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The influence of dextromethorphan on morphine analgesia in Swiss Webster mice is sex-specific

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Abstract

NMDA (*N*-methyl-D-aspartate) antagonists are known to enhance the analgesic effects of opioids. However, virtually, all studies of this phenomenon have been done using male subjects. Here, the noncompetitive NMDA receptor antagonist dextromethorphan (DEX) was tested over a range of doses (10–200 μg intracerebroventricularly [i.c.v.]) in male and female Swiss Webster mice in combination with 5 mg/kg intraperitoneal (i.p.) morphine. Males exhibited enhanced morphine analgesia following either 100 or 200 μg DEX, but there was no evidence of DEX-mediated potentiation in females at any dose. Instead, DEX attenuated morphine analgesia in females. We also evaluated the effect of 100 μg i.c.v. DEX with different doses of morphine (1, 5 and 10 mg/kg). Again, DEX significantly enhanced morphine analgesia in male mice and attenuated it in females. Next, ovariectomized (OVX) female mice were compared to males following 5 mg/kg i.p. morphine and 100 μg i.c.v. DEX. Male and OVX females exhibited equivalent maximal levels of analgesia following administration of DEX. Morphine analgesia was not enhanced by DEX in sham-treated females and OVX mice with estradiol treatment (5 μg i.p. once daily for 7 days) also did not show DEX enhancement. These experiments demonstrate that the ability of NMDA receptor antagonists to potentiate morphine analgesia is modulated by an estrogen-sensitive mechanism and suggest that sex differences may play a critical role toward a more general understanding of the potentiation of opioid-induced analgesia through NMDA receptor antagonists.

Keywords: Morphine; NMDA [*N*-methyl-D-aspartate]; Dextromethorphan; Sex; Mice; Analgesia

Morphine has remained the drug of choice for the treatment of moderate to severe pain for well over a century. However, tolerance to morphine or other opiate drugs can be profound and debilitating, and detract markedly from the intended therapeutic benefits. Tolerance, defined as a decrease in the potency of a drug following repeated administration (Cox, 1990), can occur during a single administration of an opioid (Kornetsky and Bain, 1968). In order to enhance the therapeutic efficacy of opioids such as morphine, researchers have sought ways to minimize tolerance and other adverse effects (see Bhargava, 1994 for review).

NMDA receptors are thought to play a role in the adaptation to opioids. NMDA antagonists attenuate the development of tolerance to morphine (Trujillo and Akil, 1991; Tiseo and Inturrisisi, 1993; Lufty et al., 1999) and there is a large body of research demonstrating that blockade of NMDA receptors potentiates opiate analgesic efficacy over a range of assays in a variety of species (e.g., Chapman and Dickenson, 1992; Advokat and Rhein, 1995; Mao et al., 1996; Plesan et al., 1999; Wen et al., 2004). For example, enhancement of opiate analgesia by NMDA receptor blockade has been reported in various strains of male mice (Bernardi et al., 1996; Bhargava, 1997; Lufty et al., 1999;

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Redwine and Trujillo, 2003), rats (Bespalov et al., 1998; Plesan et al., 1999; Christensen et al., 1998, 1999; Carlezon et al., 2000; Nishiyama, 2000; Kozela et al., 2001; Bulka et al., 2002; Laulin et al., 2002) and non-human primates (Allen and Dystra, 2001).

Despite what appears to be ample evidence for NMDA mediation of opioid analgesia, when NMDA receptor antagonists have been used to either enhance the analgesic effects of opiates or to attenuate tolerance, in the vast majority of studies, only male subjects have been used. This is noteworthy because there are known sex differences in pain thresholds and in the pharmacological effects of morphine and NMDA antagonists (e.g., Kepler et al., 1991; Mogil et al., 1993; Kavaliers and Galea, 1995; Cicero et al., 1996; Boyer et al., 1998; Kest et al., 1999; Sarton et al., 2000). Indeed, we recently published a broad survey of the effects of several NMDA antagonists on morphine analgesia and evaluated both male and female mice (Nemmani et al., 2004). In this study, we reported that sex, along with the site of antagonism, morphine dose and time after injection, all significantly influenced morphine response. Others have found similar results: Holtman et al. (2003) showed that sex influenced NMDA receptor-mediated effects on morphine analgesia in rats. It is also worth noting that not all studies, even using males, find unequivocal potentiation of morphine effects following NMDA antagonism (see Kozela et al., 2001 or Redwine and Trujillo, 2003 for reviews).

