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Characterization of the Antinociceptive and Pronociceptive Effects of Methadone in Rats

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Background: **Recently, it has been appreciated that in addition to their antinociceptive properties, opioid analgesics also can enhance pain sensitivity (opioid-induced hyperalgesia [OIH]). OIH may enhance preexisting pain and contribute to dose escalation, tolerance, and misuse/abuse of opioids. Better information is needed to determine which opioid or opioid combinations may be least likely to produce OIH and therefore possibly represent better choices for pain management. Herein the authors have examined the hyperalgesic and antinociceptive properties of racemic methadone and its enantiomers alone and in combination with morphine in rats. Methadone is of particular interest because it possesses both μ-receptor agonist and *N*-methyl-D-aspartate receptor antagonist activities.**

Methods: **The antinociceptive and hyperalgesic properties of *d,l*-methadone, *l*-methadone, and *d*-methadone were characterized by dose and sex using the thermal tail-flick test (high and low intensity). The responses to *l*- and *d*-methadone combinations with morphine were also determined with this model.**

Results: **Antinociceptive and hyperalgesic effects of *d,l*-methadone were demonstrated. These effects were related to dose but not to sex. The degree of hyperalgesia was greater with *l*-methadone compared with *d,l*-methadone. In contrast, *d*-methadone (*N*-methyl-D-aspartate antagonist) did not produce hyperalgesia. Furthermore, *d*-methadone blocked morphine hyperalgesia, enhanced antinociception, and abolished sex-related differences. This seems to be the result of antagonistic activity of *d*-methadone at the *N*-methyl-D-aspartate receptor.**

Conclusion: **The current findings with methadone are supportive of previous findings implicating μ-opioid and *N*-methyl-D-aspartate receptor mechanisms in OIH. Better understanding of OIH may help in choosing the most appropriate opioids for use in the treatment of pain.**

OPIOID analgesics are widely used for the treatment of moderate to severe pain. There is a line of evidence that μ-opioids (*e.g.*, morphine, fentanyl), in addition to activating a pain inhibitory system (analgesia), also activate a pain facilitatory system (hyperalgesia).[1,2] This paradoxical pain-enhancing effect has typically been demonstrated as a response observed later in the time course after analgesia both in humans[3–7] and rodents[8–15] and referred to as a delayed hyperalgesia. We have found that the pain inhibitory and pain facilitatory systems can be studied independently by using high (antinociceptive) and low (subantinociceptive) doses of opioids such as morphine and oxycodone.[16,17] Our findings on low-dose morphine–produced hyperalgesia in rats were recently confirmed in mice.[18] The mechanism of opioid-induced hyperalgesia is unclear. The *N*-methyl-D-aspartate (NMDA) receptor seems to be involved as evidenced by the fact that noncompetitive NMDA antagonists (*e.g.*, MK-801, ketamine) have been shown to block both delayed hyperalgesia (high dose of an opioid)[8,11,14,15,19] and immediate hyperalgesia (low dose of an opioid).[16–18] The potential impact of opioid-induced hyperalgesia in the clinical use of opioids has been recently appreciated.[1,20] Possible influences of opioid-induced hyperalgesia include enhancing preexisting pain, contributing to dose escalation and tolerance, as well as drug-seeking behavior and the misuse or abuse of these analgesic drugs. Thus, there is a growing clinical interest in better understanding which specific opioids, opioid combinations, and dosing regimens might be most appropriate for treating pain patients, in particular those with chronic pain, to minimize opioid-induced hyperalgesia.

Methadone is an opioid analgesic that differs from classic opioids (*e.g.*, morphine) in that in addition to μ-opioid agonist activity, it also seems to act as a noncompetitive antagonist at the NMDA receptor.[21–23] Methadone is clinically used as a racemic mixture of the levorotatory (*l*) and dextrorotatory (*d*) isomers. The opioid-like activity of the racemate seems to be almost entirely due to *l*-methadone,[24–27] while *d*-methadone has been shown to act as an NMDA antagonist.[28,29] This unique characteristic of *d,l*-methadone presented an interesting possibility for examining the interaction between its opioid and NMDA activities and how they might contribute to the antinociceptive and pronociceptive actions of this drug. Furthermore, use of the individual methadone enantiomers (*l*- and *d*-methadone) allowed a separation of these activities.

We examined the effects of methadone in rats of both sexes. This was of interest because both the antinociceptive[30–35] and pronociceptive[16,17] effects of opioids are influenced by sex. Relatively little is known about sex-related differences with methadone.[36,37] The differing responses between sexes with opioids have implicated NMDA receptor mechanisms.[38–40]

The specific purpose of this study was to further characterize the interaction between μ-opioid and NMDA receptors in antinociception and hyperalgesia. We have

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done this by using a clinically important analgesic, *d,l*-methadone, which has both μ-opioid and NMDA antagonist properties. In addition, the individual enantiomers (*d* and *l*) of methadone were also studied. First, we determined whether methadone has pronociceptive properties (hyperalgesia) and whether it was a property of the *l*- and/or the *d*-enantiomer. Next, we determined whether methadone antinociception and/or hyperalgesia were related to the sex of the rat. Finally, we examined the relation between *d*- and/or *l*-methadone and morphine on antinociception and hyperalgesia. This preclinical study may be of importance in helping to guide the appropriate use of methadone alone and in combination with other μ-opioids in the clinical setting.

