injection alone or in combination with U69,593 (s.c. or tail). For all tests, a 15 s cutoff latency was implemented, as this indicated a maximal antihyperalgesic effect (i.e., nociceptive thresholds returned to baseline levels). The doses of dextromethorphan selected for study are similar to those used in previous studies (e.g., Holtman et al. 2003; Craft and Lee 2005), as higher doses are known to alter locomotor activity as well as produce ataxia and stereotypies (Dansysz et al. 1994; Plesan et al. 1998; Redwine and Trujillo 2003).

**Antihyperalgesic activity of κ opioids at the site of inflammation**

To examine the local mediation of locally administered opioids, doses that produced high levels of antihyperalgesia in males and females were administered in the tail along with either local or i.c.v. administration of the selective κ antagonist nor-BNI. For tests with i.c.v administration of nor-BNI, nor-BNI was administered 24 hrs before capsaicin. For tests with local (in tail) administration, nor-BNI was administered 15 min before capsaicin. Due to the long duration of nor-BNI, testing for an individual rat was terminated after the completion of this test. In order to provide a comparison between the results obtained in this persistent pain model with those in an acute pain model, and to identify the dose of nor-BNI to be
administered i.c.v. in tests of local mediation of antihyperalgesia, additional tests were conducted using a warm water tail-withdrawal procedure. In this procedure, rats were placed in restraint tubes with the distal 7 cm of the tail immersed in 50°C water and latency to remove the tail measured (see Cook et al. 2000 for details). Tail withdrawal latencies were typically between 10 - 11 secs in both males and females. After baseline latencies were assessed, U69,593 was administered and the latency to remove the tail from the 50°C warm water was determined. A 15-s cut-off to tail-withdrawal was imposed to avoid tissue damage. For these tests, nor-BNI was administered i.c.v. 24 hrs before administration of U69,593.

Data analysis

For dose-effect curves examining the antihyperalgesic effects of opioids, latencies to tail-withdrawal following administration of the drug were converted to the percentage of the maximum possible effect using the following equation: % antihyperalgesic effect = [(observed-baseline)/(15 s-baseline)] x 100. When possible, the dose of each drug required to produce a 50% antihyperalgesic effect (ED\textsubscript{50}) was derived mathematically (least-squares method) using log-linear interpolation with at least three doses on the ascending limb of the dose-effect curve. For each opioid, an ANOVA was also conducted with sex and dose as between-groups factors. In instances in which there was a main effect for sex, post-hoc tests were conducted using the Fisher’s protected least significant difference test to assess the effect of sex on each dose of the opioid. For statistical analyses using ANOVA, the alpha level was set at 0.05. Relative potency estimates were also calculated by comparing the potency of s.c. administration of opioids with local administration of opioids within males and within females. For this analysis, dose ratios were calculated in a manner described by
(Tallarida and Murray 1987), in which a common slope was determined between linear regression lines representing the 2 dose-effect curves, and then the distance between the regression lines calculated. Differences in the relative potency of s.c. and tail administration of opioids were considered to be significant if the 95% confidence interval did not overlap 1.0 (by using a t test).

To calculate the % maximal antinociceptive effect, tail-withdrawal latencies for the 50°C warm water tail-withdrawal procedure were converted to percent antinociceptive effect using the following equation: % antinociceptive effect = [(test latency - baseline) / (15 s - baseline)] X 100. The time-course evaluation of a drug’s effect was measured in this assay with an ANOVA on area under the curve used for statistical analysis. Area under the curve was estimated by the Trapezoidal Rule using available statistical software (Tallarida and Murray 1987).

*Intracerebroventricular Injections*

Rats were stereotaxically implanted with a single 22-gauge cannula into the left lateral ventricle [anterior-posterior (AP) +0.9, medial-lateral (ML) +1.5, dorsal-ventral (DV) −3.2] under anesthesia induced with a 1 ml/kg injection of a 1:1 (v/v) mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Coordinates are expressed at millimeters from bregma (Paxinos and Watson 1986). Rats were given i.c.v. injections of nor-BNI in a 5 µl volume with a 28-gauge injector that protruded 1 mm beyond the tip of the cannula. The injector was connected by a length of tubing to a 10 µl Hamilton microsyringe.
Drugs

The following drugs were used: nort-binaltorphamine, trans-3,4-dichloro-N-methyl-N[2-(1-pyrolidinyl)cyclohexyl] benzeneaceamide methanesulfonate (U50,488) (all provided by the National Institute on Drug Abuse), dextromethorphan hydrobromide monohydrate, spiradoline mesylate, butorphanol tartrate, nalbuphine HCl, 1-oxaspiro[4,5]dec-8-yl benzeneacetamide (U69,593), and capsaicin (all purchased from Sigma-Aldrich Co., St. Louis, Mo.). Capsaicin was dissolved in a solution of Tween 80/95% ethanol/saline in a ratio of 1/1/8, and was diluted to lower concentrations with saline. For injection in the tail, capsaicin (0.1 ml volume when administered alone) was mixed in the same syringe as the test drugs in a 0.1 ml volume. For other tests, drugs were administered s.c. in a volume of 0.5-1.0 ml/kg.

Results

κ opioids

Fig. 1 shows that in both males and females the κ opioids spiradoline, U50,488 and U69,593 produced dose-dependent increases in antihyperalgesia, with maximal or near maximal effects obtained at the highest doses tested. As shown in Table 1, spiradoline was the most potent of the κ opioids in both males and females when administered locally, whereas U69,593 and U50,488 were approximately equally potent. In contrast, when administered systemically U50,488 was the most potent, whereas spiradoline and U69,593 were approximately equally potent. Table 2 shows that sex differences were observed in the potency of these κ opioids, with the antihyperalgesic potency of systemic and local administration of spiradoline and U50,488 being greater in males. Analyses based on
ANOVA for spiradoline confirmed these observations, indicating a main effect for sex (systemic: $F_{1,12}=10.3$, $P<0.05$; local: $F_{1,12}=4.7$, $P<0.05$) and dose (systemic: $F_{1,24}=23.9$, $P<0.05$; local: $F_{1,24}=39.5$, $P<0.05$), but no dose x sex interaction. Similarly, a main effect for dose (systemic: $F_{2,24}=61.7$, $P<0.05$; local: $F_{2,26}=27.2$, $P<0.05$) and sex (systemic: $F_{1,12}=55.8$, $P<0.05$; local: $F_{1,13}=4.5$, $P<0.05$) was observed for U50,488. A significant dose x sex interaction was observed for systemic ($F_{2,24}=20.2$, $P<0.05$) but not local, administration of U50,488. In contrast to spiradoline and U50,488, sex differences with U69,593 were observed only following local administration ($F_{1,13}=5.2$, $P<0.05$). Analyses also revealed a main effect of dose ($F_{2,26}=156.4$, $P<0.05$) and a dose x sex interaction ($F_{2,26}=3.4$, $P<0.05$). Although there was no main effect for sex with systemic administration of U69,593 or a dose x sex interaction, there was a main effect for dose ($F_{2,12}=45.5$, $P<0.05$).

Antihyperalgesic activity of κ opioids at the site of inflammation

To determine the role of κ opioid receptors at the site of inflammation in mediating the antihyperalgesic effect of κ opioids, U69,593 was administered locally in combination with both local and i.c.v. administration of the κ antagonist nor-BNI. As shown in panels “a” and “b” of Fig. 2, the effects of U69,593 were attenuated by local but not i.c.v. administration of nor-BNI, suggesting that the effects of U69,593 were mediated by κ opioid receptors at the site of inflammation. ANOVAs indicated a main effect for treatment ($F_{2,20}=61.6$, $P<0.05$) with post hoc analyses confirming attenuation by local ($P<0.05$) but not by i.c.v. administration. There was, however, no main effect for sex or a sex x condition interaction.

For comparison, and to determine if the dose of nor-BNI selected for i.c.v. administration was appropriate, similar tests were conducted in a 50°C warm water tail-
withdrawal procedure. In this procedure, systemic administration of opioids is known to produce antinociception via central and/or spinal sites. In order to equate the antinociceptive effects produced by U69,593, a higher dose was used in females (5.6 mg/kg) than males (3.0 mg/kg). In this procedure, systemic administration of U69,593 produced a time-dependent antinociceptive response, with a peak effect observed 30 to 45 minutes after administration (Fig. 2, Panels “c” and “d”). Although complete dose-response testing was not conducted, with this higher dose the peak effect of U69,593 was slightly smaller in the females. In this procedure, local administration (i.e., in the tail) of U69,593 failed to produce an antinociceptive effect in either males or females. Administration of nor-BNI via the i.c.v. route completely attenuated the effects of U69,593, suggesting that in this procedure the effects of U69,593 were mediated by spinal or supraspinal κ opioids receptors. ANOVA indicated a main effect for treatment ($F_{2,26}=27.1, P<0.05$) with post hoc analyses confirming attenuation ($P<0.05$) by i.c.v. administration of nor-BNI. Post hoc analyses also confirmed a difference ($P<0.05$) in the antinociceptive effects of systemic and local administration of U69,593 in the tail withdrawal procedure. No main effect for sex or a sex x condition interaction was found.