In order to further study the sex-dependency of opiate analgesia by NMDA antagonists, we compared the effect of dextromethorphan, a noncompetitive NMDA receptor antagonist, combined with morphine in male, female and ovariectomized female mice.

1. Materials and methods

1.1. Animals

Swiss Webster mice of both sexes were bred in-house from stock obtained from Taconic Farms (Germantown, NY) and maintained 2–4 per Thoren caging in a temperature (21±2 °C) and light (12 h reverse light/dark cycle; lights off at 0700 h) controlled colony room. Food and water were freely available at all times and corncob bedding was changed twice a week. Between 7 and 12 weeks of age, naïve mice were taken from the colony room in their home cages to a procedural room and allowed to habituate to the experimental room for at least 30 min before testing. All experiments were approved by the Furman University animal care and use committee and in accordance with NIH guidelines.

1.2. Nociceptive assay

Pain sensitivity was measured by determining the latency to reflexive withdrawal of the tail (TW) following immersion in warm water bath (Ben-Bassat et al., 1959). Animals were lightly restrained in a cloth container (voluntarily entered in all cases) and the distal half of the tail was immersed in 49 °C water. The latency to reflex withdrawal was measured using a stopwatch to the nearest 0.1 s by an experienced observer blind to drug condition. To improve accuracy, two TW latency determinations, separated by approximately 10 s, were made and averaged at each time point. A cut-off response latency was set at 15 s to prevent tissue damage.

1.3. Drug administration

Morphine sulfate (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline and delivered intraperitoneally (i.p.) using a 26 g 3/8″ needle. Dextromethorphan (DEX; Sigma-Aldrich) was also dissolved in saline but with 5% acetic acid. DEX (or vehicle, saline with 5% acetic acid) was delivered intracerebroventricularly (i.c.v.). in order to insure that equal amounts of DEX were present in the central nervous system of male and female mice. I.c.v. injections were always in a volume of 2.5 μl/mouse and were made directly into the left lateral ventricle using the method of Laursen and Belknap (1986) under isofluorane anesthesia. Isofluorane is a safe, short-acting, inhalant anesthetic with no reported interactions with morphine, DEX or gonadal steroids.

1.4. Group designations and testing procedures

Data included in this study represent three separate experiments. In the first, we evaluated the effect of various doses of DEX (10, 100 or 200 μg) on analgesia resulting from 5 mg/kg morphine. We assessed TW latencies in male and female mice, delivered i.c.v. DEX or vehicle immediately followed by i.p. injection of morphine, and then reassessed TW latencies at 30, 60, 90 and 120 min. For the second experiment, we kept the dose of DEX constant at 100 μg/mouse, but varied morphine doses (0, 1, 5 or 10 mg/kg). We chose this middle dose of DEX to minimize the likelihood of either "floor" or "ceiling" effects in our two-tailed analyses. Administration and testing procedures were the same as in the first experiment except that post-injection TW latencies were determined at 30, 60 and 120 min. In the last experiment, we evaluated the effect of 100 μg DEX in combination with 5 mg/kg morphine (again at 30, 60, 90 and 120 min post-injection) and we also included female subjects that varied with respect to hormone status (see below). There were 8–15 subjects per experimental group.