Materials and Methods

Animals

Age-matched (85–90 days old) male and female (weighing approximately 350 and 250 g, respectively) Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in this study. Rats were housed in accordance with *Guide for the Care and Use of Laboratory Animals*[41] in a humidity and temperature-controlled facility with lights on 06:00–18:00 h. Each rat was kept separately in a transparent cage with a sawdust-covered floor and free access to tap water and standard laboratory chow. Male and female rats were separately housed and were tested on alternate days. Body weights were determined on the day of experiment. The estrous cycle was not determined in female rats. The effect of possible fluctuation in baseline values was controlled by normalization of responsiveness to a drug for preinjection baseline each day in each rat. All experiments were conducted during the light phase of the cycle (09:00–14:00 h). Rats were tested each day in the same order. All rats were handled and trained before the initiation of the study. At the end of the experiment, the rats were killed with pentobarbital sodium (120 mg/kg). The experiments were performed according to a protocol approved by the University of Kentucky Animal Care and Use Committee.

Drugs

The drugs used were *d,l*-methadone hydrochloride ((±)-methadone; Sigma-Aldrich, St. Louis, MO), *d*-methadone hydrochloride (*S*(+)-methadone; Sigma-Aldrich), *l*-methadone hydrochloride (*R*(−)-methadone; National Institute on Drug Abuse, Research Triangle Institute, Research Triangle Park, NC), and morphine sulfate (Mallinckrodt, St. Louis, MO); drugs were dissolved in normal saline (0.9%) and were injected by the intraperitoneal route (1 ml/kg). Doses are expressed as the salts. A "low dose" of methadone or morphine refers to doses that are in a subantinociceptive range.

Tail-flick Test

The response to radiant heat was determined by the tail-flick test using a standard tail-flick apparatus (EMDIE Instrument Co., Roanoke, VA). Tail-flick latency (TFL) was measured by recording the time from the onset of heat stimulus to the tail to withdrawal of the tail from the heat source. The high-intensity radiant heat was used to determine responsiveness to antinociceptive doses of methadone or morphine (power intensity = 2.5; average baseline TFL = 2–3 s; a cutoff time = 10 s). To more readily observe the effects of subantinociceptive doses of methadone and morphine, a low-intensity radiant heat was used (power intensity = 1.0; average baseline TFL = 8–10 s; cutoff time = 20 s).[16,42] The TFL was measured before injection (twice, 15 min apart) and at 15, 30, 60, and 120 min after injection of the drug(s). High- and low-intensity thermal stimuli preferentially activate A-δ and C fibers, respectively.[43] Therefore, to allay concern as to using different intensities of radiant heat, it is important to note that morphine (high dose)–produced antinociception and delayed hyperalgesia were demonstrated using both high- and low-intensity tail-flick tests in rats. In addition, morphine (low dose)–produced hyperalgesia was confirmed on several assays, including low-intensity tail-flick, hot plate, and paw pressure assays[16] as well as tail-withdrawal test (47.3°C[18] and 52°C[44]).

Procedures

Male and female rats were treated as follows:

A single drug:

1. *The antinociceptive effect* (the high-intensity tail-flick assay): *d,l*-Methadone (0.5, 1, 3, 5 mg/kg) was administered in 1-week intervals until all doses were tested in each rat (8 rats/sex). A crossover paradigm was used to balance the order of doses (a Latin square design, 2 × [4 × 4]). Weekly intervals ensured washout of the drug (methadone half-life = 70–90 min in the rat).[45]
2. *The hyperalgesic effect* (the low-intensity tail-flick assay): *d,l*-Methadone (1, 10, 100 μg/kg), *d*-methadone (1, 10, 100 μg/kg), and *l*-methadone (1, 10, 100 μg/kg) were administered at weekly intervals (randomized doses; 8 rats/drug/sex). Saline (1 ml/kg) served as control. In addition, a single dose of *l*-methadone (1 μg/kg) was administered to determine the duration of its hyperalgesic activity (3 rats/sex).

Drug combination:

1. *The antinociceptive effect* (the high-intensity tail-flick assay): *d,l*-Methadone (10 μg/kg), *d*-methadone (10 μg/kg), and morphine (1 mg/kg) were given alone or in combination (8 rats/treatment/sex).
2. *The hyperalgesic effect* (the low-intensity tail-flick assay): Morphine (20 μg/kg) was given alone or in combination with *d*-methadone (10 μg/kg) or *l*-methadone (10 μg/kg) (8 rats/treatment/sex). Doses were based on our previous findings that morphine (1

mg/kg) provided a consistent but low antinociception (male > female), whereas morphine (20 μg/kg) produced hyperalgesia (female > male) in rats.[16]

Statistical Analysis

All values presented are mean ± SEM of n rats. For each rat at each time point, the responses (TFL) were normalized for preinjection baseline values. The antinociceptive and hyperalgesic effects were defined as any significant increase and decrease from baseline value, respectively. Antinociception was determined as the percent maximum possible effect (%MPE) = (TFL − baseline)/(10 − baseline) × 100. Hyperalgesia was expressed as baseline-normalized TFL. Areas under the time–action curves ($AUC_{0-120\ min}$) were calculated by the trapezoidal rule.

The valid use of parametric statistics was verified by normal distribution and equal variance (Kolmogorov-Smirnov normality test and Levine medial test; $P < 0.05$). Changes in response across time (time–action curves) were assessed by repeated-measures (RM) two-way analysis of variance (ANOVA; dose and time). Dose–response curves were assessed by regression analysis ($AUC_{0-120\ min}$ *vs.* log dose). Interaction between morphine and methadone was determined by two-way RM ANOVA (treatment and time). Between-sex differences were analyzed by two-way RM ANOVA (dose and sex). Comparisons with preinjection baseline were assessed by one-way ANOVA on ranks or *t* test. *Post hoc* multiple comparisons were performed with the Student-Newman-Keuls test. The level of significance was $P \leq 0.05$.

