Nociceptive Sensitivity and Opioid Antinociception and Antihyperalgesia in Freund’s Adjuvant-Induced Arthritic Male and Female Rats

Charles D. Cook and Michael D. Nickerson

Department of Pharmacology and Toxicology and Institute for Drug and Alcohol Studies
Running Title:  Sex Differences in Chronic Pain

To whom correspondence should be addressed:
Charles D. Cook
Department of Pharmacology and Toxicology and Institute for Drug and Alcohol Studies
Virginia Commonwealth University
410 North 12th Street
PO BOX 980613
Richmond, VA  23298-0613
Phone: 804-828-3158
Email: ccook@hsc.vcu.edu

Total number of pages:  37
Total number of tables: 2
Total number of figures: 9
Total number of references: 51
Total number of words in Abstract:  256
Total number of words in Introduction:  614
Total number of words in Discussion: 1686

Non standard abbreviations:  CFA, Complete Freund’s Adjuvant

Recommended section assignment:  Behavioral Pharmacology
Abstract

The present study was designed to examine sex differences in Complete Freund’s Adjuvant (CFA)-induced mechanical hyperalgesia and sex differences in opioid antinociception and antihyperalgesia. Female rats developed inflammation and hyperalgesia faster and exhibited greater peak hyperalgesia than male rats. In arthritic (CFA-treated) rats lower thresholds were observed during estrus and proestrus and in non-arthritic (vehicle-treated) rats lower thresholds were observed during proestrus. Morphine and oxycodone were more potent in male than female arthritic rats and butorphanol was more potent and effective in male than female arthritic rats. The potency of morphine was increased in arthritic rats, although to a greater magnitude in males. The potency of oxycodone was increased in male but not female arthritic rats. The potency of butorphanol was increased in arthritic male rats and the maximal antinociceptive effect of butorphanol was increased in arthritic female rats, but did not result in greater than 20% antinociception. Morphine, oxycodone and butorphanol all produced antihyperalgesic effects (returning thresholds of arthritic rats to the thresholds of non-arthritic rats) with greater potency in males than females. The peripherally acting opioid agonist loperamide produced intermediate levels of antinociception in male and female arthritic rats and no antinociception in non-arthritic rats. Loperamide was more potent in male than female arthritic rats at producing antihyperalgesia. These data demonstrate sex differences in arthritis-induced hyperalgesia and responsiveness to opioid analgesics. In arthritic rats the antinociceptive effects of opioid agonists are most probably mediated by both central and peripheral opioid receptors, whereas their antihyperalgesic effects are mediated primarily by actions at peripheral opioid receptors.
Recent studies using acute nociceptive test procedures in which a noxious stimulus is presented for a brief period of time (e.g., seconds) have demonstrated that the sex of the organism, in part, mediates sensitivity to nociceptive stimuli and opioid analgesics. Female rodents are often more sensitive to the nociceptive producing effects of various types of thermal (e.g., hot-plate, radiant heat, warm-water) and mechanical (e.g., pressure) stimuli following acute presentation (Barrett et al., 2002b; Kest et al., 1999; Terner et al., 2003), although this is not always the case (Bartok and Craft, 1997; Cook et al., 2000). Moreover, morphine is often a more potent antinociceptive agent in male than female rodents using such procedures (Barrett et al., 2002a; Cicero et al., 1996; Cook et al., 2000; Kest et al., 1999).

Acute nociceptive tests in which the noxious stimulus lasts for a short duration do not model clinical pain conditions in which a prolonged state of noxious stimulation occurs. Various algogenic agents, including capsaicin and formalin, can be utilized to produce an inflammatory condition that results in a persistent, prolonged nociceptive state. In rats injected with formalin in the hindpaw females exhibit a significantly greater nociceptive response than males (Aloisi et al., 1994; Gaumond et al., 2002). Similarly, following intradermal tail administration of capsaicin, females exhibit a significantly greater hyperalgesic response to a water stimulus applied to the tail of the rat and, unlike with acute tests, morphine is equally potent in reversing the hyperalgesic state in males and females (Barrett et al., 2003). Although the duration of noxious stimulation produced by both formalin and capsaicin ranges from several minutes to hours, the duration is not on the same order of magnitude as human chronic pain conditions, which last from weeks to years, nor do formalin and capsaicin model specific disease processes seen in humans.

Complete Freund’s Adjuvant (CFA) administration to rodents produces a disease-like state that is believed to most closely resemble the human rheumatoid arthritis condition, which is characterized by
inflammation of the membrane surrounding the joints as well as damage to the bone and cartilage. When administered into the base of the tail a polyarthritic state develops in both hindpaws over several days with peak signs of inflammation, joint deterioration and hyperalgesia occurring at approximately three weeks post administration. This polyarthritic state can last upwards of several weeks. Although females represent approximately 75% of all rheumatoid arthritis cases in the United States of America (Cooper and Stroehla, 2003; Jacobson et al., 1997), most studies that use CFA to model the human arthritic condition employ male rats as subjects. Therefore, the present study was designed to determine if nociceptive sensitivity in response to mechanical pressure applied to the hindpaws and responsiveness to opioid antinociception and antihyperalgesia in male and female Lewis rats are sexually dimorphic following CFA administration. Antinociception was defined as the ability of opioid agonists to increase paw pressure thresholds in both non-arthritic and arthritic rats above non-drug baseline thresholds. Antihyperalgesia was defined as the ability of opioid agonists to return thresholds of arthritic rats to control thresholds as determined in non-arthritic rats. Initial tests were conducted with morphine as it is the prototypical mu opioid receptor agonist that has been previously examined in male rats using the CFA model of chronic pain (Millan et al., 1987; Neil et al., 1986). The lower efficacy opioid butorphanol was also examined because the magnitude of opioid-induced sex differences in potency and effectiveness are greater with lower efficacy compared to higher efficacy opioid agonists (Barrett et al., 2001; Cook et al., 2000). Finally, tests with the higher efficacy agonist oxycodone, which is used in several formulations of commercially available opioid analgesics, including OxyContin®, were conducted.
Materials and Methods

Subjects. Seventy-nine male and seventy-nine female Lewis rats approximately 60 days of age were obtained from Charles Rivers Laboratories (Raleigh, NC). Rats were individually housed, had free access to food and water and were maintained on 12/12h light/dark cycle. A summary of the tests conducted in each group of rats is shown in Table 1.