1.5. Surgical procedure and hormone replacement

Female mice in the last experiment involving gonadal hormones received either ovariectomy (OVX) or sham ovariectomy (SHAM) via dorsal incision under ketamine/xylazine anesthesia as described in Mogil et al. (1993). Males were similarly anesthetized, but no surgical manipulation occurred. Animals were allowed 10–14 days between surgery

and testing in order to be sure recoveries were complete and gonadal hormones were depleted in OVX animals. On the first of 2 test days, half of each group was administered i.p. morphine+i.c.v. DEX (100 μg) and the other half received morphine+vehicle. Beginning 2–4 days after this assessment, ovariectomized mice were given daily intraperitoneal (i.p.) injections of 5 μg estradiol (Sigma-Aldrich) in 0.1 ml sesame oil for 7 days. Males and sham-treated females were administered equivolume vehicle (sesame oil) throughout this period. On the following day, all animals were re-tested for antinociception to morphine, in a crossover design following i.c.v. injection (subjects receiving saline during the first test got DEX and vice versa). Although ketamine is also an NMDA receptor antagonist, and thus could interact with subsequent DEX administration, the long intervening period (at least 10 days) decreases this possibility, but more importantly, because all mice received equal anesthetic treatment, this is especially unlikely to account for any sex differences.

1.6. Statistics

Raw TW data were analyzed using two-way (drug and sex) repeated measure analysis of variance (ANOVA). In order to determine which groups were different from their controls, within-sex (single factor) ANOVAs were conducted and pair wise comparisons were evaluated using Dunnett's two-sided post-hoc test (SYSTAT 10.2). Raw TW data were also converted to antinociceptive area under the time×TW latency curve for each animal (AUC, min * s), analyzed by two-factor ANOVA, then further evaluated in the same manner as above. In all cases, the criterion for statistical significance was set at $p<0.05$.

2. Results

The results of the first two experiments indicate that DEX influenced morphine analgesia differently in male and female mice. While there were no differences between the sexes in morphine-induced antinociception, DEX potentiated morphine analgesia in males, but attenuated it in females (indicated by a significant interaction between sex and drug condition on TW latencies: $F_{(3,46)}=8.90$, $p<0.01$). Analyzed and presented are the mean TW latencies for each group as well as the geometric areas under the TW latency curves in order to depict both the time course (duration) and overall effects of the drug treatment in each group.