Results

Antinociceptive Effect of Methadone

The antinociceptive effect of *d,l*-methadone was determined as it was related to dose (0.5–5 mg/kg) and sex of the rat, using the high-intensity tail-flick test. At baseline, there were no significant between-sex differences in response (TFL = 2.4 ± 0.09 s [male], 2.2 ± 0.10 s [female]). Responses to a noxious stimulus (TFL) were prolonged after the administration of *d,l*-methadone, which was indicative of the antinociceptive effect (figs. 1A and B). The highest dose of *d,l*-methadone (5 mg/kg) produced maximum effect (%MPE = 100%) in both male and female rats. The antinociceptive effect of *d,l*-methadone ($AUC_{0-120\ min}$) was related to dose ($P < 0.0001$; two-way RM ANOVA), but not to sex (fig. 1C). The same was found for maximum %MPE *versus* dose relation (dose: $P < 0.0001$; sex: not significant; two-way RM ANOVA). *d,l*-Methadone had a similar potency in male and female rats (ED_{50} = 1.64 ± 0.15 and 1.75 ± 0.14 mg/kg, respectively).

Hyperalgesic Effect of Methadone

The pain-enhancing (hyperalgesic) properties of *d,l*-methadone were determined in relation to dose (1–100 μg/kg) and sex, using the low-intensity tail-flick test. Baseline TFL was found to be similar in male and female rats (TFL = 8.2 ± 0.32 and 7.3 ± 0.56 s, respectively). *d,l*-Methadone, at low doses equal to 1 and 10 μg/kg, was found to enhance sensitivity to a noxious stimulus (TFL significantly decreased in comparison with baseline values; *post hoc* Student-Newman-Keuls test; $P < 0.05$), which was indicative of hyperalgesia (figs. 2A and B). At the highest dose (100 μg/kg), the effect of *d,l*-methadone was not significantly different from the effect of saline. Overall, the hyperalgesic effect of *d,l*-methadone ($AUC_{0-120\ min}$) was inversely related to dose and was similar in male and female rats (dose: $P < 0.0001$; sex: not significant; two-way ANOVA) (fig. 2C).

To determine which enantiomer contributes to the hyperalgesic activity of *d,l*-methadone, both *l*- and *d*-

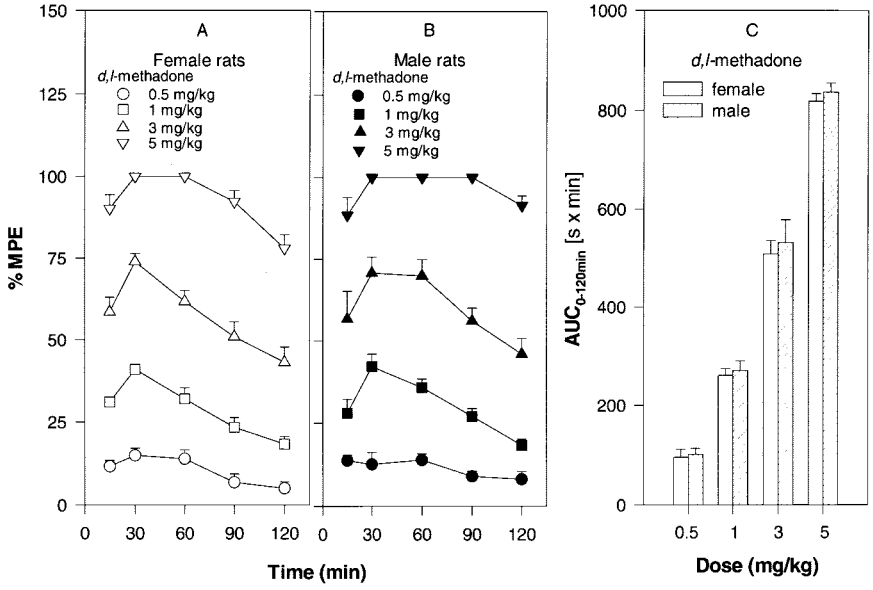

Fig. 1. Time–action curves for *d,l*-methadone–induced antinociception (0.5–5 mg/kg intraperitoneal, high-intensity tail-flick test) are shown in female rats (*A*) and in male rats (*B*). Data are presented as percentage of maximal possible effect [%MPE = (postinjection response − preinjection response/cutoff − preinjection response) × 100] and are mean ± SEM (8 rats/sex). (*C*) Dose–response curves for the antinociceptive effect of *d,l*-methadone in female and male rats are also presented. Data are presented as area under the time–action curves, 0–120 min ($AUC_{0-120\ min}$), and are mean ± SEM (8 rats/sex).