CFA Administration. On Day 1 animals were injected intradermally in the base of the tail with 0.1 ml of 5.0 mg/ml Complete Freund’s Adjuvant (CFA) (heat-killed Mycobacterium; Difco Laboratories, Detroit, MI) or 0.1 ml of vehicle (VEH) (mineral oil). Animals were weighed daily (M-F) and the day on which at least one of the hindpaws exhibited inflammation was recorded. Inflammation was operationally defined as an overt, observable increase in the size of at least one hindpaw relative to the hindpaws of VEH-treated rats.

Paw Thickness and Nociceptive Sensitivity Testing. Paw thickness (mm) of the left and right hindpaws was determined using a digital caliper. Nociceptive sensitivity was assessed by determining thresholds in response to mechanical pressure applied to the hindpaws of the rats. Rats were lightly restrained in a towel and a mechanical stimulus was applied with an analgesy meter (Ugo Basile, Varese, Italy), a device with a dome-shaped plastic tip (diameter = 1 mm) that applies a linearly increasing pressure (g) to the dorsal surface of the hindpaw, with the tip applied to the region of the paw just proximal to the third digit. A 500 g cut-off was imposed on all tests. On three separate occasions prior to Day 12 each rat was habituated to the paw pressure procedure. Habituation involved wrapping the rat lightly in a towel and placing one hindpaw on the plinth under the dome-shaped plastic tip. Increasing pressure was then applied until a withdrawal response was initiated. This habituation
procedure was repeated for the other hindpaw. On test days, paw pressure thresholds were determined for both the left and right hindpaws with the order of testing counterbalanced across animals.

**Estrous Cycle Phase Determination.** Estrous cycle phase was determined in female rats by vaginal lavage on the day of testing prior to obtaining baseline paw pressure thresholds on Days 12, 19 and 21. The stages were identified based upon the type of cells detected. Proestrus phase was signified by a majority of round, nucleated cells, and an absence of leukocytes. Estrus phase was signified by a majority of cornified cells, and an absence of leukocytes. Metestrus phase was signified by the presence of round nucleated cells, cornified cells, and leukocytes. Diestrus phase was signified by the presence of all three cell types seen in metestrus, however, the number of cells in this phase was greatly reduced.

**Drug Administration and Nociceptive Sensitivity.** Opioid agonists (morphine, butorphanol, oxycodone and loperamide) were administered using a cumulative dosing schedule. Preliminary tests indicated that in male and female VEH- and CFA-treated rats repeated threshold testing every 30 min for up to six times resulted in no change in paw pressure thresholds. Thus, immediately after baseline paw pressure thresholds were recorded rats were injected with the first dose of the opioid agonist and thresholds were re-determined 30 min later. Immediately after these tests the next drug dose was administered such that each successive dose increased the total drug concentration by 0.25, 0.5 or 1.0 log units. This cycle of drug administration followed by threshold determination continued every 30 min until maximal antinociception was achieved or until the largest dose to be tested was administered. Because butorphanol failed to produce antinociception in female rats, a dose of 10 mg/kg morphine was administered immediately following threshold determinations with 10 mg/kg butorphanol in order to determine if butorphanol could antagonize the antinociceptive effects of morphine. Although the
antinociceptive effects of 10 mg/kg morphine were not determined alone, this dose is nearly twice as large as 5.6 mg/kg morphine, which when administered alone produces 100% antinociception at 30 min post administration. For all paw pressure threshold determinations the testing order of the left and right hindpaws was counterbalanced across animals. All female rats in the opioid antinociception / antihyperalgesia tests were used irrespective of their estrous cycle status.

The effects of intrapaw administration of naloxone methiodide were examined in male and female CFA-treated rats following s.c. administration of 5.6 mg/kg morphine. Thresholds for the right hindpaw were determined 30 min post morphine administration. This was immediately followed by an injection of 30 µg naloxone methiodide into the dorsal surface of the right hindpaw. Paw pressure thresholds in the right hindpaw were re-determined 5 min post naloxone methiodide administration (35 min post morphine administration).

**Data analysis.** Differences in body weights between VEH- and CFA-treated rats were determined using a repeated measures ANOVA with treatment (i.e., CFA or VEH) as the between groups factor and days post VEH/CFA administration as the within subjects factor. Differences between male and female CFA-treated rats in the magnitude of weight loss relative to VEH-treated rats were determined using a repeated measures ANOVA. Paw pressure thresholds and paw thickness measurements for the left and right hindpaws were averaged for each rat. Paw thickness measurements for CFA-treated rats were expressed as a % increase in the paw thickness relative to the paw thickness of VEH-treated rats. Differences in the % increase in the paw thickness between male and female rats were determined using a repeated measures ANOVA. Because not all four phases of the estrous cycle were detected on each of the three test days (12, 19, 21), the data were collapsed across all three test days for analysis. Differences in paw pressure thresholds across the estrous cycle were determined using an ANOVA. All
post hoc comparisons were made with a Bonferroni or LSD adjustment. Simple effects analyses were conducted for pairwise comparisons when the interaction term was not statistically significant. The relationship between nociceptive sensitivity and paw thickness on Days 12, 19 and 21 in male and female rats that exhibited overt signs of inflammation was determined by calculating a Pearson correlation coefficient (one-tailed). The significance level for all tests was set at $P<0.05$.