Fig. 3 shows that in the capsaicin preparation, local administration of nor-BNI attenuated the antihyperalgesic effects of spiradoline in both males and females. In contrast, nor-BNI failed to attenuate the effects produced by U50,488. ANOVA indicated a main effect of drug condition for spiradoline ($F_{1,23}=22.5, P<0.05$) but no main effect for sex or a sex x condition interaction.
Mixed-action opioids

Fig. 4 shows that the mixed-action opioids butorphanol and nalbuphine produced dose-dependent increases in antihyperalgesia, with maximal or near maximal effects obtained at the highest dose tested. In both males and females, nalbuphine was more potent when administered locally, whereas butorphanol was more potent when administered locally in males but not in females (Table 2). ANOVA analyses indicated a main effect of dose for both drugs (butorphanol: systemic: $F_{3,33}=22.8$, $P<0.05$, local: $F_{3,39}=42.8$, $P<0.05$; nalbuphine: systemic: $F_{2,24}=27.3$, $P<0.05$, local: $F_{2,26}=212$, $P<0.05$), but no effect of sex or dose x sex interaction. Thus, in contrast to the κ opioids tested, there were no sex differences in the antihyperalgesic potency of either nalbuphine or butorphanol (see Table 1).

Fig. 5 shows that local administration of nor-BNI attenuated the antihyperalgesic effects of nalbuphine in both males and females, but failed to attenuate the effects produced by butorphanol. ANOVA indicated a main effect for drug condition only for nalbuphine ($F_{1,23}=660$, $P<0.05$), and no main effect for sex or a sex x condition interaction. Although nor-BNI failed to attenuate the effects of butorphanol there was a sex x condition interaction ($F_{1,36}=5.37$, $P<0.05$).

NMBA modulation of κ opioid antihyperalgesia

To evaluate the sexually-dimorphic role of the NMDA system in modulating κ opioid antihyperalgesia, the NMDA antagonist dextromethorphan was administered in combination with both local and systemic administration of U69,593. Alone, dextromethorphan (3.0 - 30 mg/kg) had no antihyperalgesic effects (data not shown). However, at the higher does tested there was a clear disruption of motor coordination and sedation. As shown in Fig. 6, in both
sexes systemic administration of dextromethorphan produced a dose-dependent attenuation of the antihyperalgesic effect of systemic administration of U69,593, with almost complete attenuation observed at the highest dose of dextromethorphan tested. ANOVA confirmed a main effect for dose \( (F_{3,46}=39.33, P<0.05) \), but no main effect for sex or a sex x dose interaction. Post hoc analysis indicated a significant \( (P<0.05) \) difference between U69,593 alone and the combination of U69,593 with 3.0 and 30 mg/kg dextromethorphan. In comparison, as shown in Fig. 7, dextromethorphan administered systemically produced a small attenuation (less than 30%) of the antihyperalgesia produced by local administration of U69,593 in both sexes. Local administration of dextromethorphan also failed to attenuate the antihyperalgesia produced by local U69,593 administration in both males and females. ANOVA analysis indicated a main effect for treatment \( (F_{2,34}=3.29, P<0.05) \), but no main effect for sex or a sex x treatment interaction. Post hoc analysis confirmed the combination of dextromethorphan administered systemically and U69,593 locally was different \( (P<0.05) \) than U69,593 alone.

Fig. 8 shows attenuation of the antihyperalgesic effects of both spiradoline and U50,488 in males and females by dextromethorphan. In these tests, the most effective dose of each opioid was combined with 30 mg/kg of dextromethorphan, with all drugs being administered systemically. ANOVA’s indicated a main effect for treatment with spiradoline alone and in combination with dextromethorphan, \( (F_{1,23}=6.87, P<0.05) \), but no main effect for sex or a sex x dose interaction. ANOVA also indicated a main effect for treatment for U50,488 alone or in combination with dextromethorphan \( (F_{1,23}=1.02, P<0.05) \), but no main effect for sex. There was a sex x treatment interaction \( (F_{1,23}=14.737, P<0.05) \).
For comparison, tests were conducted in the 50°C warm water tail-withdrawal procedure with all drugs being administered systemically. As shown in Fig. 9, equally effective doses of U69,593 were combined with a dose of dextromethorphan that produced the largest reduction in antihyperalgesia in the capsaicin preparation. In these tests, U69,593 produced near maximal effects in both males and females. Dextromethorphan attenuated the antinociceptive effect produced by U69,593 only in males ($F_{1,12}=36.2$, $P<0.05$). Comparison of difference scores in the area under the curve analysis confirmed this sexually dimorphic action. Thus, dextromethorphan attenuated the effects of U69,593 in both males and females in the capsaicin preparation, but in the 50°C warm water tail-withdrawal procedure attenuation was observed only in males.

**Discussion**

Previous studies indicate that κ opioids generally produce greater antinociception in males compared to females, and this effect has been reported in rats, mice and monkeys (Craft 2003). Unfortunately, the available animal studies have almost exclusively utilized acute nociceptive models, and thus little is known about sex differences in the effectiveness of κ opioids in persistent pain models. Consequently, one purpose of the present investigation was to examine the antihyperalgesic effects of κ opioids following administration of capsaicin, which produces a short term (30-90 mins) hyperalgesic response to mildly noxious thermal stimuli. Across the range of doses tested, spiradoline and U50,488 were more potent in males than females, and this effect was observed following both systemic and local administration at the site of inflammation. U69,593 was also more potent in males, although this effect was observed only following local administration. To date, only
two published studies have examined sex differences in κ opioids using persistent pain models. Clemente et al. (2004) reported that local administration of U50,488 was more effective at reducing formalin-induced hyperalgesia in female rats, with the magnitude of this effect being greater during diestrous than proestrous. Similarly, Binder et al. (2000) reported that the peripherally active κ-opioid asimadoline was more effective in female rats at reducing Freunds-induced hyperalgesia when tested against a thermal stimulus. Sex differences were not observed in this study when asimadoline was tested against a mechanical stimulus or with PNU50488H. Given that distinct mechanisms contribute to the persistent hyperalgesia induced by injections of formalin, Freunds and capsaicin (McCall et al. 1996, Holzer 1991, LeBars 2001), identifying the sexually-dimorphic action of κ opioids in these persistent pain models will require extensive investigation. However, the present study provides preliminary evidence to suggest marked sex differences in responsiveness to κ opioids and that the direction of these sex differences may be dependent upon the mechanisms underlying the different types of persistent nociception.

The present finding that spiradoline, U50,488 and U69,593 were on average 147, 9.9 and 57-fold more potent when administered locally than systemically, respectively, suggests that in both males and females the antihyperalgesia induced by local administration of κ opioids was mediated predominantly by activity in the site of inflammation (Ko et al. 1999). Local κ opioid activity was confirmed by the demonstration that in males and females local, but not i.c.v., administration of the κ-opioid antagonist nor-BNI attenuated the effects of local administration of both U69,593 and spiradoline. These data suggest that when administered locally U69,593 and spiradoline attenuated capsaicin-induced hyperalgesia actions via activity at peripheral κ opioid receptors. These findings extend previous studies
by demonstrating sex differences in κ opioid antihyperalgesia mediated by opioid activity at the site of inflammation.

Several possible mechanisms have been demonstrated to account for the greater effectiveness of local administration of opioids in persistent pain models. For example, inflammation is associated with an increased expression of opioids receptors in the dorsal root ganglia and upregulation of opioid receptors in small primary afferent neurons (Ji et al. 1995; Zollner et al. 2003). Inflammatory conditions can also disrupt the perineurial barrier, allowing for greater access of peripherally administered opioids to the opioid receptor population (Antonijevic et al. 1995). Although the present investigation did not directly compare these processes in males and females, the observation that κ opioids were effective when administered locally suggests that these peripheral processes are active in both males and females. Such findings are consistent with previous reports indicating that similar opioid/pain circuitry exists in males and females, but the characteristics of this circuitry can be altered by the gonadal hormones (e.g., Kavaliers and Galea 1995; Sternberg et al. 2004).

As with U69,593 and spiradoline, sex differences were observed with U50,488. In contrast to these κ opioids, local administration of nor-BNI failed to attenuate the effects of U50,488. It is unlikely that the effects produced by local injections of U50,488 were mediated by activity at the μ receptor, as in this preparation μ opioids are slightly more potent in females (Barrett et al. 2003). Although considered a selective κ agonist, U50,488 can produce antinociception by activating non-opioid receptor sites. For example, the antinociception produced by local injections of the (+)-enantiomer of U50,488 as well as high doses of the (-)-enantiomer are not reversed by nor-BNI (Joshi and Gebhart 2003). This antihyperalgesic effect is believed to be mediated by a direct blockade of sodium channels.
and not activation of κ opioid receptors. The current findings contrast with those reported previously in capsaicin preparations. In rhesus monkeys local administration of nor-BNI antagonized the antihyperalgesic effects produced by U50,488 (Ko et al. 1999), and in rats the effects of U50,488 were reversed by doses of the opioid antagonist quadazocine considerably larger than those required to attenuate the effects of the μ agonist fentanyl (Ko et al. 2000). Although it is difficult to reconcile these findings, it does confirm that under some conditions the local antihyperalgesic actions of U50,488 are not mediated directly by activity at the κ opioid receptor.