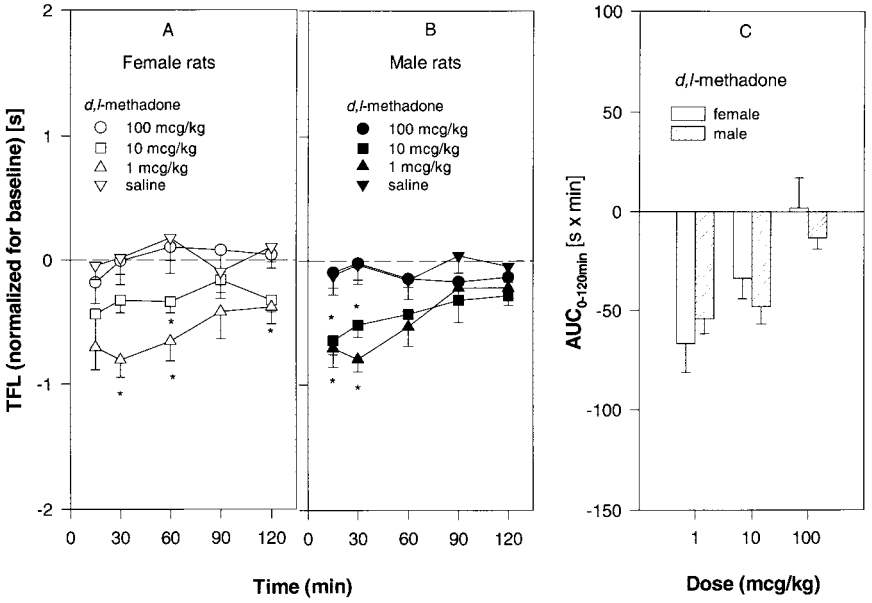

Fig. 2. Time–action curves for *d,l*-methadone–induced hyperalgesia (1–100 μg/kg intraperitoneal, low-intensity tail-flick test) in female rats (*A*) and in male rats (*B*). Data are presented as tail-flick latency (TFL; in seconds) normalized for baseline value (postinjection TFL − preinjection TFL). Data are mean ± SEM (8 rats/sex). * Significantly different from saline ($P < 0.05$; *post hoc* Student-Newman-Keuls test). (*C*) Dose–response curves for the hyperalgesic effect of *d,l*-methadone in male and female rats. Data are presented as area under the time–action curves, 0–120 min ($AUC_{0–120\ min}$), and are mean ± SEM (8 rats/sex).

methadone (1–100 μg/kg) were administered separately in rats. The data demonstrated that methadone enantiomers markedly differ in their pain-enhancing properties. First, *l*-methadone produced hyperalgesia (dose: $P < 0.025$, 0.001; time: $P < 0.001$, 0.001 in male and female rats, respectively; two-way RM ANOVA) (figs. 3A and B). Second, the overall effect ($AUC_{0-120\ min}$) was inversely related to dose (*i.e.*, a lower dose produced a greater hyperalgesia) and was significantly greater in female than in male rats (dose: $P < 0.001$; sex: $P < 0.001$; two-way RM ANOVA) (fig. 3C). Third, repeated exposure to *l*-methadone (1–100 μg/kg, randomized doses; weekly intervals) resulted in a progressive increase in pain sensitivity (decrease of baseline TFL values) in female rats, whereas baseline TFL did not change in male rats (time: $P < 0.001$; sex: $P < 0.001$; two-way RM ANOVA) (female: 7.3 ± 0.6, 6.5 ± 0.3, 4.5 ± 0.4, 3.8 ± 0.6 s; male: 8.2 ± 0.3, 8.9 ± 0.5, 7.2 ± 0.9, 7.4 ± 0.4 s at the first [naive rats], second, third, and fourth weeks, respectively). Fourth, a long-lasting hyperalgesia was observed after a single low dose (1 μg/kg) of *l*-methadone in female rats (time: $P < 0.001$; one-way ANOVA) but not in male rats (TFL: 7.25 ± 0.89, 3.1 ± 1.3, 4.4 ± 0.88, 4.6 ± 0.75 s [female]; 8.9 ± 1.14, 8.5 ± 0.4, 8.7 ± 0.5, 8.7 ± 0.54 s [male] before and 24, 48, and 72 h after injection, respectively). Fifth, compared with *d,l*-methadone, *l*-methadone had greater hyperalgesic properties (fig. 2C *vs.* fig. 3C). In striking contrast, *d*-methadone (1–100 μg/kg) produced prolongation of TFL, which was indicative of weak antinociception (%MPE[100 μg/kg] = 5.7 ± 0.84% [male], 7.6 ± 1.5% [female]) (figs. 4A and B). Next, the overall effect ($AUC_{0-120\ min}$) of

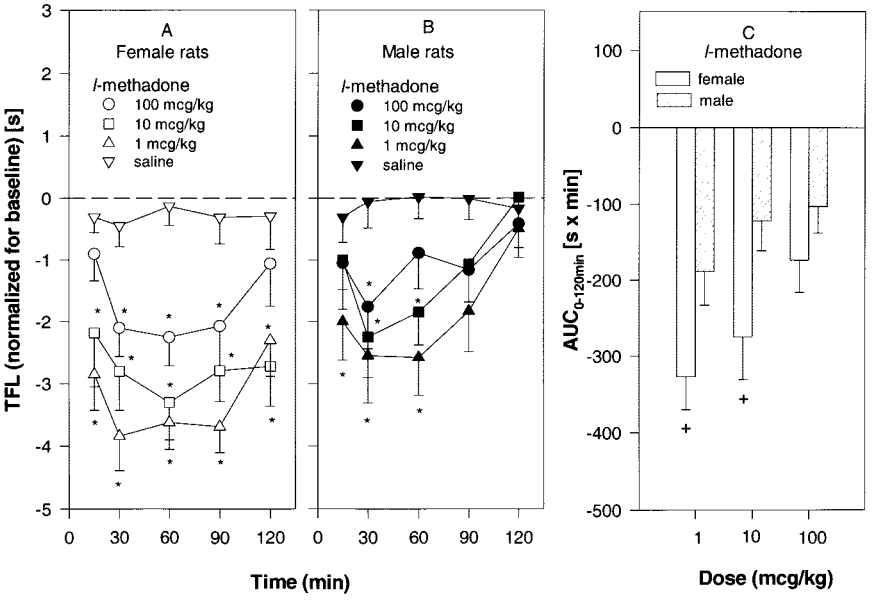

Fig. 3. Time–action curves for *l*-methadone-induced hyperalgesia (1–100 μg/kg intraperitoneal, low-intensity tail-flick test) in female rats (*A*) and in male rats (*B*). Data are presented as tail-flick latency (TFL; in seconds) normalized for baseline value (postinjection TFL − preinjection TFL). Data are mean ± SEM (8 rats/sex). * Significantly different from saline ($P < 0.05$; *post hoc* Student-Newman-Keuls test). (*C*) Dose–response curves for the hyperalgesic effect of *l*-methadone in male and female rats. Data are presented as area under the time–action curves, 0–120 min ($AUC_{0–120\ min}$), and are mean ± SEM (8 rats/sex). + Significantly different from identically treated male rats ($P < 0.05$; *post hoc* Student-Newman-Keuls test).