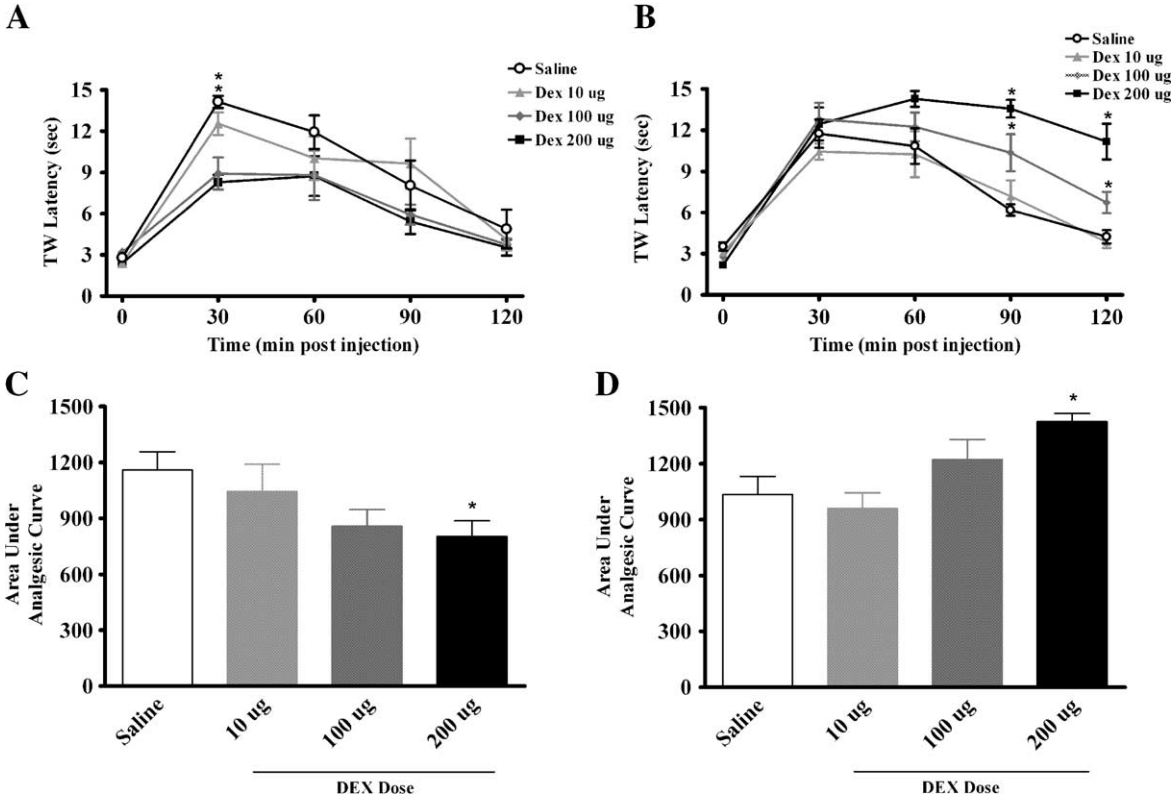

Fig. 1. The top panels of this figure depict the antinociceptive responses of female (A) or male (B) Swiss Webster mice to intracerebroventicular (i.c.v.) administration of either 10, 100 or 200 μg dextromethorphan (DEX) followed immediately by intraperitoneal (i.p.) administration of 5 mg/kg morphine. For males given 100 and 200 μg DEX, statistically significant differences in TW latencies compared to saline alone occurred at 90 and 120 min (*$p<0.05$, Dunnett's t). The lower panels summarize the same data collapsed for time in order to compare the areas under the analgesic curve (AUC) as a function of different doses of DEX in females (C) or males (D). DEX produced a dose-dependent attenuation of the AUCs in females, while a dose-dependent enhancement of the AUCs was produced in males. In both cases at 200 μg DEX, AUCs were statistically different from saline (*$p<0.05$, Dunnett's t).

The top panels of Fig. 1 show the time course for the effects of different doses of DEX on morphine analgesia in both females (Fig. 1A) and males (Fig. 1B). Overall, there was more analgesia in males, due to potentiation and prolongation of morphine effects by DEX. Fig. 1C and D show the area under the analgesic curves (AUC) for each dose of DEX. In both males and females, there was a dose-dependent effect of DEX in which DEX induced an attenuation of analgesia in females at 200 μg i.c.v. and an enhancement of morphine analgesia in males at this same dose (Dunnett's test $p<0.05$ in both cases).

In a [2 (sex)×4 (drug dose)] analysis, a main effect of sex ($F_{(1,46)}=10.93$, $p<0.01$) was obtained supporting the observation that male mice were more analgesic overall. In addition, DEX changed the shape of the analgesia curve in both sexes as it altered the early effect of morphine in females and prolonged the effect of morphine in males. Thus, there was an interaction between time and sex ($F_{(4,184)}=2.6$, $p<0.05$) and an interaction between time and drug condition ($F_{(12,184)}=2.24$, $p<0.05$) as well as a triple interaction between time, sex and drug indicating that the effect of DEX on the analgesic curve was dependent upon sex. These results are depicted again in the lower panel, showing the AUC data and borne out by analysis: a main effect of sex ($F_{(1,46)}=8.37$, $p<0.05$), and an interaction between sex and drug ($F_{(3,46)}=7.70$, $p<0.01$). The potentiation seen in males following either of the higher two doses was still present 120 min after morphine administration (Dunnett's post-hoc test, $p<0.05$ in both cases).