For opioid agonist dose-effect testing in VEH- and CFA-treated rats paw pressure thresholds following administration of the agonist were converted to a percentage of a maximum possible effect based upon a maximal 500g of pressure using the following equation: $\% \text{ Antinociception} = \left[\frac{(\text{observed} - \text{baseline})}{(500g - \text{baseline})}\right] \times 100$. Antinociception was operationally defined as opioid agonist-induced increases in paw pressure thresholds above non-drug baseline thresholds with 100% antinociception equal to 500g. The dose-effect data from CFA-treated rats were also analyzed to determine the antihyperalgesic effects of each opioid agonist. Antihyperalgesia was operationally defined as opioid agonist-induced increases in paw pressure thresholds in CFA-treated rats above non-drug baseline thresholds with 100% antihyperalgesia equal to the non-drug baseline paw pressure threshold of the respective VEH-treated group. For mean threshold calculations when the resulting threshold following drug administration was greater than the mean threshold for the non-drug baseline of the respective VEH-treated group (e.g., maximal antihyperalgesia cut-off) this value was replaced with the mean VEH-treated threshold value for calculation of the mean threshold in CFA-treated rats. Differences in drug-induced increases in paw pressure thresholds were determined using a repeated measures ANOVA. The $\% \text{ Antihyperalgesia}$ effect of each opioid was also determined based upon the following equation: $\% \text{ Antihyperalgesia} = \left[\frac{(\text{observed} - \text{CFA baseline})}{(\text{VEH baseline} - \text{CFA baseline})}\right] \times 100$. The $\% \text{ Antihyperalgesia}$ data were used to determine differences in the potency of the agonist between male and female CFA-treated rats.
ED$_{50}$ values were obtained using least squares linear 50 regression analysis, followed by calculation of 95% confidence limits by the method of Bliss (1967). Potency ratio values with a 95% confidence interval were calculated by the method of Colquhoun (1971). Differences in the relative potency of morphine were considered to be significant if the 95% confidence interval did not overlap 1.0. ED$_{50}$ values for the antihyperalgesic effects of morphine and butorphanol in male rats and of loperamide in female rats were calculated using extrapolation as the lowest dose of each agonist produced slightly greater than 50% antihyperalgesia. Instances in which an ED$_{50}$ value was not calculated, a repeated measures ANOVA with treatment (i.e., CFA or VEH) or sex as the between groups factor and drug dose as the within subjects factor was conducted to determine differences in the level of antinociception/hyperalgesia across treatment groups. For rats tested with naloxone methiodide, differences in paw pressure thresholds of the right hindpaws were determined using a repeated measures ANOVA. Simple effects analyses were conducted for pairwise comparisons when the interaction term was not statistically significant.

**Drugs.** The following drugs were used: morphine sulfate, oxycodone (supplied by the National Institute on Drug Abuse; Rockville, MD); butorphanol, loperamide and naloxone methiodide (purchased from Sigma-Aldrich Co.; St. Louis, MO). All drugs were dissolved in sterile water, except for loperamide. Loperamide was first dissolved in 20% w/v hydroxypropyl-β-cyclodextrin (ENCAPSIN HPB, American Maize Products, Hammond, IN) in sterile water, at a maximal concentration of 7 mg/ml, to form a stock solution. Appropriate amounts of stock solution were added to sterile water to form test doses. All drugs were administered s.c. in an injection volume of 0.5-1.0 ml/kg except for naloxone methiodide which was administered in a volume of 25 µl.
Results

Body Weight. On Day 1 male rats weighed approximately 293g and females weighed approximately 200g. For male rats there was a main effect of day ($F(17,221)=13.91, P=0.0001$), but not of treatment ($F(1,13)=2.34, P=0.15$) and a day by treatment interaction ($F(17,221)=26.00, P=0.0001$) such that by Day 18 CFA-treated rats weighed significantly less than VEH-treated rats. On Day 12 CFA-treated males weighed 5g more than CFA-treated males, whereas on Days 19 and 26 CFA-treated males weighed 40g and 65g less than VEH-treated males, respectively. For female rats there was a main effect of day ($F(17,221)=8.98, P=0.0001$), treatment ($F(1,13)=31.30, P=0.0001$) and a day by treatment interaction ($F(17,221)=48.84, P=0.0001$) such that by Day 11 CFA-treated rats weighed significantly less than VEH-treated rats. On Days 12, 19 and 26, CFA-treated females weighed 16g, 32g and 48g less than CFA-treated females, respectively. The magnitude of the weight loss on Days 12, 19 and 26 between male and female CFA-treated rats relative to the mean weight of their respective same day VEH-treated rats was not different ($F(1,16)=0.03, P=0.86$) indicating no sex difference in the magnitude of CFA-induced weight loss.

Nociceptive Sensitivity in Male and Female Rats. Figure 1 shows paw pressure thresholds determined on Days 12, 19 and 26 in male and female rats treated with CFA or VEH. For male and female VEH-treated rats there was no difference in thresholds across test days (sex: $F(1,10)=0.65, P=0.44$; day: $F(2,20)=0.47, P=0.63$; sex by day interaction: $F(2,20)=0.25, P=0.78$) (Fig. 1A). For male VEH- and CFA-treated rats thresholds on Days 19 and 26 were significantly lower in CFA-treated relative to VEH-treated males (treatment: $F(1,13)=14.00, P=0.002$; day $F(2,26)=3.09, P=0.06$; treatment by day interaction: $F(2,26)=1.47, P=0.25$) (Fig. 1B). For female VEH- and CFA-treated rats thresholds on Days 12, 19 and 26 were significantly lower in CFA-treated relative to VEH-treated females.
Effect of Estrous Cycle Phase on Nociceptive Sensitivity. Phase determination and paw pressure thresholds were obtained in 12 VEH- and 20 CFA-treated rats on Days 12, 19 and 21 for a total of 36 and 60 observations in VEH- and CFA-treated rats, respectively (Figure 2). In VEH-treated rats paw pressure thresholds were significantly lower in females in proestrus compared to females in metestrus and no differences in thresholds were observed among females in estrus, metestrus and diestrus \((F(3,32)=2.93, P=0.05)\). In CFA-treated rats paw pressure thresholds were significantly lower in females in estrus compared to females in metestrus and in diestrus and in proestrus compared to females in metestrus \((F(3,32)=2.77, P=0.05)\).