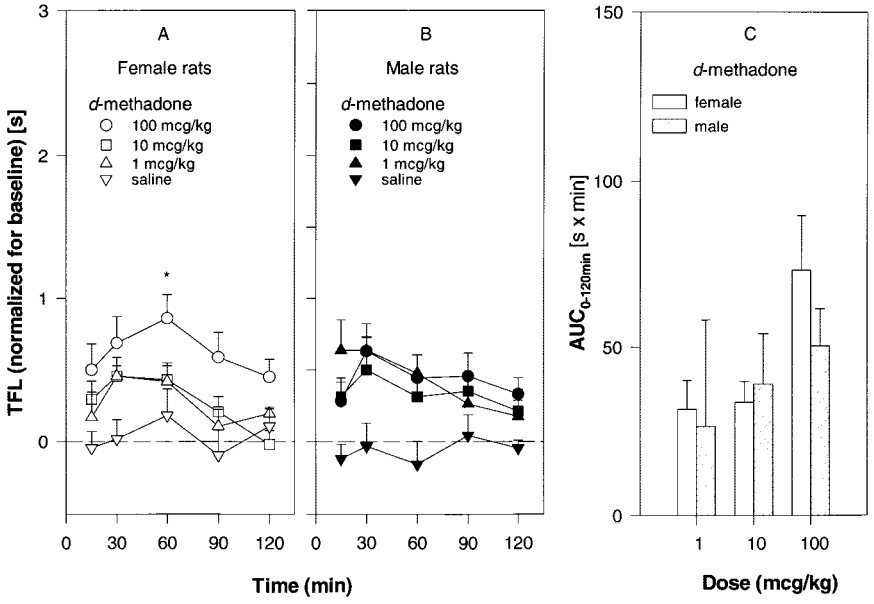

Fig. 4. Time–action curves for *d*-methadone (1–100 μg/kg intraperitoneal, low-intensity tail-flick test) in female rats (*A*) and in male rats (*B*). Data are presented as tail-flick latency (TFL; in seconds) normalized for baseline value (postinjection TFL − preinjection TFL). Data are mean ± SEM (8 rats/sex). * Significantly different from saline ($P < 0.05$; *post hoc* Student-Newman-Keuls test). (*C*) Dose–response curves for *d*-methadone in male and female rats. Data are presented as the area under the time–action curves, 0–120 min ($AUC_{0-120\ min}$), and are mean ± SEM (8 rats/sex).

d-methadone was related to dose ($P < 0.005$; two-way RM ANOVA) but not to sex (fig. 4C). Finally, no changes in baseline TFL were observed in rats receiving *d*-methadone or *d,l*-methadone.

Effect of Methadone on Morphine Antinociception

The effect of methadone (subanalgesic dose) on morphine antinociception was also determined. For this purpose, morphine (1 mg/kg), *d,l*-methadone, and *d*-methadone (10 μg/kg) were administered separately and in combination. Responses to high-intensity radiant heat (tail-flick assay) were measured. First, we confirmed our previous findings[16,39] that the antinociceptive effect of morphine was greater in male than in female rats (sex: $P < 0.0001$; time: $P < 0.0001$; two-way ANOVA) (figs. 5A and B). Second, we demonstrated that *d,l*-methadone, in a dose that was without antinociceptive activity (%MPE[10 μg/kg] = 1.4 ± 1.1% [male], 1.8 ± 1.1% [female]), significantly enhanced morphine antinociception (treatment: $P < 0.01$, $P < 0.0025$; time: $P < 0.0001$, $P < 0.0001$ in male and female rats, respectively; two-way RM ANOVA) (figs. 5A and B). Third, we found that