Recent studies suggest that sex differences in κ opioid antinociception may result from differential NMDA receptor activity. Indeed, NMDA antagonists selectively attenuate κ opioid antinociception in male and ovariectomized female mice and rats (e.g., Mogil et al. 2003; Sternberg et al. 2004; Holtman and Wala 2006). Whether these sex-dependent effects are apparent in models of persistent pain have yet to be determined. In the present investigation, the NMDA antagonist dextromethorphan attenuated the antihyperalgesic effect of U69,593, spiradoline and U50,488, and this effect was observed in both males and females. In contrast, dextromethorphan attenuated the effects of U69,593 in the 50ºC warm water tail-withdrawal procedure only in males, a finding congruent with other studies using acute pain models (Kavaliers and Choleris 1997). Indeed, NMDA antagonists have been shown to selectively antagonize the effects of various κ opioids (e.g., U69,693, U50,488) in rats and mice using the warm water tail-withdrawal and tail-flick procedures (e.g., Mogil et al. 2003; Holtman and Wala 2006). Collectively, these findings provide evidence to suggest that the NMDA receptor system plays a critical role in mediating sex differences in the effects produced by κ opioids in acute but not persistent pain models.
Direct comparisons between the effects produced by κ opioids in acute and persistent pain models should be interpreted with caution. Hyperalgesia associated with persistent nociception can be distinguished from acute nociception in both the transmission of nociceptive information, presence vs absence of inflammation, type of nociceptive response, as well as the underlying neurochemical substrates (Le Bars et al. 2001; McCall et al. 1996). Even though in the present investigation the same type of nociceptive response (i.e., tail withdrawal from warm water) was utilized with capsaicin in the 50ºC warm water tail-withdrawal procedure, there were still numerous procedural and parametric differences between these procedures. Moreover, in these procedures different doses of U69,593 were required to obtain maximal effects.

With models of inflammation, like capsaicin, opioids can produce their effects via both local at the site of inflammation and central sites, whereas in acute models of pain the activity of opioids appears to be restricted to central sites (Yaksh 1997, LeBars 2001). As such, it was possible that the interaction between the NMDA and opioid systems could be apparent at both the central and local level. In the present investigation, systemic administration of dextromethorphan produced minimal attenuation of the antihyperalgesia induced by local administration on U69,593, which contrasts with the almost complete reversal observed following systemic administration of U69,593. A relatively high dose of dextromethorphan administered locally also failed to attenuate the antihyperalgesic effects of local administration of U69,593. These findings suggest that in some pain models, attenuation of κ opioid antihyperalgesia can only be obtained when both the NMDA antagonist and κ opioid are acting centrally.
An additional purpose of the present investigation was to examine the effects of mixed-action opioids with relatively low efficacy at κ receptors (e.g., Dykstra 1990; Butelman et al. 1998). Both butorphanol and nalbuphine were more potent when administered locally at the site of inflammation than systemically. The antihyperalgesia produced by nalbuphine was reversed by local administration of nor-BNI, establishing the contribution of κ opioid receptors at the site of inflammation. In contrast, the finding that nor-BNI failed to antagonize the antihyperalgesia produced by butorphanol suggests receptors other than κ receptors are involved in its antihyperalgesic effects. Despite the differential action of butorphanol and nalbuphine, sex differences were not observed in their antihyperalgesic effects. To some extent these findings contrast with previous studies indicating that sex differences with butorphanol and nalbuphine are typically larger that those observed with more efficacious opioids in both acute and persistent pain models (Cook et al. 2000; Craft and Bernal 2001; Cook and Nickerson 2005). Although the effects of selective κ opioids have not been examined in humans, some studies do suggest that mixed-action opioids with κ opioids activity have greater antihyperalgesic effects in females (Craft 2003). The discrepancies across these studies may be a consequence of the type of pain model employed, suggesting further that the relative potency of opioids in males and females is specific to certain inflammatory pain models. The factors that make these models different may be critical in understanding sex differences in opioid antinociception.
Table 3.1 ED50 values (95% confidence limits) and relative potency ratios (95% confidence limit) for male and female rats \((n = 6–8)\) in tests conducted with spiradoline, U50,488, U69,593, butorphanol and nalbuphine when administered systemically (s.c.; mg/kg) and locally (tail; µg).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Males</th>
<th>Females</th>
<th>Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiradoline</td>
<td>0.019 (0.013-0.266)</td>
<td>0.028 (0.019-0.45)</td>
<td>1.84 (1.23-2.92)</td>
</tr>
<tr>
<td>Tail</td>
<td>2.82 (1.41-5.64)</td>
<td>11.2 (4.58-27.2)</td>
<td>3.19 (1.57-14.85)</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U69,593</td>
<td>0.11 (0.09-0.14)</td>
<td>0.16 (0.11-0.22)</td>
<td>1.32 (0.94-1.88)</td>
</tr>
<tr>
<td>Tail</td>
<td>6.92 (5.49-8.74)</td>
<td>6.5 (4.7-8.98)</td>
<td>0.88 (.609-1.24)</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U50,488</td>
<td>0.099 (0.068-0.15)</td>
<td>0.19 (0.14-0.28)</td>
<td>1.98 (1.11-4.05)</td>
</tr>
<tr>
<td>Tail</td>
<td>0.30 (0.157-0.58)</td>
<td>4.36 (1.95-9.75)</td>
<td>18.6 (6.67-69.3)</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butorphanol</td>
<td>0.031 (0.022-0.444)</td>
<td>0.05 (0.37-0.07)</td>
<td>1.51 (0.88-2.76)</td>
</tr>
<tr>
<td>Tail</td>
<td>0.203 (0.087-0.486)</td>
<td>0.113 (.004-0.36)</td>
<td>1.48 (0.40-5.76)</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>0.35 (0.28-0.44)</td>
<td>0.38 (0.31-0.45)</td>
<td>1.09 (0.82-1.49)</td>
</tr>
<tr>
<td>Tail</td>
<td>7.86 (4.54-13.6)</td>
<td>3.05 (1.21-7.75)</td>
<td>1.91 (0.89-4.72)</td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) More potent (P <.05) in males than females as determined by relative potency ratio.
Table 3.2 Relative potency ratios (95% confidence limit) for spiradoline, U50,488, U69,593, butorphanol and nalbuphine when administered systemically (s.c.) and locally (tail) in male and female rats ($n = 6-8$).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiradoline</td>
<td>192.2 (102.8-364.8)</td>
<td>102.0 (42.1-205.9)</td>
</tr>
<tr>
<td>U50,488</td>
<td>3.30 (1.17-6.61)</td>
<td>16.38 (7.032-74.1)</td>
</tr>
<tr>
<td>U69,593</td>
<td>69.23 (49.2-98.8)</td>
<td>44.02 (26.1-71.5)</td>
</tr>
<tr>
<td>Butorphanol</td>
<td>9.83 (4.01-27.9)</td>
<td>3.15 (0.579-12.2)</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>19.82 (11.7-34.0)</td>
<td>11.54 (7.34-17.4)</td>
</tr>
</tbody>
</table>