Paw Thickness and Nociceptive Sensitivity Measures. The paws of VEH-treated male rats were significantly thicker than the paws of VEH-treated female rats on Days 12 (4.79 mm vs. 4.41 mm, \(P=0.007\)), 19 (4.95 vs. 4.30, \(P=0.0001\)) and 21 (4.99 vs. 4.48, \(P=0.0001\)). In CFA-treated rats on Day 12, no sex difference in the % increase in paw thickness was observed (male: 20% (+4.04); female: 16% (+3.01); \(P=0.41\)) (Figure 3). On Day 19, paw thickness in CFA-treated female rats was increased by...
97% and in CFA-treated male rats by 76% \( (P=0.02) \). Similarly, on Day 21 the increase in CFA-treated female rats was 94% and in CFA-treated male rats was 78% \( (P=0.08) \).

The relationship between paw thickness and nociceptive sensitivity on Days 12, 19 and 21 was determined in male and female rats. Increases in inflammation in females were correlated with increased nociceptive sensitivity as evidenced by a significant correlation between paw thickness and threshold on Day 12 \( (r=-0.64, P=0.001) \) and Day 19 \( (r=-0.49, P=0.02) \) but not on Day 21 \( (r=-0.34, P=0.07) \). In male rats a significant correlation between paw thickness and threshold was obtained on Day 19 \( (r=-0.62, P=0.003) \) but not on Day 12 \( (r=-0.09, P=0.36) \) and Day 21 \( (r=-0.23, P=0.19) \). On Day 12, fourteen of twenty females (70%) and eight of twenty males (40%) had developed overt signs of inflammation. Two of twenty male rats failed to exhibit any inflammation throughout the entire 21 day experiment.

**Opioid Antinociception.** The antinociceptive effects of morphine were examined in the same VEH- and CFA-treated male and female rats on Days 12 (Figure 4, top panel) and 19 (Figure 4, bottom panel). On both days, morphine produced dose-dependent increases in antinociception in male and female rats. On Day 12, the potency of morphine in VEH-treated male and female rats was similar (Figure 4, top panel; Table 2). Morphine was equally potent in male VEH- and CFA-treated rats, whereas morphine was 1.48-fold more potent in CFA- than VEH-treated female rats (Figure 4, top panel; Table 2). On Day 19, the potency of morphine was increased by 2.12- and 1.50-fold in male and female CFA-treated rats compared to male and female VEH-treated rats, respectively (Figure 4, bottom panel; Table 2). Comparison of morphine’s potency in male and female rats indicated that morphine was significantly more potent (1.51-fold) in male compared to female CFA-treated rats and equally potent male and
female VEH-treated rats (Figure 4, bottom panel; Table 2). Thus, the magnitude of the increase in morphine’s potency was significantly greater in male than female CFA-treated rats.

In a separate group of rats the antinociceptive effects of morphine were examined on Day 26 (data not shown). Morphine was equally potent in male and female VEH-treated rats (Table 2). The potency of morphine was increased in CFA-treated compared to VEH-treated male rats by 2.21-fold. In CFA-treated rats morphine was 1.53-fold more potent in male than female rats. This was similar to the 1.51-fold potency difference observed on Day 19.

The antinociceptive effects of butorphanol were examined in VEH- and CFA-treated male and female rats on Days 12 (Figure 5, top panel) and 19 (Figure 5, bottom panel). On Day 12, butorphanol produced dose-dependent increases in antinociception in VEH- and CFA-treated males with no difference in potency (ED$_{50}$ value (+SEM), VEH: 0.63 (0.21-1.91); CFA: 0.67 (0.43-1.05)) (Figure 5, top panel). In contrast, less than 20% antinociception was observed in VEH- and CFA-treated female rats (Figure 5, top panel). Tests of simple effects indicated that the level of antinociception at a dose of 1.0 mg/kg butorphanol was greater in CFA- compared to VEH-treated female rats. On Day 19, the potency of butorphanol was increased by 5.82-fold in male CFA-treated compared to VEH-treated rats (VEH: 1.01 (0.51-1.97); CFA: 0.17 (0.11-0.25)) (Figure 5, bottom panel). In female rats butorphanol produced less than 20% antinociception although tests of simple effects indicated that the magnitude of antinociception at each dose of butorphanol was greater in CFA- than VEH-treated rats (Figure 5, bottom panel). Therefore, butorphanol was more effective in male than female CFA-treated rats.

The failure to obtain greater than 20% antinociception in female rats suggested that butorphanol possesses low intrinsic efficacy at the mu opioid receptor. As such, when combined with an effective antinociceptive dose of morphine butorphanol should antagonized morphine’s effects. Indeed, when a dose of 10 mg/kg morphine was administered following the determination of thresholds in response to
the 10 mg/kg butorphanol dose the resulting antinociception at 30 and 60 min post morphine administration was less than 5% (data not shown).

The antinociceptive effects of oxycodone were tested on Day 21 in the rats that were tested with butorphanol (Figure 6). In all rats tested, oxycodone produced dose-dependent increases in antinociception with maximal effects obtained at a dose of 3.0 mg/kg. The potency of oxycodone was increased by 1.80-fold in CFA-treated male rats compared to VEH-treated male rats (VEH: 1.22 (1.05-1.43); CFA: 0.69 (0.60-0.78)). In contrast, the potency of oxycodone in female VEH- and CFA-treated rats was similar (VEH: 1.38 (1.10-1.73); CFA: 1.27 (1.22-1.33)). Comparison of the potency of oxycodone in CFA-treated rats indicated that oxycodone was 1.84-fold more potent in male than female rats.