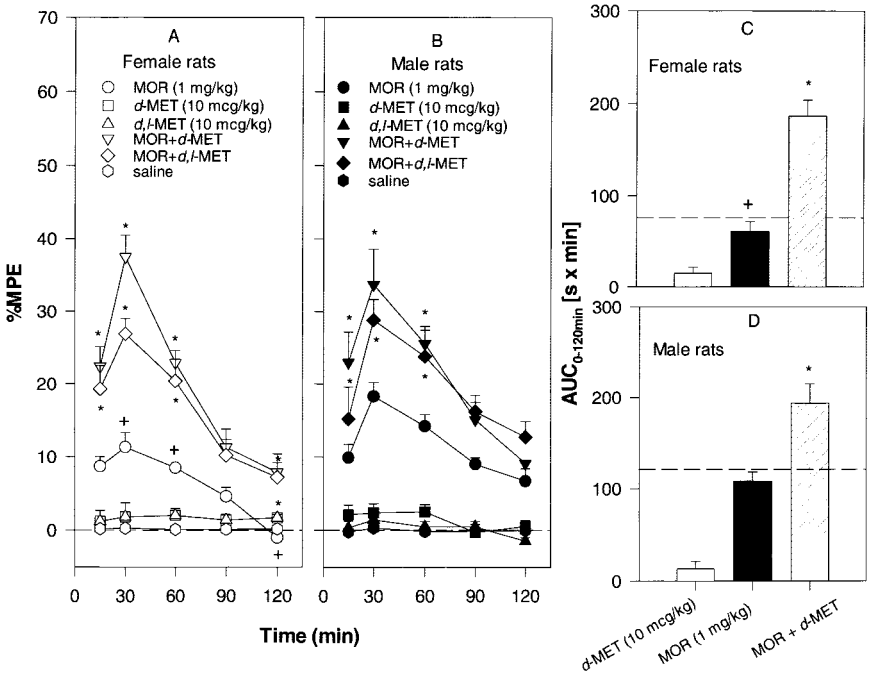

Fig. 5. Time–action curves for morphine (MOR; 1 mg/kg intraperitoneal), *d*-methadone (*d*-MET; 10 μg/kg intraperitoneal), and *d,l*-methadone (*d,l*-MET; 10 μg/kg intraperitoneal) alone; MOR (1 mg/kg intraperitoneal) plus *d*-MET (10 μg/kg intraperitoneal); and MOR (1 mg/kg intraperitoneal) plus *d,l*-MET (10 μg/kg intraperitoneal) in female (*A*) and male (*B*) rats (high-intensity tail-flick test). Antinociception is presented as percentage of maximal possible effect [%MPE = (postinjection response − preinjection response/cutoff − preinjection response) × 100]. Data are mean ± SEM (8 rats/sex/treatment). * Significantly different from MOR alone ($P < 0.05$; *post hoc* Student-Newman-Keuls test). + Significantly different from identically treated male rats ($P < 0.05$; *post hoc* Student-Newman-Keuls test). Enhancement of MOR (1 mg/kg intraperitoneal) antinociception in the presence of *d*-methadone (10 μg/kg intraperitoneal) in female (*C*) and in male (*D*) rats. Data are presented as areas under the time–action curves, 0–120 min ($AUC_{0-120\ min}$), and are mean ± SEM (8 rats/sex/treatment). The anticipated values for additivity are indicated by the *dashed lines*. * Significantly different from MOR alone ($P < 0.05$; *t* test). + Significantly different from identically treated male rats ($P < 0.05$; *t* test).

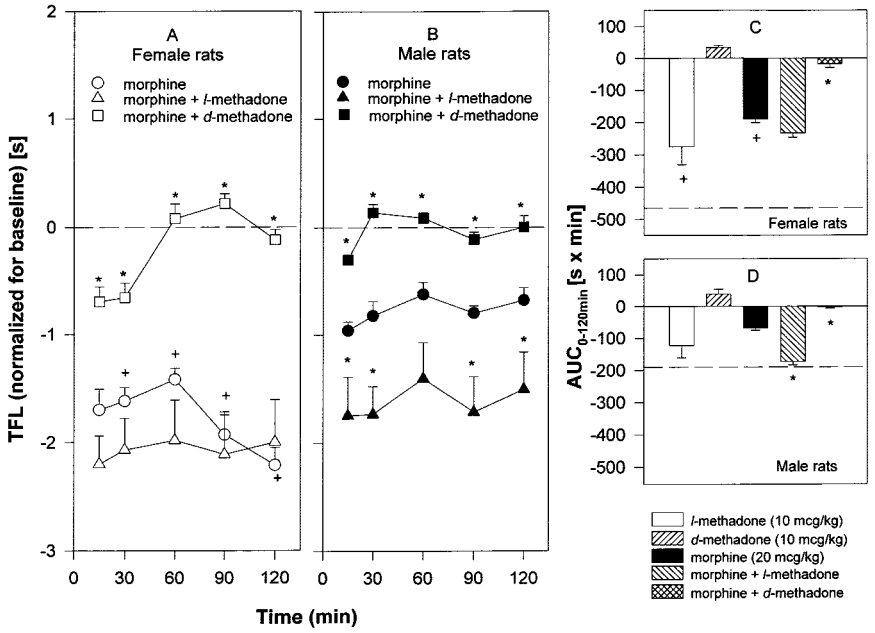

Fig. 6. Time–action curves for morphine (MOR; 20 μg/kg intraperitoneal) alone and in combination with *d*-methadone (*d*-MET; 10 μg/kg intraperitoneal) or *l*-methadone (*l*-MET; 10 μg/kg intraperitoneal) in female (*A*) and in male (*B*) rats (low-intensity tail-flick test). Data are presented as tail-flick latencies (TFL; in seconds) normalized for preinjection baseline (postinjection TFL − preinjection TFL) and are mean ± SEM (8 rats/sex/treatment). * Significantly different from MOR alone ($P < 0.05$; *post hoc* Student-Newman-Keuls test). + Significantly different from identically treated male rats ($P < 0.05$; *post hoc* Student-Newman-Keuls test). The overall effects of *d*-MET (10 μg/kg intraperitoneal) and *l*-MET (10 μg/kg intraperitoneal) on MOR (20 μg intraperitoneal) hyperalgesia in female (*C*) and male (*D*) rats. Data are presented as the area under the time–action curves, 0–120 min ($AUC_{0-120\ min}$), and are mean ± SEM (8 rats/sex/treatment). The anticipated values for additivity are indicated by the *dashed lines* (*l*-MET + MOR). * Significantly different from MOR alone ($P < 0.05$; *t* test). + Significantly different from identically treated male rats ($P < 0.05$; *t* test).

the enhancement of morphine antinociception was even more pronounced when morphine was combined with a low dose of *d*-methadone that was without antinociceptive activity (%MPE[10 μg/kg] = 2.5 ± 1.0% [male], 2.0 ± 0.9% [female]) (treatment: $P < 0.0025$, 0.0001; time: $P < 0.0001$, $P < 0.0001$ in male and female rats, respectively; two-way RM ANOVA) (figs. 5A and B). Fourth, the AUC values were not affected by kinetics because neither *d*- or *d,l*-methadone prolonged the time–action curve of morphine. Fifth, potentiation of morphine antinociception by *d*-methadone seemed to be greater in female (approximately threefold) than in male (approximately twofold) rats (fig. 5C *vs.* fig. 5D). This was solely because of the low potency of morphine in the female rats. Sex-related differences in responsiveness to morphine were abolished in the presence of a low dose of *d*-methadone (fig. 5C *vs.* fig. 5D).