In the second experiment, testing the effect of DEX combined with different doses of morphine, DEX again affected morphine analgesia differently in males and females (Fig. 2). A repeated measure ANOVA indicated significant effects of drug ($F_{(5,92)}=5.23$, $p<0.01$), sex ($F_{(1,92)}=5.96$, $p<0.05$) and their interaction ($F_{(5,92)}=3.39$, $p<0.01$). There was also a time×drug interaction ($F_{(15,276)}=4.62$, $p<0.01$), and again, a triple interaction between time, drug and sex ($F_{(15,276)}=1.85$, $p<0.05$) reflecting the fact that DEX prolonged morphine analgesia in male mice (Fig. 2B). In males given 5 or 10 mg/kg of morphine in combination with DEX, analgesia lasted at least 2 h. This was not the case in males without DEX treatment, or in females receiving DEX (Fig. 2A), as these groups were all back to baseline nociceptive values at this time point. Using Dunnett's test to compare each of the drug treatment groups to a control group receiving neither morphine nor DEX (S-S), DEX was found to enhance morphine-induced analgesia in males only at 10 mg/kg. Moreover, DEX was found to obviate the analgesia seen in female mice receiving either 5 or 10 mg/kg DEX as neither of these doses produced significant analgesia when combined with 100 μg DEX. When combined with 1