a More potent ($P < .05$) when administered locally than systemically as determined by relative potency ratio.
Figure 3.1 Antihyperalgesic effects of spiradoline, U50,488 and U69,593 administered systemically (s.c.) and locally (tail) in male and female rats (n=6-8). Data for systemically administered drugs are expressed in mg/kg and for locally administered drugs in µgs. A warm water tail-withdrawal procedure was used for testing in which the distal 7 cm of the tail was immersed in water maintained at 45°C. Equally effective doses of capsaicin were injected 3.5 cm from the tip of the tail 15 min prior to the test with all opioids administered at the same time as capsaicin. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 3.2 **Panels a and b.** Antihyperalgesic effects of local (tail) administration of U69,593 (300 µg) alone and in combination with central (i.c.v.) or local (tail) administration of nor-BNI (10 µg) in male and female rats (n=5) in the capsaicin preparation. Additional details are as described in Fig. 1. Asterisks (*) indicate a significant difference between the antihyperalgesic effects of U69,593 alone (tail) vs in combination with nor-BNI (tail). Crosses (+) indicate a significant difference in the antihyperalgesic effect of the combination of U69,593 administered in the tail and nor-BNI administered i.c.v. vs to the combination of U69,593 and nor-BNI administered in the tail. **Panels c and d.** Antinociceptive effects of U69,593 alone when administered systemically (3.0 mg/kg males; 5.6 mg/kg females: these doses were determined to be equally effective in males and females) and in combination with central (i.c.v.) administration of nor-BNI (10 µg) in the warm water tail-withdrawal procedure in males and females. In this procedure, the distal 7 cm of the tail was immersed in water maintained at 50°C and latency to withdrawal the tail from warm water was recorded. Antinociceptive effects of U69,593 administered locally (tail; 300 µg) are also shown. All tests were conducted in male and female rats (n=5). For all panels, vertical bars represent the standard error; where not indicated, the standard error fell within the bar or the data point. Asterisks (*) indicate a significant difference in the antinociceptive effects of U69,593 alone (s.c.) vs in combination with nor-BNI (i.c.v.).
Figure 3.3 Antihyperalgesic effects of local (tail) injections of spiradoline (100 µg), U50,488 (300 µg males, 1000 µg females: these doses were determined to be equally effective in males and females), alone and in combination with local injections of nor-BNI (10 µg) in male and female rats (n=6-8) in the capsaicin preparation. Procedural details are as described in Fig. 1. Vertical bars represent the standard error; where not indicated, the standard error fell within the bar. Asterisks (*) indicate a significant difference between the antihyperalgesic effects of opioids alone vs in combination with nor-BNI.
Figure 3.4 Antihyperalgesic effects of the mixed-action opioids butorphanol and nalbuphine administered both systemically (s.c.) and locally (tail) in male and female rats (n=6-8) in the capsaicin preparation. Data for systemically administered drugs are expressed in mg/kg and for locally administered drugs in µg. Other procedural details are as described in Fig. 1. Vertical bars represent the standard error; when not indicated, the standard error fell within the bar.
Figure 3.5 Antihyperalgesic effects of local (tail) administration of nalbuphine (1000 µg), butorphanol (100 µg males, 300 µg females: these doses were determined to be equally effective in males and females) alone and in combination with local (tail) administration of nor-BNI (10 µg) in male and female rats (n=6-8) in the capsaicin preparation. Procedural details are as described in Fig. 1. Vertical bars represent the standard error, where not indicated, the standard error fell within the bar. Asterisks (*) indicate a significant differences in the antihyperalgesic effect when the opioid was administered alone vs in combination with nor-BNI.
Figure 3.6 Antihyperalgesic effects of systemically (s.c.) administered U69,593 (10 mg/kg) alone and in combination with systemically (s.c.) administered dextromethorphan in male and female rats (n=6–8) in the capsaicin preparation. Procedural details are as described in Fig. 1. Alone, dextromethorphan (3.0-30 mg/kg) had no antihyperalgesic effects (data not shown). Vertical bars represent the standard error; when not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of U69,593 alone vs in combination with dextromethorphan.
Figure 3.7  Antihyperalgesic effects of U69,593 (300 µg) administered locally (tail), and in combination with dextromethorphan (30 mg/kg) administered systemically (s.c.) and locally (tail) in the capsaicin preparation in male and female rats (n=6–8). Procedural details are as described in Fig. 1. Vertical bars represent the standard error; when not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of local administration of U69,593 alone vs in combination with dextromethorphan administered systemically.
Figure 3.8 Antihyperalgesic effects of spiradoline (17.5 mg/kg) and U50,488 (10 mg/kg, males; 17.5 mg/kg, females: these doses were determined to be equally effective in males and females) alone and in combination with dextromethorphan (30 mg/kg) in male and female rats (n=6–8) in the capsaicin preparation. Procedural details are as described in Fig. 1. All drugs were administered systemically (s.c.). Vertical bars represent the standard error; where not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of opioid alone vs in combination with dextromethorphan.
Figure 3.9 **Panel A.** Antinociceptive effects of U69,593 (3.0 mg/kg males; 5.6 mg/kg females: these doses were determined to be equally effective in males and females) alone and in combination with dextromethorphan (30 mg/kg) when administered systemically (s.c.) in both male and female rats (n=6-8) in the warm water tail-withdrawal procedure. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point. **Panel B.** Area under the curve (AUC) difference score for systemic administration of U69,593 in combination with dextromethorphan. Data represent the difference in AUC for U69,593 alone - AUC for U69,593 in combination with dextromethorphan in both males and females. Asterisks (*) indicate a significant sex difference in AUC difference score.
CHAPTER 4
SEX DIFFERENCES IN NMDA ANTAGONIST ENHANCEMENT OF MORPHINE ANTIHYPERALGESIA IN A CAPSAICIN MODEL OF PERSISTENT PAIN: COMPARISONS TO TWO MODELS OF ACUTE PAIN

Introduction

Sex differences in opioid antinociception have been studied extensively in acute pain models (e.g., hotplate), with the majority of reports indicating that male rodents and monkeys are more sensitive to the antinociceptive effects of µ opioids than their female counterparts (Negus and Mello, 1999; Cook et al., 2000; Craft and Bernal, 2001). Several recent lines of physiological, pharmacological and behavioral evidence suggest that this sexual dimorphism may extend to the manner in which NMDA antagonists modulate the actions of µ opioids. For example, dizocilpine has been shown to reduce morphine-induced c-Fos expression to a greater extent in female rats, with castrated males displaying a level of c-Fos expression similar to that of females (D’Souza et al., 1999; D’Souza et al., 2002). In a hotplate procedure, dizocilpine selectively blocks the development of morphine tolerance in male mice, whereas in a warm water tail-withdrawal procedure it selectively facilitates the development of tolerance in males (Bryant et al. 2006). Of particular clinical relevance is the finding that NMDA antagonists produce a sexually-dimorphic effect on acute µ opioid antinociception. Indeed, Grisel et al. (2005) reported that dextromethorphan enhanced morphine antinociception in male and ovariectomized female mice, but attenuated morphine
antinociception in intact females. Similarly, Craft and Lee (2005) reported that
dextromethorphan enhanced morphine antinociception to a greater extent in male rats, with
this effect being most evident in a hotplate procedure. Not all reports indicate that NMDA
antagonists produce sexually-dimorphic effects on morphine antinociception (e.g., Bryant et
al., 2006; Holtman et al., 2003), with these discrepancies most likely explained by
differences across studies in type of NMDA antagonist, dose of NMDA antagonist,
procedure, and rodent genotype. The dose of morphine tested is also critical, as some NMDA
antagonists enhance low levels of antinociception produced by morphine and attenuate high
levels (Nemmani et al., 2004; Craft and Lee, 2005).

In contrast to these findings in acute pain models, there are no investigations
evaluating sex differences in NMDA-opioid interactions using persistent pain models.
Nociception induced by persistent and acute nociception differ along a number of critical
dimensions, including their neurochemical and anatomical substrates, duration, frequency,
and type of nociceptive response, as well as the type of nociceptive fibers they activate
(LaMotte et al. 1992; McCall et al. 1996; Le Bars et al. 2001; Kayser et al 2007). The distinct
mechanisms underlying acute vs persistent pain have been shown to be critical determinants
of sex differences in both opioid sensitivity and NMDA-opioid interactions. Indeed, recent
studies suggest that the sex differences in µ opioid antinociception apparent in acute pain
models may not be apparent in all persistent pain models (Barrett et al, 2003: Cook and
Nickerson, 2004). Moreover, NMDA antagonists have been shown to selectively block the
antinociceptive effects κ opioids in males when examined in various models of acute pain,
but not in models of persistent pain (Mogil et al., 2003; Holtman and Wala, 2006; Lomas et
al., 2007). Clearly, while acute nociceptive assays are a beneficial starting point, it is
essential to determine if the sexually-dimorphic manner in which NMDA antagonists modulate the effects opioids is apparent in persistent pain models.

The purpose of the present study was to examine sex differences in the extent to which the NMDA antagonist dextromethorphan alters the antihyperalgesic effects of morphine in a model of persistent pain. In the model selected for study, the chemical irritant capsaicin was administered directly into the tail resulting in inflammation, vasodilatation and a hyperalgesic response localized at the site of administration (Caterina et al., 1997; Holzer et al., 1991; Winter et al., 1995). The hyperalgesia induced by capsaicin was then measured by a decrease in the latency to tail-withdrawal from an acute presentation of a mildly noxious thermal stimulus (45°C water), and in both males and females this hyperalgesia persists for 60-90 mins (Barrett et al., 2003). Unlike some models used to examine persistent pain (e.g., formalin-induced hyperalgesia), in the capsaicin procedure NMDA antagonists do not alter the development or persistence of the hyperalgesic response (Sakurada et al., 1998; Lomas et al., 2007). As such, this model provides a unique opportunity to examine sex differences in NMDA-opioid interactions against a persistent form of nociception. In order to facilitate comparisons across studies, as well as provide a direct comparison across acute and persistent pain models using the same drugs, doses and rodent strain, tests were also conducted using a hotplate (52°C) and warm-water tail-withdrawal (52°C) procedure.

Methods

Animals

Intact male and female F344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). All testing occurred between 3 and 6 months of age, and rats were
individually housed in a colony on a 12-h/12-h light/dark cycle. All rats had unlimited access to food and water.

Testing

Capsaicin: A warm-water tail withdrawal procedure was used to assess hyperalgesia induced by an injection of capsaicin in the tail. During testing, each rat was lightly restrained, with the distal 7 cm of the tail immersed in water maintained at 45°C, a relatively innocuous nociceptive stimulus (Lynn and Carpenter, 1982). Baseline tail-withdrawal latencies were determined before administration of any drugs, and rats that failed to maintain their tails in the water for 15 sec were excluded from subsequent testing. Following determination of baseline latencies, capsaicin was injected 3.5 cm from the tip of the tail. All injections of capsaicin were made under light halothane anesthesia, with rats recovering from this procedure within 2-3 mins. After administration of capsaicin, tail-withdrawal latencies decreased from 15 sec to an average of 3.5 - 4.5 sec with this effect being comparable in both males and females.

For tests of the antihyperalgesic effects of morphine and dextromethorphan, a 3.0 and 1.0 µg dose of capsaicin was chosen for males and females, respectively, as these doses produce a comparable magnitude and duration of hyperalgesia (Barrett et al., 2003). Before initiating testing, a capsaicin baseline was assessed in each rat. Approximately 1 week later, capsaicin was administered in the tail, followed by an intraperitoneal (i.p) injection of saline, morphine or dextromethorphan alone or in selected combinations. Dextromethorphan was chosen as it is used clinically and has previously been shown to enhance morphine antinociception in a number of acute nociceptive procedures (Grisel et al., 2005; Pleasan et al
1995). Although a number of pretreatment times and doses were examined for both dextromethorphan and morphine, tail-withdrawal latencies were always determined 15 min after the capsaicin tail injection, which is the time point corresponding to the peak effect of capsaicin. For all tests, a 15 sec cutoff latency was implemented, as this indicated a maximal antihyperalgesic effect (i.e., nociceptive thresholds returned to baseline levels). Rats were tested approximately once per week with no more than 5 tests per animal. Previous studies conducted in our laboratory indicated that this frequency of testing and the number of tests produced reliable and consistent tail-withdrawal latencies (Barrett et al., 2003).