Effect of Methadone on Morphine Hyperalgesia

The last objective was to determine the effect of methadone enantiomers on morphine hyperalgesia. A low dose (subantinociceptive) of morphine (20 μg/kg) was administered alone or in combination with a low dose (10 μg/kg) of *d*- or *l*-methadone. Responsiveness to low-intensity radiant heat was assessed. First, we confirmed our previous findings[16] that morphine, in a low dose, enhanced sensitivity to noxious stimuli (hyperalgesia) in a sex-related fashion (female > male) in rats (sex: $P < 0.05$; time: not significant; two-way RM ANOVA). Second, we demonstrated that the *d*- and the *l*-enantiomers of methadone interacted with morphine (a low dose) in an opposite manner (treatment: $P < 0.001$, 0.001; time: $P < 0.001$, not significant in male and female rats, respectively; two-way RM ANOVA). The hyperalgesic effect of morphine was blocked by a low dose of *d*-methadone in both male and female rats (figs. 6A–D). *d,l*-Methadone also attenuated morphine hyperalgesia; however, the effect was less for *d,l*- than for *d*-methadone (approximately twofold). Conversely, morphine hyperalgesia was either unchanged (female) or enhanced (male: the additive effect) by a low dose of *l*-methadone. This was likely due to the low hyperalgesic effect of morphine in the male rats (figs. 6A–D).

Discussion

The purpose of this study was to characterize the antinociceptive and pronociceptive properties of methadone and its enantiomers in rats. First, using a wide range of doses as well as high and low intensities of radiant thermal stimulus (tail-flick assay), we demonstrated both pain-inhibitory (antinociception) and pain-facilitatory (hyperalgesia) properties of *d,l*-methadone (μ-opioid/NMDA antagonist). Second, we showed that one methadone enantiomer (*l*-methadone) had greater pain-enhancing properties than the other enantiomer (*d*-methadone). Third, we found that the hyperalgesic effect of *l*-methadone (μ-opioid), but not *d,l*-methadone, was related to sex of the rat (female > male). Fourth, we demonstrated that *d*-methadone (NMDA antagonist) significantly enhanced morphine (μ-opioid) antinociception and attenuated morphine hyperalgesia.

d,l-*Methadone–induced Antinociception and Hyperalgesia*

Previous data from our laboratory clearly showed that pain-inhibitory and pain-facilitatory effects of opioids

(*e.g.*, morphine, oxycodone) can be separately demonstrated by utilizing high (antinociceptive) and very low (subantinociceptive) doses in rats.[16,17] Recently, this was also shown in mice.[18] The involvement of an NMDA mechanism is suggested because MK-801 and ketamine enhanced morphine antinociception (high doses) and blocked morphine hyperalgesia (low doses).[16,18,39,40] Whereas MK-801 is a specific NMDA antagonist, ketamine has also been noted to activate the inhibitory descending monoaminergic system, which could contribute to its enhancement of morphine antinociception.[46] The current study with methadone (μ-opioid/NMDA antagonist) confirmed and extended these findings. Dose-related antinociception and hyperalgesia were separately demonstrated by the respective administration of high (0.5–5 mg/kg) and low (1–100 μg/kg) doses of *d,l*-methadone. This bidirectional effect of *d,l*-methadone suggests that the NMDA antagonistic effect may not fully compensate its opioid-induced hyperalgesic activity. This is the first (to the best of our knowledge) preclinical evidence for low-dose methadone–produced hyperalgesia. Enhanced sensitivity to pain has been previously shown in previous opioid addicts maintained on methadone.[47,48] In this regard, opioid-induced hyperalgesia (*e.g.*, morphine, fentanyl) has been repeatedly demonstrated in several different settings characterized by dose (very low, high) and administration (acute, long-term, withdrawal).[20]

Contribution of the l*-Enantiomer and the* d*-Enantiomer to Methadone-induced Hyperalgesia*

Methadone-produced hyperalgesia was further characterized by using its optical isomers, *l-* and *d*-methadone. The opioid-like effects were repeatedly demonstrated with *l*-methadone, whereas *d*-methadone was found to be weak or inactive as an opioid in humans and rodents.[25,26,49,50] The current data demonstrated marked differences in the pain-enhancing properties (hyperalgesia) of methadone enantiomers. In this regard, at low doses (1–100 μg/kg), *l*-methadone produced a dose-related (inverse), long-lasting hyperalgesia. Furthermore, once-weekly *l*-methadone administration induced a persistent increase of baseline pain sensitivity. Similar alteration in baseline response was demonstrated during repeated administration of morphine (low dose) in mice.[44] A long-lasting sensitization (a single dose), as well as progressive enhancement of delayed hyperalgesia (repeated doses) were produced by several μ-opioids (high doses), morphine, fentanyl, and heroin.[9,10,12,15] Therefore, the pain-enhancing property of *l*-methadone seems to be due to its action at the μ-opioid receptor. The absence of hyperalgesia after administration of *d*-methadone (1–100 μg/kg) may likely be explained by its weak opioid activity. As expected, hyperalgesia was greater for the *l*-enantiomer than for the *d,l*-racemate. In summary, these data clearly demonstrated that the pain-enhancing effect of *d,l*-methadone was entirely due to the presence of the *l*-enantiomer (μ-opioid). Recent work has demonstrated the presence of a pronociceptive PLCβ_3/PKCγ/NMDA pathway stimulated by extremely low concentrations of morphine, through the μ-opioid receptor, in mouse brain.[51]