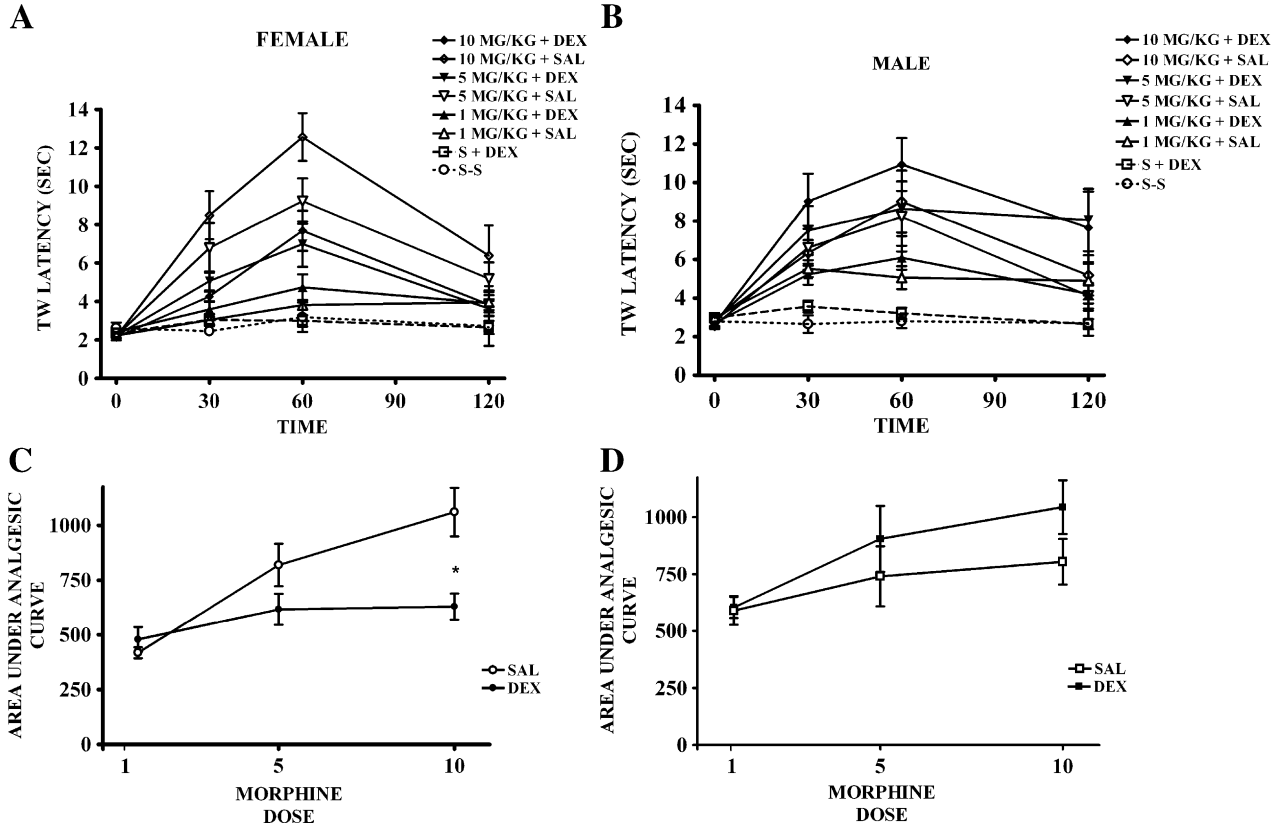

Fig. 2. Panels A and B illustrate the effect for different doses of parental morphine when DEX was co-administered i.c.v. at a single dose of 100 μg. DEX was given in combination with 1, 5 or 10 mg/kg i.p. morphine. DEX enhanced and prolonged morphine antinociception in males and attenuated it in females. The lower panels, showing the area under the analgesic curves (AUC), summarize these differential effects of DEX in males and females.

mg/kg morphine, there was no effect of DEX on morphine-induced analgesia in either sex. AUC data are shown in Fig. 2C (female) and D (males).

In the third experiment, ovariectomy reversed and estrogen replacement in ovariectomized mice restored—the female-specific phenotype. Again, DEX potentiation of morphine analgesia was evident in males, as well as in ovariectomized female mice, but not in other groups (Fig. 3). ANOVA of the raw TW data for Experiment 3 (see Fig. 3A), analyzed in a 2 (drug condition; saline vs. 100 μg DEX) × 4 (sex/hormone status) design showed a significant difference between the drug conditions ($F_{(1,130)}=12.45$, $p<0.01$) and a significant effect of sex ($F_{(3,130)}=11.86$, $p<0.01$). Again, the interaction was significant, indicating that the effect of drug was dependent upon sex ($F_{(3,130)}=7.43$, $p<0.01$). Within subjects, there was an effect of time ($F_{(4,520)}=279.60$, $p<0.01$) and a significant interaction between time and drug ($F_{(4,520)}=3.38$, $p<0.05$) as well as a triple interaction ($F_{(12,520)}=2.41$, $p<0.01$). The pattern of results is evident in the summarized AUC data shown in Fig. 3B: OVX mice were indistinguishable from males (DEX enhanced analgesia in both cases), which were both different from either sham-treated or OVX females given estradiol replacement. Although the specific comparison between sham females receiving either DEX or saline was not significant, there was a tendency ($p=0.06$, see # in Fig. 3B) for DEX to reduce analgesia in these intact females.

3. Discussion

These data indicate that ovarian hormones modulate the effect of dextromethorphan on morphine analgesia. Enhancement of morphine analgesia occurred in male mice following co-administration with DEX, but the opposite was generally true in females. These differences are probably mediated by estrogen or its metabolite(s) because ovariectomized (OVX) females, like males, showed a robust DEX-mediated enhancement of morphine analgesia. Moreover,