**Opioid Agonist Antihyperalgesia.** Antihyperalgesia was operationally defined as opioid agonist-induced increases in paw pressure thresholds in CFA-treated rats above non-drug baseline thresholds with maximal antihyperalgesia equal to the non-drug baseline paw pressure threshold of the respective VEH-treated group. In Figure 7 the antihyperalgesic effects of morphine (top panel) and butorphanol (middle panel) on Day 19 and of oxycodone (bottom panel) on Day 21 are expressed in g. In both male and female rats each opioid agonist produced dose-dependent increases in paw pressure thresholds with significantly greater increases in thresholds occurring at lower doses in male compared to female rats (Morphine: Male \((F(5,45)=12.59, P=0.0001), \) Female \((F(5,45)=16.37, P=0.0001); \) Butorphanol: Male \((F(4,36)=23.15, P=0.0001), \) Female: \((F(4,36)=25.27, P=0.0001); \) Oxycodone: Male: \((F(5,45)=33.63, P=0.0001), \) Female: \((F(5,45)=33.04, P=0.0001))). Sex differences in the potency of each agonist to reverse CFA-induced hyperalgesia were determined by converting the raw paw pressure thresholds in Figure 7 to % Antihyperalgesia (see data analysis section of Materials and Methods). Morphine was
approximately 5-fold more potent in male than female rats (Male: 0.024 (0.02-0.03); Female: 0.196 (0.12-0.33)). Butorphanol was approximately 62-fold more potent in male than female rats (Male: 0.0008 (0.0004-0.001); Female: 0.04 (0.02-0.10)). Because an ED$_{50}$ value was not calculated in male rats tested with oxycodone, the % antihyperalgesia at each dose was compared between males and females. Doses of 0.03, 0.1, 0.3, and 1.0 mg/kg oxycodone produced significantly greater % antihyperalgesia in male than female rats (sex: $F(1,18)=64.94$, $P=0.0001$; dose: $F(3,54)=20.07$, $P=0.0001$; sex by dose interaction: $F(2,26)=3.95$, $P=0.03$).

**Effects of Loperamide and Naloxone Methiodide.** Data within the literature indicate that the pain relieving effects of opioid agonists in CFA-treated male rats are, in part, mediated by opioid receptors located in the peripheral nervous system (Stein et al., 1989; Zollner et al., 2003). As such, loperamide, which is a peripherally active opioid agonist that possesses high intrinsic efficacy at the mu opioid receptor and has minimal access to the central nervous system (DeHaven-Hudkins et al., 1999), was examined for its ability to produce antinociception and antihyperalgesia. Figure 8 shows the antinociceptive (left panel) and antihyperalgesic (right panel) effects of loperamide administered to male and female VEH- and CFA-treated rats on Day 19. In male CFA-treated rats loperamide produced dose-dependent increases in % antinociception with maximal effects (~60% antinociception) obtained at a dose of 1.0 mg/kg loperamide. In VEH-treated male rats loperamide produced no antinociception (dose: $F(4,56)=11.11$, $P=0.0001$; treatment: $F(1,14)=24.86$, $P=0.0001$; dose by treatment interaction ($F(4,56)=13.36$, $P=0.0001$). In VEH-treated female rats loperamide produced no antinociception, whereas in female CFA-treated rats loperamide produced up to 25% antinociception at the doses tested. A dose of 0.1 mg/kg loperamide produced significantly greater antinociception in CFA- than VEH-treated female rats and doses of 1.0 (P=0.052) and 3.0 (P=0.051) mg/kg approached significance (dose:
Comparison of the antinociceptive effects of loperamide between male and female CFA-treated rats indicated that doses of 0.1 – 3.0 mg/kg loperamide produced significantly greater antinociception in male than female rats (dose: $F(4,72)=23.75, P=0.0001$; sex: $F(1,18)=9.91, P=0.006$; dose by sex interaction: $(F(4,72)=5.56, P=0.001$).

Loperamide produced dose-dependent antihyperalgesic effects in both male and female CFA-treated rats (Figure 8, right panel), although the dose required to produce maximal effects (>80% antihyperalgesia) was larger in female CFA-treated rats. Loperamide was approximately 4-fold more potent in male (0.09 (0.06-0.12)) than female (0.36 (0.23-0.58)) CFA-treated rats.

Intrapaw administration of naloxone methiodide, which is an opioid antagonist with limited access to the central nervous system (Brown and Goldberg, 1985), was examined for its ability to block the antinociceptive effects of morphine in CFA-treated rats. As shown in Figure 9, a dose of 5.6 mg/kg morphine increased paw pressure thresholds in male and female CFA-treated rats to approximately 500g and 30 µg naloxone methiodide significantly antagonized the antinociceptive effects of morphine in both male and female rats (dose: $F(1,18)=29.53, P=0.0001$; sex: $F(1,18)=4.11, P=0.06$; dose by sex interaction: $(F(1,18)=3.31, P=0.09$).
Discussion

CFA-induced Mechanical Hyperalgesia. The present study demonstrated that sex is an important variable in mediating the onset of CFA-induced inflammation and the magnitude of hyperalgesia. Female rats developed inflammation at a faster rate and exhibited greater hyperalgesia than male rats. The polyarthritic state was also associated with a significant retardation in weight gain, although the magnitude of weight loss did not differ between male and female rats. These results are consistent with previous reports in which the onset of an arthritic state in male rats is associated with changes in paw volume, body weight, joint stiffness and mechanical hyperalgesia (Millan et al., 1987; Nagakura et al., 2003). The increased nociceptive sensitivity in female arthritic rats is consistent with studies demonstrating that females are more sensitive than males to the nociceptive producing effects of the algogenic agents formalin and capsaicin (Aloisi et al., 1994; Barrett et al., 2003; Gaumond et al., 2002), and are in agreement with studies demonstrating that females develop a more severe arthritic state based on articular indices and gross observational measures (Allen et al., 1983; Holmdahl, 1995; Wilder et al., 1982).

