d-Methadone Is Antinociceptive in the Rat Formalin Test

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ABSTRACT

The l-isomer of methadone possesses opioid activity, whereas the d-isomer is weak or inactive as an opioid. Both d- and l-methadone have been shown to bind to the N-methyl-D-aspartate (NMDA) receptor. To determine whether d-methadone has functional, in vivo NMDA receptor antagonist activity, the antinociceptive effects of d-methadone were evaluated in the rat tail-flick and formalin tests. Cumulative dose-response analysis in the tail-flick test revealed an ED50 value for intrathecal (spinal) l-methadone of 15.6 μg/rat. In contrast, spinal d-methadone produced no antinociception at a cumulative dose of 460 μg/rat. d-Methadone in a dose range from 32 to 320 μg/rat dose-dependently reduced formalin-induced flinching behavior during phase 2 but not during phase 1 of the formalin test. These antinociceptive effects of d-methadone were not blocked by a spinal dose of naloxone that effectively antagonized an antinociceptive (tail-flick test) dose of l-methadone. d-Methadone at an intrathecal dose of 250 μg shifted the ED50 value for NMDA-induced nociceptive behaviors more than 3-fold to the right, which indicates an antagonism of these NMDA receptor-mediated effects. These results indicate that d-methadone is antinociceptive as a result of its NMDA receptor antagonist activity.

Like morphine, methadone binds preferentially to the mu type of the opioid receptor (Neil, 1984), and produces behavioral effects similar to morphine in animal tests of opioid activity (Smits and Myers, 1974). The clinically available and commonly used laboratory form of methadone is the racemic mixture (dl-methadone). The l-isomer is responsible for the opioid properties, whereas the d-isomer is weak or inactive as an opioid (Horng et al., 1976; Olsen et al., 1976; Smits and Myers, 1974). Ebert et al. (1995) have reported that racemic methadone possesses affinity for the NMDA receptor in rat cortical membranes, and reduces NMDA-induced depolarization in vitro in rat spinal cord and cortical wedge preparations. We found that both the d- and l-isomers of methadone can bind to the noncompetitive (MK-801) site on the NMDA receptor in rat forebrain and spinal cord membranes with an affinity approximately equal to that of dextrorotamorph, an established NMDA receptor antagonist (Gorman et al., 1997; Elliott et al., 1995). The NMDA receptor plays an important role in pain transmission by modulating both “wind-up,” a physiological phenomenon whereby sensory neurons decrease activation thresholds, enlarge receptive field size and fire spontaneously in the aftermath of noxious peripheral stimulation (Woolf and Thompson, 1991; Dubner and Ruda, 1992). Blockade of the NMDA receptor with an NMDA receptor antagonist produces antinociception in a variety of animal pain models. Thus, d-methadone, although it has little opioid activity, may be antinociceptive if it possesses in vivo NMDA receptor antagonist activity and may contribute to the analgesic effects of racemic methadone. In the present study, we examined the effects of intrathecal (spinal) d- and l-methadone on the rat tail-flick test, an opioid-sensitive pain model, and d-methadone on the rat formalin test, a pain model involving central sensitization. Furthermore, to determine whether the antinociceptive effects of spinal d-methadone involve opioid receptors we examined the effects of the coadministration of naloxone plus d-methadone on the formalin test.

Materials and Methods

Animals and preparations. Male Sprague-Dawley rats weighing 300 to 350 g were used. For the spinal administration of drugs to the rat, a catheter was placed in the intrathecal space 2 to 4 days before the experiments. Under halothane anesthesia, a PE-10 tube was inserted through a small hole made in the atlanto-occipital membrane, and threaded 9 cm down the intrathecal space to the lumbosacral level of the spinal cord (Shimoyama et al., 1997). A catheterized rat with any signs of paralysis was excluded from the experiments.

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; d, dextrorotatory; l, levorotatory; IT, intrathecal; ED50, median effective dose
study. At the end of the study, 5 µl of a 1% methylene blue solution was introduced into the catheter followed by 10 µl of saline to confirm the position of the catheter and the spread of the dye in the intrathecal space.

**Drugs.** The enantiomers, d-methadone [(S)-(+) -methadone] and l-methadone [(R)-(−)-methadone] were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). The free base of each isomer was dissolved in saline with the aid of sodium hydroxide and the final pH was adjusted to 7.0. The naloxone and NMDA solutions were prepared alone and in a solution with d- or l-methadone, as indicated below, to limit the total volume administered.