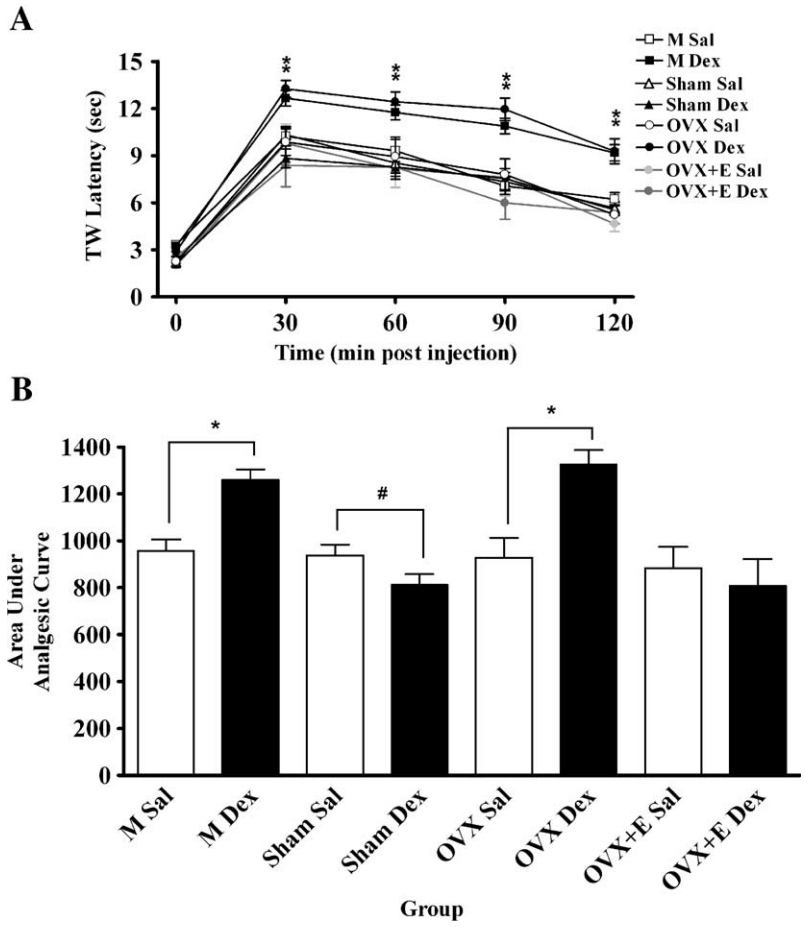

Fig. 3. This figure illustrates the effect of saline or 100 μg i.c.v. DEX, followed by 5 mg/kg i.p. morphine on TW latencies in either intact males, intact (sham-treated) females, ovariectomized (OVX) females or OVX females with estradiol replacement. Both males and OVX females given DEX and morphine exhibited a significant increase in duration and amount of antinociception as compared to intact females or OVX females receiving estradiol treatment (Panel A). Panel B summarizes these data by showing the area under the analgesic curves (AUC) for each group. Asterisks signify significant differences between DEX- and saline-treated subjects within each sex condition, while the # sign reflects a non-significant ($p=0.06$) tendency for DEX attenuation of analgesia in the sham surgery group.

OVX mice receiving estradiol replacement also failed to exhibit DEX-enhanced morphine analgesia.

In order to evaluate the influence of DEX directly and to avoid possible sex differences in the peripheral metabolism of DEX (e.g., Ramachander et al., 1978: Labbe et al., 2000), central (i.c.v.) administration was used. There are two other studies reporting DEX effects on opioid analgesia interacting with sex (Holtman et al., 2003; Nemmani et al., 2004), but both of these evaluated the effects of NMDA antagonists delivered systemically. This procedural difference may account for conflicting results across experiments. In our previous report (Nemmani et al., 2004), males showed effects of DEX akin to those we found here, but females did not show any alteration in morphine analgesia by systemic (oral) administration. Following intraperitoneal administration, Holtman et al. (2003) found slightly more DEX-mediated enhancement of morphine analgesia in female than male rats, a discrepancy that may be due to species influences. Unfortunately, there are so few studies that include both male and female subjects that general conclusions about the mechanisms underlying sex-specific modulation of NMDA-opioid interactions are not possible at this time. We would predict that cyclic changes in ovarian hormones might influence the way that DEX interacts with morphine, and also may explain why the attenuation of morphine analgesia by DEX in females was variable (clearly present in Experiment 1, nearly evident in Experiment 2 and absent in Experiment 3) though this hypothesis needs to be tested directly.

Clinical trials using NMDA antagonists (Mercadante et al., 2000; Wu et al., 2000a,b; Heiskanen et al., 2002) have also found enhancement of opiate effects and, as a matter of regulatory requirement, do include both sexes. However, the average age in each of these studies is between 50 and 60 years (i.e., post-menopausal). Moreover, the authors neither analyze the data by sex or age, so it is impossible to discern the relative efficacy in females or how this might depend upon hormonal status. However, Bell (1999) evaluated the effect of adding ketamine to the treatment regimen of opiate tolerant humans in case studies of 3 subjects: 2 males and 1 female. Both males showed dramatic improvement in analgesia levels when morphine was combined with ketamine, but Bell reports that in the female subject analgesia was "insufficient".