These present results examining the influence of the estrus cycle on nociceptive sensitivity must be interpreted with caution as the number of observations during estrus and proestrus in arthritic and in non-arthritic rats were markedly less than the number of observations during metestrus and diestrus. Thresholds were lower in non-arthritic rats during proestrus and in arthritic rats thresholds were lower during estrus and proestrus. Although a definitive conclusion regarding the influence of circulating hormones can not be made, these results complement those demonstrating greater thermal hyperalgesia in arthritic rats during the proestrus phase (Bradshaw et al., 2000).
Opioid-induced Antinociception. The potency and effectiveness of the opioid agonists to produce antinociception were influenced by the sex of the rat and the relative efficacy of the agonist for the mu opioid receptor. In arthritic rats, the higher efficacy agonists morphine and oxycodone were significantly more potent in male than female rats, and butorphanol, which acts primarily as a lower efficacy mu opioid agonist (Cook et al., 2000; Morgan et al., 1999), was more potent and effective in male than female arthritic rats. In fact, butorphanol antagonized the antinociceptive effects of a large dose of morphine in female arthritic and non-arthritic rats suggesting that butorphanol has lower relative intrinsic efficacy than morphine at the mu opioid receptor in female rats and that the relative intrinsic efficacy of butorphanol is greater in male than female rats. In rats experiencing chronic pain sex differences in opioid antinociception were most evident with the lower efficacy agonist butorphanol. This is in agreement with previous studies demonstrating marked sex differences in the potency and effectiveness of lower compared to higher efficacy mu opioid agonists using acute nociceptive pain tests (Barrett et al., 2002b; Cook et al., 2000). In contrast with preclinical findings in rats in which butorphanol produces antinociception primarily through mu opioid receptors (e.g., Garner et al., 1997; Smith et al., 1999), in humans butorphanol as well as other mixed action opioids exhibit analgesic profiles indicative of greater kappa than mu opioid receptor involvement (Gear et al., 1996a; Gear et al., 1996b; Miller and Ernst, 2004). This potential greater involvement of kappa receptors with mixed action opioids in humans may, in part, explain the findings that in humans females are more sensitive than males to the analgesic effects of butorphanol, nalbuphine and pentazocine (Gear et al., 1996a; Gear et al., 1999; Gear et al., 1996b).

The potency and effectiveness of the opioid agonists tested were also dependent upon the pain state of the animal. For example, the potency of morphine was increased in both male and female arthritic rats, which is consistent with previous studies using only male rats (Millan et al., 1987; Neil et
The increase in the potency of morphine was only obtained when the rats were exhibiting hyperalgesia such as on Day 12 in female rats and on Days 19 and 26 in male and female rats. Interestingly, the magnitude of the hyperalgesia did not appear to influence the magnitude of the increase in opioid potency. That is, even though female CFA-treated rats were exhibiting greater hyperalgesia on Day 19 compared to Day 12 the magnitude of the increase in morphine’s potency in female rats was the same. Similarly, increases in potency and effectiveness were observed with butorphanol as well as oxycodone (only in males). The failure to observe a change in potency in female arthritic rats is not clear. Some researchers suggest that oxycodone’s antinociceptive effects are mediated by kappa and not mu opioid receptors (Nielsen et al., 2000; Ross and Smith, 1997; Ross et al., 2000). However, the potency of kappa opioid agonists have been shown to be increased in CFA-treated rats (Hylden et al., 1991; Neil et al., 1986), although others find their potency to be decreased (Millan et al., 1987). If oxycodone’s antinociceptive effects are mediated by both mu and kappa opioid receptors than the failure to detect a change in oxycodone’s potency may be the net effect of an increase and a decrease in potency mediated by mu and kappa receptors, respectively. Changes within the peripheral and central opioid systems (Millan et al., 1986; Stein et al., 1989; Zollner et al., 2003) in addition to alterations in other neurotransmitter systems, including the noradrenergic, serotonergic and glutamatergic systems (Guo et al., 2002; Hama et al., 2003; Hylden et al., 1991; Okamoto et al., 2002; Xie et al., 2002) may be responsible for the increased potency.

**Opioid-induced Antihyperalgesia.** All of the opioid agonists tested produced antihyperalgesic effects in both male and female rats. Each agonist returned the pain threshold of arthritic rats to the threshold level of non-arthritic rats and, each agonist was more potent at this effect in male than female rats. Importantly, this demonstrates that even lower efficacy agonists, like butorphanol, which fail to produce
antinociception, are capable of treating the hyperalgesia associated with a chronic pain state. Such a finding may be of clinical importance in that lower efficacy opioid agonists are associated with fewer side effects, including less respiratory suppression and less tolerance and dependence (Gringauz et al., 2001; Liguori et al., 1996; Pircio et al., 1976), compared to higher efficacy agonists like morphine. Therefore, treatment of chronic pain patients, who are non responsive to the pain relieving effects of nonsteroidal anti-inflammatory agents, but do not require the level of pain relief induced by the higher efficacy agonist morphine may receive adequate relief with lower efficacy opioid agonists. Although studies have investigated the effectiveness of opioid analgesics in human rheumatoid arthritis patients (e.g., Hardin and Kirk, 1979; Herrero-Beaumont et al., 2004; Tanaka et al., 2001), there have been no studies comparing the effectiveness of higher, intermediate and lower efficacy opioid agonists nor are there studies examining sex differences in responsiveness to opioid analgesics.