**Warm water tail-withdrawal and hotplate procedures:** Prior to testing, rats were habituated to restraint tubes, baseline tests were conducted in both the warm water tail-withdrawal and hotplate procedures, and each rat was administered a 2.5 mg/kg dose of morphine (no data were collected for this test). This habituation protocol was designed, in part, to limit the impact of stress-induced antinociception which has previously been reported to be influenced by sex but minimized by habituation (Mogil and Belknap, 1997; Dhabhar et al., 1997).

During both habituation and testing in the warm water tail-withdrawal procedure, rats were placed in restraint tubes, the distal 7 cm of the tail immersed in 52°C water, and the latency to tail-withdrawal recorded. An increase in the latency to remove the tail from the warm water was taken as a measure of nociception, with an upper cutoff limit of 15 sec to minimize tissue damage. In this procedure, baseline tail-withdrawal procedures ranged from 7-10 sec in males and 6.5-9 sec in females. After a baseline tail-withdrawal latency was determined, rats were placed on the hotplate which was set at 52°C. Latency to hind-paw lick or an escape response was then determined, with an upper cutoff time of 60 sec. In this
procedure, baseline to hind-paw lick or an escape latencies ranged from 20-35 sec in males and 17-35 sec in females. In each series of tests, two baseline latencies were determined prior to drug administration. Subsequently, rats were administered an i.p injection of saline, morphine and dextromethorphan alone or in selected combinations. Data for both the warm water tail-withdrawal and hotplate procedures were collected across a 2 hr interval. No rat was exposed to the testing procedures more than 6 times and at least 6 days separated each test.

Data Analysis

Capsaicin: For the antihyperalgesic effects of drugs, latencies to tail-withdrawal following administration of drug were converted to the percentage of the maximum possible effect using the following equation: % antihyperalgesic effect = [(observed-baseline)/(15 s-baseline)] x 100. An ANOVA analysis was then used to determine differences across dose/drug combinations and sex. In instances in which there was a main effect for drug condition and sex, post-hoc tests were conducted using the Fisher’s protected least significant difference test to compare dose combinations within sex. For statistical analyses, the alpha level was set at 0.05. The dose of morphine required to produce a 50% antihyperalgesic effect (ED$_{50}$) was derived mathematically (least-squares method) using log-linear interpolation with at least three doses on the ascending limb of the dose-effect curve.

Warm water tail-withdrawal and hotplate procedures: In order to calculate % maximal antinociceptive effects, tail-withdrawal latencies (warm water) and lick/escape latencies (hotplate) were converted to percent antinociceptive effect using the following
equation: \% antinociceptive effect = [(test latency - baseline) / (cutoff - baseline)] X 100. The time-course evaluation of a drug effects was measured in these procedures and thus area under the curve was used for statistical analysis. Area under the curve was estimated by the Trapezoidal Rule using available statistical software (Tallarida and Murray 1987\textsuperscript{©}). An ANOVA analysis was then used to determine differences across doses and sexes. Post hoc tests and ED\textsubscript{50} values were calculated in a manner similar to that described above. For statistical analyses, the alpha level was set at 0.05.

**Drugs**

The following drugs were used: morphine sulfate (provided by the National Institute on Drug Abuse), dextromethorphan hydrobromide monohydrate, and capsaicin (both purchased from Sigma-Aldrich Co., St. Louis, Mo.). Capsaicin was dissolved in a solution of Tween 80 / 95\% ethanol / saline in a ratio of 1/1/8, and was diluted to lower concentrations with saline. Capsaicin was injected alone in the tail in a 0.1-ml volume. Saline, morphine and dextromethorphan were administered i.p. in a volume of 0.5 to 1.0 ml/kg. Small amounts of lactic acid were added to dextromethorphan to promote solubility. As dextromethorphan occasionnally caused lesions and severe adverse effects in both male and female rats at the 30 mg/kg dose, testing at this dose was limited.

**Results**

Fig. 1 shows that in males and females morphine produced dose-dependent increases in antihyperalgesia, with near maximal effects obtained at the highest dose tested. ANOVA indicated a main effect for dose ($F_{4,64}=26.9$, $P<0.05$), no main effect for sex, or a dose x sex
interaction. ED\text{50} values for morphine in females 6.22 mg/kg (95% CL: 3.95-9.79) and males 4.45 mg/kg (95% CL: 2.57-7.86) were also similar. Across the dose range examined, dextromethorphan produced only minimal levels of antihyperalgesia in both males and females, with no dose tested producing greater than an 11% effect. The higher doses of dextromethorphan did, however, produce sedation and disruption of motor performance, although these effects were typically short lived and did not interfere with testing.

Fig. 2 shows that in males dextromethorphan enhanced the antihyperalgesic effect of doses of morphine that produced minimal (1.0 and 2.5 mg/kg) to moderate (5.0 mg/kg) antihyperalgesic effects when administered alone. At the two lowest doses of morphine tested, 20 and 30 mg/kg dextromethorphan increased the antihyperalgesic effect of morphine from 11% to 74%, and from 13% to 86%, respectively. At the highest dose of morphine tested, 10 mg/kg dextromethorphan increased levels of antihyperalgesia from 73% to 100%. In females, no dose of dextromethorphan altered the antihyperalgesic effects produced by morphine. For 1.0 and 2.5 mg/kg morphine, ANOVA confirmed a main effect for sex (1.0: \( F_{1,48}=12.5, P<0.05; 2.5: F_{1,41}=64.6, P<0.05 \)), drug (1.0: \( F_{3,48}=13.9, P<0.05; 2.5: F_{3,41}=8.69, P<0.05 \)), and a sex x drug interaction (1.0: \( F_{3,48}=6.4, P<0.05; 2.5: F_{3,41}=6.45, P<0.05 \)). At 5.0 mg/kg morphine, ANOVA indicated a main effect for sex, (\( F_{1,32}=7.57, P<0.05 \)), but no main effect for drug and no sex x drug interaction. Post hoc tests confirmed that, with the exception of 1.0 mg/kg morphine / 10 mg/kg dextromethorphan combination, in males all dose combinations enhanced (\( P<0.05 \)) the antihyperalgesic effect of morphine. In contrast, enhancement of the antihyperalgesic effects of morphine was not obtained in females regardless of the dose combination tested.
In order to determine if these sex-specific effects of dextromethorphan were a consequence of a sexual dimorphism in the time-course of morphine or dextromethorphan actions, tests were conducted in which selected doses of these drugs were administered at different pretreatment times. Fig. 3 shows that in males dextromethorphan enhanced the antihyperalgesic effect of morphine at all pretreatment times tested. In females, varying the pretreatment time of dextromethorphan failed to alter the antihyperalgesic effectiveness of morphine. ANOVA confirmed a main effect for sex \((F_{1,48}=97.3, P<0.05)\), pretreatment time \((F_{3,48}=9.106, P<0.05)\), and a sex x pretreatment time interaction \((F_{4,48}=9.75, P<0.05)\). Post hoc tests also confirmed enhancement \((P<0.05)\) of the antihyperalgesic effect of morphine at all dextromethorphan pretreatment times in males, but at no pretreatment time in females.

Fig 4 shows the antihyperalgesic effect of morphine when administered at various pretreatment times before dextromethorphan. For males, all pretreatment times produced an enhancement of morphine antihyperalgesia, whereas in females enhancement was not observed at any pretreatment time. ANOVA confirmed a main effect for sex \((F_{1,66}=55.6, P<0.05)\), pretreatment time \((F_{4,66}=2.8, P<0.05)\), and a sex x pretreatment time interaction \((F_{4,66}=9.7, P<0.05)\). Post hoc tests confirmed an enhancement \((P<0.05)\) of the antihyperalgesic effect of morphine at all dextromethorphan pretreatment times in males, whereas in females enhancement was not observed at any pretreatment time.

Fig. 5 shows the effects of morphine and dextromethorphan alone in males and females in two acute pain models, the warm water tail-withdrawal and hotplate procedures. Across the dose range examined, dextromethorphan failed to produce an antinociceptive effect. Alone, morphine produced dose-dependent increases in antinociception in both males and females. Based on morphine ED\(_{50}\) values in females (6.47 mg/kg, 95% CL: 4.87-8.60)
and males (6.03 mg/kg, 95% CL: 5.08-7.17) there were no sex differences in the potency of morphine in the warm water tail-withdrawal procedure. Time-course analyses of morphine (2.5 - 10 mg/kg morphine) in males and females (data not shown) indicated that the peak effects of morphine were typically seen at the 30 min test interval, and thereafter the magnitude of morphine antinociception gradually decreased, returning to baseline levels between 90 and 120 mins. ANOVA of these time-course data indicated no main effect for sex, a main effect for dose ($F_{3,43} = 41.2$, $P<0.05$) with no dose x sex interaction. Similarly, in the hotplate procedure sex differences were not observed in the potency of morphine, as comparable ED$_{50}$ values were obtained in females (12.36 mg/kg, 95% CL: 5.69-26.9) and males (8.06 mg/kg, 95% CL: 5.74-11.3). Peak effect and the duration of morphine (2.5 - 10 mg/kg) antinociception in this procedure were similar to that observed in the warm water tail-withdrawal procedure (data not shown). ANOVA of these time-course data indicated no main effect for sex, a main effect for dose ($F_{3,44} = 13.5$, $P<0.05$) with no dose x sex interaction.