Multiple mechanisms no doubt underlie the behavioral and neural modifications associated with chronic opiate use. How precisely NMDA-receptors influence morphine effects is not entirely understood, but over the past several years a dominant model has emerged that suggests opioid potentiation and reversal of tolerance by NMDA receptor antagonists may be due to the same cellular mechanisms (prevention of opioid receptor desensitization; see Trujillo, 2002 for a review). For example, NMDA receptor activation contributes to acute tolerance following a single exposure to an opiate (Larcher et al., 1998) and a lack of acute opioid tolerance (see Wang and Ho, 1994 for review or cf.: Kornetsky and Bain, 1968) may mediate the potentiation of morphine analgesia seen in the presence of NMDA antagonists. Thus, antagonism of NMDA receptor activity may attenuate opioid receptor desensitization, preventing acute tolerance after a single dose of opioid. This protection against acute opioid tolerance may be manifest in behavioral assays as analgesic potentiation. Again, there is ample evidence that NMDA receptor antagonists alter the development and/or expression of tolerance in male mice (Kolesnikov et al., 1988; Kolesnikov and Pasternak, 1999; Lufty et al., 1999; Popik and Kozela, 1999; Belozertseva et al., 2000; Redwine and Trujillo, 2003) and rats (Mao et al., 1996; McNally and Westbrook, 1998; Houghton et al., 2001; Quartaroli et al., 2001; or see Mao, 1999; Trujillo, 2000 for reviews). NMDA receptor antagonists have also been shown to block opiate withdrawal in males (see Mao, 1999; Trujillo, 2000 for reviews). We are currently investigating the ability of NMDA-receptor antagonists to prevent tolerance in females, but for now this is an open question.

The mechanisms underlying estrogen's effect on NMDA receptors have not been fully elucidated, but there is a substantial literature on estrogen modulation of NMDA receptors (see McEwen et al., 1997 for review). For instance, Cyr et al. (2001) review evidence showing that both OVX and estradiol treatment alter NMDA receptor specific binding in various brain regions. Estrogen has also been shown to alter expression of NMDA receptors (D'Souza et al., 2003) and to inhibit both NR1 and NR2a subunit mRNA levels (Gore et al., 2002). In fact, steroids can modulate a variety of voltage and ligand-gated ion channels and, in particular, estrogenic compounds directly inhibit NMDA receptor-mediated neuronal responsiveness (Park-Chung et al., 1997; Weaver et al., 1997). Estrogenic steroids can also affect NMDA receptor-mediated behaviors. For example, Kalkbrenner and Standley (2003) lowered NMDA-induced seizure threshold, as well as hippocampal damage resulting from seizures, in females through ovariectomy. Estrogen also interacts with central NMDA receptors to enhance LTP in the hippocampus (Good et al., 1999) and to provide neuroprotection against antagonist interference with LTP. Gureviciene et al. (2003) report that the blockade of LTP and lowered acquisition of water maze learning by NMDA antagonism, are both ameliorated by estrogen treatment. Taken together with the present results, these studies support a role for estrogen in negative modulation of NMDA receptor activity.

It is worth noting, however, that DEX is not a specific antagonist, and its interaction with opiates could occur through its effects on other systems. For example, nicotinic receptors have been implicated in pain transmission and are also under control of estrogen (Lapchak et al., 1990; Nakazawa and Ohno, 2001; Curtis et al., 2002). In particular, DEX and its metabolite dextrorphan have been

found to act as antagonists at the $\alpha_3\beta_4$ neuronal nicotinic receptor (Hernandez et al., 2000; Damaj et al., 2005).

The fact that we saw no evidence of DEX-mediated potentiation of morphine analgesia in intact female subjects suggests that estrogen may mediate qualitative sex differences in the adaptive response to morphine. A better understanding of NMDA-modulation of opiate analgesia and tolerance, as well as the way that these mechanisms interact with sex steroids, may aid in the development of new and better pharmacotherapies to alleviate chronic or severe pain (Wiesenfeld-Hallin, 1998). To help expedite these advances, both basic studies and clinical trials ought to pay particular attention to variables such as sex and hormonal status in order to facilitate understanding of pain sensitivity, antinociception and opiate tolerance in both males and females.

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