**Central Versus Peripheral Antinociception and Antihyperalgesia.** The results with loperamide suggest that the antihyperalgesic effects of opioid agonists are peripherally mediated. For example, loperamide was completely ineffective at producing antinociception in non-arthritic rats, yet morphine, which readily crosses the blood-brain-barrier, produced profound antinociception in these animals. Furthermore, that loperamide completely blocked CFA-induced hyperalgesia provides additional evidence that actions at peripheral opioid receptors are responsible for reversing hyperalgesia. Indeed, CFA treatment results in increased mu opioid receptor binding in the hindpaw and in the dorsal root ganglia of primary afferent neurons as well as increases in mu opioid receptor G-protein coupling in the dorsal root ganglia (Hassan et al., 1993; Mousa et al., 2001; Zollner et al., 2003). Zollner at al. (2003) also demonstrated that intrapaw administration of buprenorphine produces no antinociception in the non-CFA-injected paw, but profound antinociception in the CFA-injected paw. Interestingly,
loperamide also produced intermediate levels of antinociception in arthritic rats suggesting that the chronic-pain-induced changes within the peripheral nervous system were involved in mediating the increases in paw pressure thresholds above the VEH-treated thresholds. This demonstrates that changes within the peripheral nervous system of CFA-treated rats are enough to not only to mediate the reversal of the hyperalgesia but also to, in part, mediate increases in CFA thresholds above the baseline thresholds of VEH-treated rats (e.g., antinociception) under the current experimental conditions. This provides evidence that the mechanisms mediating antinociception and antihyperalgesia are not necessarily dissociable and that antihyperalgesia and antinociception fall along a continuum. The present results demonstrate that the central opioid system is involved in mediating the antinociceptive effects of morphine, butorphanol and oxycodone in VEH-treated rats, whereas their antinociceptive effects in CFA-treated rats are mediated by both the central and peripheral opioid systems. That intrapaw administration of naloxone methiodide antagonized the antinociceptive effects of s.c. morphine administration in CFA-treated rats provides additional evidence that morphine’s effects in arthritic rats are, in part, mediated by the peripheral opioid system. Taken together, the results demonstrate prominent sex differences in both the central and peripheral nervous systems that influence sensitivity to opioid agonists. The sex differences in the antinociceptive effects of opioid agonists can not be explained, however, by differences in mu opioid receptor G-protein coupling efficiency within the brain (Selley et al., 2003), yet, it remains to be determined if sex differences in G-protein coupling efficiency in the peripheral opioid system exist.

Summary. The present study demonstrated that profound sex differences exist in the magnitude of hyperalgesia as well as in the sensitivity to opioid-induced antinociception and antihyperalgesia in arthritic rats. The level of hyperalgesia was correlated with the magnitude of inflammation in both male
and female arthritic rats. Because female arthritic rats exhibited greater hyperalgesia than male arthritic rats, they may have required larger amounts of the opioid agonists to produce antinociception and hyperalgesia. That is, animals suffering from greater pain may be more resistant to the effects of opioid agonists. However, such conclusions may be dependent upon the type of algogenic agent employed, the duration of the nociception and the type of nociceptive stimulus. For example, no sex differences in the potency of opioid-induced antihyperalgesia were observed in rats injected with capsaicin in the tail even though female rats exhibited significantly greater thermal hyperalgesia (Barrett et al., 2003).
References


Footnotes

This work was supported by a National Institute on Drug Abuse grant (K12-DA14041) as well as funds from Virginia Commonwealth University’s Institute for Drug and Alcohol Studies. CDC is a Building Interdisciplinary Research Careers in Women’s Health (BIRCWH) Scholar and is supported by a National Institute on Drug Abuse career development award (K12-DA14041). Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee Virginia Commonwealth University, and the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press, 1996).
Legends for Figures

Fig. 1. Effects of VEH (N=6) and CFA (N=9) treatment in male and female rats on paw pressure thresholds on Days 12, 19 and 26. A. Comparison of male and female VEH-treated rats. B. Comparison of male VEH- and CFA-treated rats. C. Comparison of female VEH- and CFA-treated rats. D. Comparison of male and female CFA-treated rats. Brackets indicate SEM. * Indicates a significant difference (Bonferroni post-hoc) from same day VEH treatment group (panels B. & C.) or male rats (panel D.).

Fig. 2. Effect of estrous cycle phase on paw pressure thresholds in female VEH- and CFA-treated rats. Phase determination and thresholds were obtained in 12 VEH- and 20 CFA-treated rats on Days 12, 19 and 21 for a total of 36 and 60 observations in VEH- and CFA-treated rats, respectively. Numbers within bars indicate the number of observations that make up the mean for that phase. E: estrus, M: metestrus, D: diestrus and P: proestrus. Brackets indicate SEM. * Indicates a significant difference (LSD post-hoc) between stages.

Fig. 3. Percent increase in paw thickness of male (N=18) and female (N=20) CFA-treated rats on Days 12, 19 and 21. Brackets indicate SEM. * Indicates a significant difference (Bonferroni post-hoc) between male and female rats.

Fig. 4. Antinociceptive effects of morphine in male and female VEH- (N=6) and CFA-treated (N=10) rats on Day 12 (top panel) and Day 19 (bottom panel). Brackets indicate SEM; where not visible the SEM fell within the data point.
Fig. 5. Antinociceptive effects of butorphanol in male and female VEH- \( (N=6) \) and CFA-treated \( (N=10) \) rats on Day 12 (top panel) and Day 19 (bottom panel). * Indicates a significant difference (Bonferroni post-hoc) between female CFA- and VEH-treated rats. Other details as in Fig. 4.

Fig. 6. Antinociceptive effects of oxycodone in male and female VEH- \( (N=6) \) and CFA-treated \( (N=10) \) rats on Day 21. Other details as in Fig. 4.

Fig. 7. Antihyperalgesic effects of morphine (Day 19, top panel), butorphanol (Day 19, middle panel) and oxycodone (Day 21, bottom panel) in male (left side) and female (right side) CFA-treated rats \( (N=10) \) expressed in g. This graph represents the ability of each mu opioid agonist to return baseline thresholds of CFA-treated rats (BL) to the baseline thresholds of VEH-treated rats (VEH). Data above VEH indicate the non-drug baseline paw pressure thresholds of the control VEH-treated rats. The VEH-treated thresholds represent the maximal antihyperalgesic effect obtainable. Numbers on the X-axis indicate doses of the test drug in mg/kg. The dashed lines represent the thresholds of the VEH-treated control groups (e.g., maximal antihyperalgesia). Brackets indicate SEM. * Indicates a significant difference (Bonferroni post-hoc) relative to BL condition. Numbers within the bars represent the % Antihyperalgesic Effect with 100 % antihyperalgesia equal to the respective non-drug VEH-treated baseline thresholds.