Fig. 6 shows the antinociceptive effect of selected doses of morphine when combined with dextromethorphan in the warm water tail-withdrawal procedure. Alone, in both males and females 2.5 and 5.0 mg/kg morphine produced only low levels of antinociception (less than 24%). When administered in combination, dextromethorphan produced a dose- and time-dependent enhancement of morphine antinociception, with this effect observed in both males and females. At the higher doses of dextromethorphan, morphine produced near maximal levels of antinociception with a peak effect and duration of action that was consistently larger in males. When enhancement was observed in males, low to moderate levels of antinociception were generally apparent even at the 120 min test interval. In
contrast, in females antinociception was not typically observed beyond the 60 or 90 min test intervals. ANOVA of area under the curve data for 2.5 and 5.0 mg/kg morphine indicated a main effect for sex (2.5: $F_{1,65}=39.4$, $P<0.05$; 5.0: $F_{1,49}=6.78$, $P<0.05$), drug (2.5: $F_{4,65}=23.3$, $P<0.05$; 5.0: $F_{3,49}=22.3$, $P<0.05$), and a sex x drug interaction (2.5: $F_{4,65}=3.13$, $P<0.05$; 5.0: $F_{3,49}=4.12$, $P<0.05$).

Post hoc tests conducted on AUC data revealed that in males 5.0 and 10 mg/kg dextromethorphan were the lowest doses that enhanced ($P>0.05$) the effects produced by 2.5 and 5.0 mg/kg morphine, respectively. In females, enhancement of morphine antinociception was observed at the 20 and 10 mg/kg dextromethorphan doses, respectively. These analyses also confirmed a significantly ($P<0.05$) larger antinociceptive effect in males for all doses of dextromethorphan in combination with 2.5 mg/kg morphine and at the two highest doses of dextromethorphan in combination with 5.0 mg/kg morphine.

Similar effects were obtained in the hotplate procedure. Alone, both doses of morphine produced only low levels of antinociception, less than 11% in males and 15% in females. As shown in Fig. 7, the antinociceptive effects of morphine were enhanced by the two higher doses of dextromethorphan in males, and only at the highest dose in females. This effect was evident both in terms of the peak effect and duration of morphine antinociception. For both doses of morphine, ANOVA of area under the curve data indicated a main effect for sex (2.5: $F_{1,65}=13.9$, $P<0.05$; 5.0: $F_{1,51}= 7.45$, $P<0.05$) and drug (2.5: $F_{4,65}=14.8$, $P<0.05$; 5.0: $F_{3,51}=7.7$, $P<0.05$). A sex x drug condition interaction was evident only at 2.5 mg/kg morphine ($F_{4,65}=5.98$, $P<0.05$). Post hoc tests confirmed that the two highest doses of dextromethorphan enhanced the antinociceptive effect of both 2.5 and 5.0 mg/kg morphine to a greater extent ($P<0.05$) in males. For both doses of morphine, enhancement of morphine
antinociception was observed at the two highest doses of dextromethorphan in males, and only at the highest of dextromethorphan in females.

Discussion

One purpose of the present investigation was to examine the effects of the non-competitive NMDA antagonist dextromethorphan on morphine antinociception in two acute pain models, the hotplate (52°C) and warm water tail-withdrawal (52°C) procedures. The failure to observe sex differences in morphine antinociception in these procedures provided an opportunity to evaluate potential interactions with NMDA antagonists under conditions in which baseline levels of morphine antinociception were comparable in both males and females. Under these conditions, dextromethorphan produced a dose- and time-dependent enhancement of morphine antinociception, with this effect observed in both males and females. In a number of instances, the combination of dextromethorphan and low doses of morphine produced maximal antinociceptive effects. The magnitude of these dextromethorphan-induced enhancements of morphine antinociception, however, was consistently larger in males and observed at lower doses of dextromethorphan. Such finding are consistent with previous studies indicating that in acute pain models NMDA antagonists enhance the peak effect and duration of morphine antinociception in both rats and mice (Grisel et al 2005, Nemmani et al., 2004, Craft and Lee, 2005). These findings extend those reports to a strain of rats (F344) known to display large sex differences in μ opioid antinociception (Terner et al. 2003), and confirm findings indicating that dextromethorphan can produce relatively large increases in morphine antinociception (e.g., Plesan et al. 1999).
In the hotplate procedure, the enhancement observed in male and female rats was smaller than that observed in the warm-water tail-withdrawal procedure. These findings extend previous reports of differences across acute nociceptive assays in the extent to which NMDA antagonists enhance morphine antinociception. For example, Craft and Lee (2005) reported greater enhancement in male Sprague-Dawley rats in a hotplate procedure, yet failed to observe enhancement in a warm water tail-withdrawal procedure. Although greater effects were observed in the present investigation in the warm water tail-withdrawal procedure with F344 rats, this discrepancy could reflect a rodent strain-dependency, and there is evidence that rat strain and substrain are critical determinants of sensitivity to µ opioid antinociception (Terner et al., 2003, Kest et al., 1999), sex differences in µ opioid antinociception (Terner et al., 2003, Kest et al., 1999), and the extent to which dextromethorphan enhances the antinociceptive effects of µ opioids in males (Bulka et al., 2002, Plesan et al. 1999).

The major purpose of the current investigation was to evaluate NMDA-opioid interactions in a model of persistent pain. In the procedure selected for study, administration of the chemical irritant capsaicin in the tail produces a 60-90 min hyperalgesic response to mildly noxious warm water (45°C). In this procedure, systemically administered morphine was equally potent at reducing capsaicin-induced antihyperalgesia in males and females (Barrett et al., 2003). Dextromethorphan, which had no antihyperalgesic effect when administered alone, produced a dose-dependent enhancement of morphine antihyperalgesia in males. The higher doses of dextromethorphan tested increased morphine antihyperalgesia by 63 - 73%. In contrast, in females dextromethorphan failed to enhance the antihyperalgesic effect of morphine. This sexually-dimorphic effect was observed across doses of morphine that produced both low and moderate levels of morphine antihyperalgesia. Such findings
markedly contrast with those reported in acute pain models, where enhancement is typically observed in both males and females (Craft and Lee, 2005; Grisel et al 2005; present investigation).

While studies suggest that sex differences in µ opioid antinociception are not due to opioid pharmacokinetics, binding affinity, receptor density or µ receptor-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding (Kepler et al., 1991; Candido et al., 1992; Cicero et al., 1996; Selley et al., 2003), some studies suggest a longer onset and shorter offset of morphine antinociception in females (Sarton et al, 2000). Moreover, some NMDA antagonists and their major metabolites have a longer plasma half-life in female rats (e.g., Ramachander et al., 1978; Shelnutt et al., 1999). Consequently, it was possible that the failure to observe enhancement in females was a consequence of the time-course of the drugs examined. To evaluate this possibility, pretreatment times ranging from 15 - 60 mins for both morphine and dextromethorphan were examined. Across the pretreatment times examined, dextromethorphan-induced enhancement of morphine antihyperalgesia was observed only in males. These findings suggest that the sexual dimorphism in NMDA-opioid interactions observed in the present investigation was not a consequence of sex differences in the pharmacokinetics of dextromethorphan or morphine, but rather reflects sex differences in the extent to which the NMDA system modulates the effects produced by µ opioids.

The mechanism underlying this sexual dimorphism in NMDA-opioid interactions and why differences are more pronounced in models of persistent than acute pain have not been determined. Previous studies have shown a sex-dependency in NMDA-κ opioid interactions that is apparent in acute but not persistent pain models (Holtman and Wala, 2006; Lomas et al., 2007). Moreover, a sex-dependency in NMDA-µ opioid interactions have been observed
in stress-induced analgesia (e.g., Mogil et al 1993), the development of morphine tolerance (Bryant et al. 2006) and morphine-induced c-Fos expression (D’Souza et al., 1999; D’Souza et al., 2002). Some evidence also suggests that these sex-dependent effects may be mediated by estrogen, as estrogen can modulate NMDA receptors in various brain regions (Cyr et al., 2001).

Although the present investigation represents a preliminary investigation of NMDA-opioids interactions in a persistent pain model, a weakness in our approach was the failure to determine estrous cycle or the potential influence of gonadal hormones. Indeed, the recent finding that the level of dextromethorphan-induced enhancement of morphine antinociception in ovarectomized female mice was comparable to that of male mice suggests that gonadal hormones may play a role in mediating NMDA-opioid interactions (Grisel et al 2005). This finding is supported by evidence that female rats in diestrous show a greater enhancement of morphine antinociception by the NMDA antagonist LY235959 than in other estrous phases (Craft and Lee, 2005). Moreover, estrous cycle has been shown to influence the development of nociception in a variety of pain models (Aloisi and Ceccarelli, 2000; Ren et al., 2000), including the capsaicin procedure (Barrett et al., 2003). Given the pervasive nature of the influence of gonadal hormones on both nociception and opioid activity, it would not be surprising if gonadal hormones influenced NMDA-opioid interactions in persistent pain models.