Sex-related Differences

An important characteristic of opioid analgesia is its dependence on sex. Although this aspect of opioid pharmacology has received an increasing attention in recent years, the mechanisms involved (effects of gonadal hormones, pharmacokinetics, physiologic factors) remain poorly understood.[30–35] Typically, the antinociceptive responsiveness to μ-opioids, such as morphine, is greater in male than in female rats.[30,31,34] In addition, we have previously found that sex-related differences in morphine (low dose) hyperalgesia were opposite (female > male).[16] An NMDA mechanism is likely involved because both ketamine[16,39] and *d*-methadone (current study) were found to abolish sex differences in morphine antinociception and hyperalgesia. Sex-related modulation of opioid antinociception by an NMDA mechanism has also been demonstrated by other laboratories (using site-specific NMDA antagonists).[38,40] No sex differences in the antinociceptive effect of *d,l*-methadone were demonstrated by us (tail-flick test: ED_{50} = 1.64 ± 0.15 *vs.* 1.75 ± 0.14 mg/kg in male and female rats, respectively) or in recent work from another laboratory (warm-water tail-withdrawal assay: ED_{50} = 1.49 ± 0.20 *vs.* 1.81 ± 0.37 mg/kg in male and female rats, respectively).[37] The hyperalgesic effect of *d,l*-methadone also was similar in male and female rats. This seems to be related to the presence of the NMDA antagonist properties of the *d*-enantiomer. Likewise, responsiveness to *d*-methadone (limited or no opioid activity) was not related to the sex of the rat. Conversely, as it was expected with a μ-opioid, *l*-methadone (low dose) produced greater hyperalgesia in female than in male rats. Taken together, these findings provide additional support for a sex-related (female > male) NMDA receptor mechanism in μ-opioid–produced hyperalgesia in rats. This may be a reason that the antinociceptive effect of a μ-opioid is typically greater in male *versus* female rats.

Methadone and Morphine Interaction on Antinociception and Hyperalgesia

We have also studied the interaction between methadone and morphine in the current study. This was of interest both from a basic pharmacology aspect as well as having potential clinical application in pain management. There is evidence for the existence of multiple subtypes of μ-opioid receptors that differ in their functional activation by opioids as well as by their cellular and central nervous system localization.[52] Therefore, the use of low-dose combinations of different opioids has

been suggested as a way to reduce overall opioid toxicity, improve analgesia, and reduce opioid tolerance.[53,54] The study of the combination of methadone with morphine was of interest based not only on potential difference in interaction on μ-receptors,[55] but also given the NMDA receptor antagonistic properties of this drug.[21–23] NMDA receptor antagonism has been linked to enhancement of opioid efficacy, delay in opioid tolerance development, and the prevention of enhanced pain sensitivity (opioid-induced hyperalgesia).[1,19]

The current data demonstrated the opposite effects of *d*-methadone on morphine-produced antinociception (enhancement) and hyperalgesia (attenuation). These data replicated previous work with noncompetitive NMDA antagonists (MK-801, ketamine) and morphine (enhancement and blockade of antinociception and hyperalgesia, respectively)[16,18,39,40] and also were in agreement with previous findings that *d*-methadone blocked morphine tolerance and NMDA-induced hyperalgesia in rats.[28,56] Therefore, the mechanism by which μ-opioid antinociception was enhanced and hyperalgesia was attenuated seems to be the same (likely NMDA mediated). Nevertheless, beside its antagonist action on the NMDA receptor, *d*-methadone may have other pharmacologic properties as demonstrated by inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid–induced hyperexcitation in spinal neurons,[57] inhibition of the L-calcium currents,[58] and blockade of nicotinic acetylcholine receptors.[59] In addition, although *d*-methadone produced naloxone-insensitive antinociception in the formalin test (phase 2),[29] it also showed naloxone-reversible neuronal inhibitory effects in the rat spinal cord.[60]

Importantly, the current low-dose combination study strongly suggested antinociceptive synergy between *d*-methadone and morphine. Therefore, additional study is warranted (isobologram). It would also be of interest to determine whether the *d*-methadone–morphine interaction is unique or applies to other opioids. It is important to note that although enhancement of opioid antinociception by NMDA antagonists (*e.g.*, MK-801, ketamine, dextromethorphan) has been demonstrated by several laboratories,[39,61–66] this phenomenon seems to depend on several factors, such as type of NMDA antagonist, dose, pain test, species, and strain. Therefore, differences in *d*-methadone doses (200-fold) and species (rats *vs.* mice) may account for the discrepancy between the current study and a previous study[55] where a high dose of *d*-methadone (4 mg/kg) was found without effect on morphine (1 mg/kg) antinociception in male mice (tail-flick test). Surprisingly, in the latter study, the *l*-enantiomer of methadone (0.5 mg/kg) synergized with morphine but not with other μ-opioid agonists (*e.g.*, fentanyl) in mice.[55]

The effect of *l*-methadone (subantinociceptive dose) on morphine antinociception was not tested herein. Nevertheless, we demonstrated that the hyperalgesic effect of morphine was not reversed by a low dose of *l*-methadone. This was expected because both drugs seem to act primarily on the μ-opioid receptors. Assuming that the overall effect of morphine is equal to the sum of two opposite processes (antinociception and pronociception), potentiation of morphine antinociception by a subantinociceptive dose of *l*-methadone is unlikely. Indeed, enhancement of morphine antinociception was greater in the presence of *d*-methadone than in the presence of *d,l*-methadone. In summary, although there is evidence that both methadone enantiomers bind with similar affinities to the noncompetitive site of the NMDA receptor in rat forebrain and spinal cord synaptic membranes,[21–23] the current data suggest that the functional NMDA antagonist property of methadone may reside primarily with the *d*-enantiomer.

In conclusion, opioids are now being used more frequently for pain, in particular chronic nonmalignant pain, despite a continuing controversy regarding their efficacy and safety with long-term use. Recent preclinical and clinical studies suggest that another potentially important consequence of treatment with opioids is the phenomenon of an increase in pain sensitivity secondary to opioid exposure, which has been termed opioid-induced hyperalgesia. Better information is needed to understand this phenomenon and to determine which opioids or opioid combinations may be most appropriate for long-term use. In the current study, we have examined methadone, a commonly used opioid analgesic unique in possessing both μ-opioid and NMDA antagonist properties. Opioid-induced hyperalgesia resulted from the presence in the racemate of the *l*-enantiomer (likely μ-agonist) and was antagonized by the presence of the *d*-enantiomer (NMDA antagonist). Although there are many considerations in regard to opioid-induced hyperalgesia, methadone may represent a better choice for long-term opioid treatment than other pure opioid agonists.

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