Fig. 8. Antinociceptive (left panel) and antihyperalgesic (right panel) effects of loperamide in male and female VEH- \( (N=6) \) and CFA-treated \( (N=10) \) rats on Day 19. * Indicates a significant difference (Bonferroni post-hoc) between CFA- and VEH-treated male rats. # Indicates a significant difference (Bonferroni post-hoc) between male and female CFA-treated rats. Other details as in Fig. 4.
Fig. 9. Paw pressure thresholds (g) obtained in the right hindpaw of male (N=10) and female (N=10) CFA-treated rats following the administration of 5.6 mg/kg morphine alone (MS, s.c.) and in combination with 30 µg naloxone methiodide (NLX METH) administered into the right hindpaw. Following determination of thresholds 30 min post morphine administration naloxone methiodide was administered into the hindpaw with thresholds being re-determined 5 min later. Brackets indicate SEM. * Indicates a significant difference (Bonferroni post-hoc) between the morphine and morphine plus naloxone methiodide conditions.
Table 1.

Summary of tests conducted in each group of male and female rats and where the test results are presented in the Results section. Each group contains 6 VEH-treated rats and 10 CFA-treated rats unless otherwise noted.

<table>
<thead>
<tr>
<th>Group</th>
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<tr>
<td>Male⁴</td>
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<td></td>
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<tr>
<td></td>
<td>Day 26 morphine</td>
<td>Table 2</td>
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<td>Female⁴</td>
<td>body weight</td>
<td>-- ⁵</td>
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<td></td>
<td>Day 12, 19, 26 thresholds</td>
<td>Fig. 1</td>
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<td></td>
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<tr>
<td>Male⁵</td>
<td>Day 12, 19, 21 paw thickness</td>
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<td>Day 21 oxycodone</td>
<td>Figs. 6, 7</td>
</tr>
<tr>
<td>Female</td>
<td>Day 19 loperamide</td>
<td>Fig. 8</td>
</tr>
</tbody>
</table>
Day 21 morphine + naloxone methiodide

Male

Day 19 loperamide

Day 21 morphine + naloxone methiodide

Fig. 9

Fig. 8

Fig. 9

\(^a\) \(N = 9\) for CFA-treated rats

\(^b\) Body weight data are described in the Body Weight section of the Results

\(^c\) \(N = 8\) for CFA-treated rats. Two rats failed to develop any signs of inflammation and were excluded from the analysis.
Table 2.

ED$_{50}$ values (95% confidence limits) for morphine$^a$ on Days 12, 19 and 26 in male and female VEH- and CFA-treated rats.

<table>
<thead>
<tr>
<th>Day</th>
<th>Sex</th>
<th>VEH</th>
<th>CFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 12</td>
<td>Male</td>
<td>2.84 (2.14-3.78)</td>
<td>2.64 (2.02-3.43)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.67 (2.62-5.14)</td>
<td>2.41 (2.06-2.83)$^b$</td>
</tr>
<tr>
<td>Day 19</td>
<td>Male</td>
<td>2.95 (2.17-4.01)</td>
<td>1.51 (1.15-1.99)$^{b,c,d}$</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.50 (2.66-4.61)</td>
<td>2.37 (2.05-2.74)$^b$</td>
</tr>
<tr>
<td>Day 26</td>
<td>Male</td>
<td>3.05 (2.42-3.83)</td>
<td>1.43 (1.07-1.92)$^{b,c}$</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.37 (2.44-4.64)</td>
<td>2.38 (1.93-2.93)</td>
</tr>
</tbody>
</table>

$^a$ Morphine (0.03-5.6 mg/kg) was tested in the same groups of rats on Days 12 and 19. A separate group of rats was tested with morphine (1.0-10 mg/kg) on Day 26.

$^b$ More potent in CFA-treated than VEH-treated rats as determined by relative potency ratios.
c More potent in male than female CFA-treated rats as determined by relative potency ratios.

d More potent in male CFA-treated rats on Day 19 than Day 12 as determined by relative potency ratios.
Figure 1
Figure 2

This figure shows a bar graph comparing the effect of VEH (vehicle) and CFA (complete Freund's adjuvant) on paw pressure across different stages of the estrous cycle. The cycle is divided into four phases: E (estrus), M (metestrus), D (diestrus), and P (proestrus). The graph displays the mean paw pressure (in grams) for each phase, with error bars indicating the standard error of the mean. The number of observations for each phase is indicated at the bottom of the bars: E (3), M (12), D (18), and P (3) for VEH; and E (4), M (26), D (23), and P (7) for CFA. Asterisks denote statistically significant differences between the VEH and CFA groups within each phase.
Figure 3

% Increase in Paw Thickness

- Male - CFA
- Female - CFA

Day 12: Male - CFA (20 ± 5%), Female - CFA (10 ± 3%)
Day 19: Male - CFA (80 ± 10%), Female - CFA (100 ± 5%)*
Day 21: Male - CFA (80 ± 10%), Female - CFA (100 ± 5%)

* Significant difference between male and female groups.
Figure 4: Graphs showing % Antinociception over different doses of Morphine (mg/kg) for Day 12 and Day 19, with different symbols representing Male - VEH, Female - VEH, Male - CFA, Female - CFA.
Day 21

% Antinociception

Oxycodone (mg/kg)

Figure 6
Figure 7
Figure 8
Day 21

![Graph showing paw pressure comparison between male and female subjects at 5.6 MS and +30 NLX METH.](image_url)

- Male
- Female

Paw Pressure (g)

500
450
400
350
300
250
200
150
100
50
0

5.6 MS
+ 30 NLX METH

Figure 9