It is well established that categorization of pain as being either acute or persistent represents an oversimplified construct. Numerous types of persistent pain have been well characterized (e.g., inflammatory, neuropathic), with each type mediated by distinct neurochemical substrates. For example, whereas activation of non-NMDA excitatory amino
acid receptors modulate the development and persistence of nociception in a plantar incision model of pain (Zahn et, 1998), NMDA receptors are involved in the hyperalgesia induced by Freund’s adjuvant and formalin (e.g., Ren and Dubner, 1993) as well as that produced by following neuropathic injuries (Mao et al, 1992). In contrast, capsaicin-induced hyperalgesia involves activation of neuorokinin receptors, but not NDMA receptors (Lao et al. 2003). As such, it is possible that the sexually dimorphic manner in which NMDA antagonists enhance µ opioid antihyperalgesia may be specific to certain pain models, and thus to certain types of pain. While it is important to examine the effects of the combination of NMDA antagonists and morphine in multiple persistent pain models, such experiments may prove difficult as NMDA antagonists can have a direct effect on the development of nociception. The lack of NMDA involvement in the development of the hyperalgesic response in the capsaicin model made this an effective model in which to initiate study of the effects of NMDA antagonist on µ opioid antihyperalgesia.

While acute pain models represent a beneficial starting point to examine the effects of NMDA antagonists on µ opioid antinociception, ultimately in the clinical population these drugs will be used in conditions of persistent or chronic pain. In clinical studies, the extent to which NMDA antagonists enhance the analgesic effects of opioids has produced mixed results (e.g., Dudgeon et al., 2007). For example, in cancer pain patients titrating doses of morphine or morphine/dextromethorphan combinations, the combination therapy provided satisfactory pain control at half the morphine dose (Katz et al, 2000). In contrast, in patients with chronic, non-neuropathic pain (e.g., low back pain, osteoarthritis), enhancement was not observed (Galer et al., 2005). The current findings may help explain some of these discrepant findings as they demonstrate that NMDA-opioid interactions are dependent upon the type of
nociception as well as the sex of the subject. Although sex has not been systematically examined in clinical studies, a recent set of case reports indicated that the NMDA antagonist ketamine was more effective in enhancing opioid analgesia in 2 male chronic pain patients then in a female patient (Bell, 1999). Determining the mechanism by which sex and the type of persistent pain mediate NMDA-opioid interactions should shed light on the potential clinical utility of combining NMDA antagonists with μ opioids.
Figure 4.1 Antihyperalgesic effects of morphine and dextromethorphan administered systemically in male and female rats (n=6-8). A warm water tail-withdrawal procedure was used for testing in which the distal 7 cm of the tail was immersed in water maintained at 45°C. Equally effective doses of capsaicin were injected 3.5 cm from the tip of the tail 15 min prior to the test with all drugs administered at the same time as capsaicin. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point. Asterisks (*) indicate significant difference in the antihyperalgesic effects of morphine alone vs in combination with selected doses of dextromethorphan.
Figure 4.2 Antihyperalgesic effects of morphine alone and in combination with dextromethorphan in male and female rats (n=6–8) in the capsaicin-induced hyperalgesia procedure. Procedural details are as described in Fig. 1. Vertical bars represent the standard error; when not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of morphine alone vs in combination with selected doses of dextromethorphan.
Figure 4.3 Antihyperalgesic effects of morphine alone and in combination with dextromethorphan in male and female rats (n=6–8) in the capsaicin-induced hyperalgesia procedure. The dose combination of morphine and dextromethorphan were selected as they produced high levels of antihyperalgesia in males (see Fig. 2). Dextromethorphan was administered at various pretreatment times before testing, while morphine was always administered 30 min before testing. Additional procedural details are as described in Fig. 1. Vertical bars represent the standard error; when not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of morphine alone vs in combination with dextromethorphan.

![Graph showing antihyperalgesic effects of 2.5 mg/kg morphine + 20 mg/kg dextromethorphan](image)
Figure 4.4 Antihyperalgesic effects of morphine alone and in combination with dextromethorphan in male and female rats (n=6–8) in the capsaicin-induced hyperalgesia procedure. The dose combination of morphine and dextromethorphan were selected as they produced high levels of antihyperalgesia in males (see Fig. 2). Morphine was administered at various pretreatment times before testing, while dextromethorphan was always administered 30 min before testing. Additional procedural details are as described in Fig. 1. Vertical bars represent the standard error; when not indicated, the standard error fell within the bar. Asterisks (*) indicate significant difference in the antihyperalgesic effects of morphine alone vs in combination with dextromethorphan.
Figure 4.5 Antinociceptive effects of morphine and dextromethorphan administered systemically in male and female rats (n=6-8) in a warm water tail-withdrawal procedure (left panel) and a hotplate procedure (right panel). All data reflect the effects of these drugs at a 30 min pre-session injection time. In the warm water tail-withdrawal procedure, the distal 7 cm of the tail was immersed in water maintained at 52°C and latency to withdrawal the tail from warm water was recorded. In the hotplate procedure rats were placed on the 52°C hotplate and latency to hind paw withdrawal/escape was recorded. Control data (SAL) indicate the effects of saline administration. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point.
Figure 4.6 Time-course assessment of the antinociceptive effects of selected doses of morphine administered alone (dotted lines) and in combination with dextromethorphan in female and male rats (n=6-8) in a warm water tail-withdrawal (52°C) procedure. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point. Data in the right most panels reflect area under the curve (AUC) analyses for administration of morphine alone and in combination with dextromethorphan over 2 hr time course. Asterisks (*) indicate a significant sex difference in AUC.

**Warm Water Tail-Withdrawal**

<table>
<thead>
<tr>
<th>Dextromethorphan (mg/kg)</th>
<th>Females</th>
<th>Males</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ 5.0</td>
<td>105</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>+ 10</td>
<td>80</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>+ 20</td>
<td>50</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>+ 30</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

% Antinociceptive Effect

Time Course (mins)

Graphs showing the time-course assessment of antinociceptive effects for 2.5 mg/kg and 5.0 mg/kg of morphine alone and in combination with 5.0 mg/kg of dextromethorphan for both females and males.
Figure 4.7 Time-course assessment of the antinociceptive effects of selected doses of morphine administered alone (dotted lines) and in combination with dextromethorphan in female and male rats (n=6-8) in a hotplate (52°C) procedure. Vertical bars represent the standard error; where not indicated, the standard error fell within the data point. Data in the right most panels reflect area under the curve (AUC) analyses for administration of morphine alone and in combination with dextromethorphan over 2 hr time course. Asterisks (*) indicate a significant sex difference in AUC.
CHAPTER 5
GENERAL DISCUSSION

Experimental Results

The present series of experiments examined several factors that effect the direction and magnitude of sex differences in opioid antinociception, with a particular focus on the role of the modulatory actions of the NMDA system. Experiment 1 utilized a behavioral procedure to induce temporal summation of pain in rats. This short-lasting change in nociceptive sensitivity has been used as a model to examine the mechanisms underlying the development and persistence of chronic pain. In the current research, this model of temporal summation was evaluated for its sensitivity to parametric manipulations, sex differences, modulation by NMDA receptor antagonists, and sensitivity to reversal by opioids. The findings suggest a number of similarities in the characteristics and receptor modulation of temporal summation in humans and rats. Interestingly, in this model of chronic pain there were no sex differences in opioid potency. While this finding is consistent with those observed in some chronic pain models, it contrasts with those observed in acute pain models. Results also showed that the NMDA system played a modulatory role in the development of temporal summation, as pretreatment with selected NMDA antagonists greatly reduced the development of hypersensitivity to the nociceptive stimulus.
Sex differences in the potency of the antinociceptive effects of both κ and μ opioids have been reported in acute pain models using thermal, chemical and mechanical nociceptive stimuli (Kelpler et al. 1989; Cicero et al 1996, 1997; Barrett et al. 2002). Importantly, the majority of studies suggest that the sex differences observed in these models may be mediated by activity in the NMDA receptor system. To date, this relationship has not been examined in more clinically relevant models of persistent pain. Experiment 2 evaluated sex differences in the antihyperalgesic actions of select κ and mixed-action opioids in the capsaicin model of persistent pain. Results of this experiment showed that while κ opioids were generally more potent in males, sex differences were not observed with the mixed-action opioids that have activity at both the μ and κ receptors. In addition, κ opioids were more potent when administered locally, in the tail than systemically in both sexes, suggesting that the antihyperalgesia induced by local administration was mediated predominantly by activity at the site of inflammation (Ko et al. 1999). The NMDA antagonist dextromethorphan attenuated the development of κ opioid antihyperalgesia equally in males and females. These findings contrast with those observed in acute pain models, where NMDA antagonists selectively block κ opioid-induced antinociception in males (Mogil et al. 2003; Sternberg et al. 2004; Holtman and Wala 2006). Together these findings suggest that sex differences NMDA system activity may be dependent upon the mechanisms underlying the different types of nociception.

The purpose of Experiment 3 was to evaluate the role of the NMDA system in modulating sex differences in μ opioid-induced hyperalgesia in a model of persistent pain. In acute pain models, NMDA antagonists have been shown to enhance the antinociceptive effects of the μ opioid morphine to a greater extent in males than females, but to date there
are no published reports of utilizing persistent pain models (Craft and Lee 2003; Grisel et al 2005 but see Holtman et al. 2003 ). In Experiment 3, both acute (warm water tail-withdrawal and hot-plate) and persistent (capsaicin) pain models were utilized. In the capsaicin preparation, the NMDA antagonist dextromethorphan enhanced the antihyperalgesic effect of low to moderate doses of morphine in males, but had no effect in females. This result was seen across different pretreatment times for both morphine and dextromethorphan. In contrast, enhancement of morphine antinociception was observed in both males and females in the acute pain models, with the magnitude of this effect being greater in males. These findings demonstrate a sexually dimorphic interaction between NMDA antagonists and morphine in a persistent pain model that can be quantitatively distinguished from those observed in acute pain models. Taken together, the present series of studies highlight the importance of the type/duration of nociceptive stimulation when examining the role of diverse receptor systems in modulating sex-specific opioid activity.

**Pain models**

The pursuit of the mechanisms underlying the effectiveness of opioids can, in part, be traced to a seminal study by Lasagna and Beecher (1954) in which it was reported that standard doses of morphine failed to produce postsurgical pain relief in up to 35% of patients. While numerous factors have been found to contribute to these individual differences, sex is perhaps one of the most critical. Interestingly, while this study of the effectiveness of morphine focused on postoperative pain that can persist for days to weeks, research on the influence of sex on opioid antinociception and the mechanisms underlying this relationship have focused almost exclusively on models of acute, short acting pain. In
these models a noxious stimulus is applied to a portion of the body until a withdrawal response is observed (Fillingim and Ness, 2000; Mogil et al., 2000). Results from experiments utilizing acute pain models have identified an extensive list of factors that influence sex differences in opioid sensitivity, including type of noxious stimulus, genetic factors and opioid efficacy. This line of research has also led to the demonstration of a limited role for pharmacokinetic factors, as systemic injections of morphine produce comparable blood and brain concentrations in male and female rats (Craft et al. 1996; Cicero et al. 1997). However, as clinical pain is more often persistent or chronic in nature a complete picture of sex difference in opioid sensitivity must encompass more models of long lasting pain.

Persistent and acute models of pain vary greatly in many aspects, including the duration of nociception, activation of \(\text{A}_\delta\) (phasic, acute) vs \(\text{C}\) (tonic, persistent) fibers, presence of inflammation, and tissue damage as well as the neurochemical substrates underlying these effects (Abbott et al. 1981, Coderre and Melzack, 1992, Le Bars 2001). There is even evidence to suggest that pain models are sensitive to genetic factors, as rodent lines selected for high opioid sensitivity in a thermal nociceptive assay have been shown to be relatively insensitive to opioid antinociception in a persistent formalin assay (Mogil et al., 1996).

Although opioids represent the typical treatment option for persistent pain in the clinical population, their effectiveness has been described as highly variable and often unpredictable (Przewlocki and Przewlocka, 2001). While some of this variability can be attributed to individual differences it is becoming increasing clear that different types or manifestations of persistent pain may be responsible for some of this variability. It is evident
from the limited research in this area that even at the level of persistent pain conditions, the
type of persistent pain model utilized has a direct effect on the magnitude of sex differences
in the response to opioid analgesics. For example, while the present set of studies found, a
greater antihyperalgesic effect in males with κ opioids in the capsaicin preparation, Binder et
al. (2000) reported that the peripherally active κ-opioid asimadoline was more effective in
female rats at reducing Freunds-induced hyperalgesia. The involvement of the NMDA
system in the persistent model is one critical factor that varies within persistent pain models.
For example, experimental results show in the carrageenin model of acute inflammation,
NMDA receptor antagonists reduced thermal hyperalgesia (Ren et al., 1992), while in a
tissue injury model of post-operative pain, NMDA receptor antagonists had no effect on
mechanical hyperalgesia (Zahn and Brennan, 1998). In the capsaicin preparation, the current
and previous research (Sakurada et al., 1998) indicates the NMDA system does not influence
the development and persistence of capsaicin-induced hyperalgesia. It is this characteristic
which made the capsaicin preparation an excellent candidate for the examination of the
relationship between sex differences, opioids and the NMDA system in persistent pain. As
administration of the NMDA antagonist dextromethorphan alone had no effect on the
development of the hyperalgesic response, any changes in the effects produced by
opioids/dextromethorphan could be attributed to the direct effects of the NMDA system on
opioid activity rather than changes in basal levels of nociception.

In contrast, the NMDA system is more actively involved in other models of persistent
pain. For example, the formalin model of pain nociception is distinguished by 2 phases, the
latter of which is characterized ongoing stimulation of nociceptors, inflammation, as well as
NMDA receptor activation (Fu et al., 2001). Similar to the formalin model, the nociceptive
response in the temporal summation model developed and tested in experiment 1 was determined to be NMDA dependent. In this temporal summation produced administration of various non-competitive NMDA antagonists decreased the level of temporal summation in both males and females at doses that failed to produce an antinociceptive effect in the acute tail-withdrawal procedure. This result was not surprising as temporal summation in humans is thought to mimic wind-up, a central mechanism implicated in the development of some chronic pain conditions (Dickenson and Sullivan 1987; Price et al. 1994; Arendt-Nielsen et al. 1995; Graven-Neilson et al. 2000; Guirimand et al. 2000). It is important to note, however, that parametric analyses indicated nociceptive stimulus intensity greatly affected the degree to which NMDA antagonists reduced the temporal summation response, as greater attenuation was observed at the lower stimulus intensities. It is thus possible that high nociceptive stimulus intensities may require activation of NMDA and non-NMDA systems in this model.

Interestingly, in Experiments 2 and 3 it was the data obtained in the female animals that did not correspond to results reported in studies of sex differences in acute pain models. The NMDA antagonist dextromethorphan significantly attenuated the antihyperalgesic effect of the κ opioids U69,593, spiradoline and U50,488, and this effect was observed in both males and females. In contrast, NMDA antagonists have been shown to selectively antagonize the effects of κ opioids in male rats and mice using acute nociceptive procedures (e.g., Mogil et al. 2003; Holtman and Wala 2006, Kavaliers and Choleris 1997). Similarly, a dose-dependent enhancement of morphine antihyperalgesia was selectively obtained in males, whereas in acute models NMDA antagonists enhance the peak effect and duration of
morphine antinociception in both male and female animals (Grisel et al. 2005, Nemmani et al., 2004, Craft and Lee, 2005).

**Implications and Future Directions**

Future investigation of the NMDA system modulation of sex differences should focus on the specific role of the numerous binding sites located on the NMDA receptor. The NMDA receptor is a large complex consisting of an ion channel and a number of binding sites that allow for various compounds to activate and regulate its activity (Mao et al., 1999). Antagonism of these specific receptor sites has become of interest partly due to their apparent ability to modulate opioid antinociception. Indeed, modulation has been achieved through competitive (i.e., LY-235959) and non-competitive channel blocking antagonism (i.e., dextromethorphan, MK-801) or actions at different sites such as the strychnine-insensitive glycine (i.e., (+)-HA-966) or polyamine site (i.e., ifenprodil) (Parsons, 2001). There is evidence that the antagonism of various binding sites on the NMDA receptor may have differential effects on opioid antinociception, with non-competitive channel blocking antagonists producing the most consistent enhancement of opioid antinociception (Trujillo and Akil, 1991, Pleasan et al., 1998; Redwine and Trujillo, 2003).

It is extremely difficult at this time to begin to postulate the mechanisms underlying these diverse actions as the effects of NMDA antagonism varied across opioids (μ vs κ), sexes, and pain models. These diverse results do, however, rule out a number of simple explanations, such as estrous cycle, drug kinetics, drug pharmacokinetics (binding sites). While physiological studies have demonstrated an influence of gonadal hormones on the density of NMDA binding sites as well as NMDA antagonist induced c-fos expression...
(Gazzaley et al. 1996) the current experiments did not systematically address the role of gonadal hormones in NMDA modulation of opioid antinociception/antihyperalgesia. It is possible that gonad hormone play an indirect role in this relationship. Based on the current findings, however, identification of the mechanisms underlying sex difference in NMDA modulation of opioid antinociception/antihyperalgesia requires extensive research examining different pain models that have distinct mechanisms underlying the development of pain. In this sense, animal research has followed a similar path as pain research in humans. For a number of years the pervasive belief was indicated that opioids were more effective in females, as demonstrated in studies of oral surgery (Gordon et al 1995). However, more recently there many studies indicating the picture has changed considerably, as these differences have not been consistently observed. It appears that the type of pain experienced has an effect on the efficacy of the opioid in the general population as women were found to experience more severe postoperative pain and required a greater dose of morphine than men in the immediate postoperative period (Abrun et al., 2005). In acute laboratory pain conditions Fillingim et al (2005) found no sex difference in effectiveness of morphine analgesia (see also Zacny, 2001). In addition in conditions of chronic cancer pain no sex differences in morphine analgesia (Kaiki et al, 1983).

Ultimately the goal of studying sex differences in antinociception is the identification of variables that can be used and manipulated in such a way as to increase the effectiveness of pain treatment in the general clinical population. Understanding the physiology of sex differences in pain and opioid effectiveness may also aid in understanding the origin of chronic pain disorders. While there is not a perfect relationship between findings in animal
studies and humans, it is the commonality of factors and the examination of differences that will ultimately aid in the treatment of various pain conditions.
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