**Study 1.** The antinociceptive potency of spinal d- and l-methadone were determined by the tail-flick test and cumulative dose-response analysis. Intrathecal doses of each drug were delivered in a volume of 5 µl followed by 10 µl of saline to flush the catheter. Because of the limited solubility of the isomers, the highest cumulative dose tested was 460 µg/rat. A tail-flick apparatus (EMDIE, Richmond, VA) was used to apply radiant heat at 5 to 8 cm from the tip of the tail. The time from the onset of the heat stimulus to the withdrawal of the tail (tail-flick latency) was measured. The intensity of the radiant heat was adjusted so that the base-line latencies were between 2.5 and 3.5 sec. Subsequent response latencies were determined at 15 min after spinal d- or l-methadone. This pretreatment time was selected from a time course study after 40 µg/rat of spinal d-methadone, which revealed a peak analgesic effect at 15 min after drug administration. To avoid tissue damage the heat stimulus was turned off after 10 sec (cut-off latency). After measuring the base-line latencies, increasing doses of d- or l-methadone were administered until each animal became an analgesic responder (cumulative dose-response assessment, Elliott et al., 1994; Shimoyama, N. et al., 1996) or reached the highest test dose (see above). An analgesic responder was defined as one whose response tail-flick latency was 2 or more times the value of the base-line latency. The latency data were converted to a quantal form by determining the percentage of analgesic responders in each group for each cumulative dose, and a dose-response curve was constructed for each isomer of methadone. The treatment groups averaged nine animals.

**Study 2.** The time course of the antagonist action of spinal naloxone on the antinociceptive (tail-flick test) effects of spinal l-methadone were determined by coadministering l-methadone at 80 µg/rat and naloxone at 30 µg/rat. Other groups received l-methadone at 80 µg/rat or naloxone at 30 µg/rat.

**Study 3.** To examine the effects of d-methadone on formalin-induced flinching behavior, d-methadone at a dose of 32, 160 or 320 µg/rat or saline in a volume of 10 µl was administered intrathecally 15 min before the intraplantar injection of formalin. Formalin was diluted to 5% from a stock solution of 100% (formaldehyde solution 37% w/v, Fisher Scientific Company, Fairlawn, NJ) and was injected subcutaneously into the right hindpaw in a volume of 50 µl with the use of a 50-µl glass syringe and a new disposable 30-gauge needle. Immediately after the formalin injection, the rat was placed in a test chamber and was observed continuously by a blinded observer for the next 60 min. The number of flinches, defined as quick shakes of the injected hindpaw, were recorded. The formalin injection resulted in a biphasic reaction of flinching behaviors (phase 1, 0–10 min; phase 2, 10–60 min). Each rat was observed for overt central nervous system behavioral effects throughout the experiment and tested for its ability to negotiate a 60-degree mesh (Shimoyama, M. et al., 1996) immediately before the injection of formalin.

**Study 4.** The effects of spinal d-methadone on the formalin test were evaluated with or without the concurrent administration of naloxone. Naloxone at a dose of 30 µg/rat completely blocked the effects of an approximately ED₅₀ antinociceptive dose of spinal l-methadone (80 µg/rat) on the tail-flick test for at least 75 min (see fig. 2). Saline, d-methadone at 250 µg/rat or d-methadone at 250 µg/rat + naloxone 30 µg/rat was administered spinally to rats 15 min before intraplantar formalin, and the formalin-induced flinching behavior was observed by a blinded observer as in study 3.

**Study 5.** The ability of spinal d-methadone to antagonize the nociceptive behavioral responses to intrathecal NMDA was determined by estimating the ED₅₀ values for NMDA-induced behaviors after pretreatment with saline or d-methadone at 250 µg/rat. Spinal NMDA produces a short-lasting behavioral response which consists of intense caudally directed biting, licking and scratching behaviors that are usually accompanied by vocalization (Okano et al., 1993). Doses of NMDA from 0.6 to 7.3 nmol/rat were administered intrathecally with a 3-min interinjection interval. A responder was defined as a rat in which NMDA produced a scratching, biting and licking of the caudal dermatomes that was at least 30 sec in duration. Once an animal became a responder, it was not subjected to further testing.

**Statistical analysis.** The quantal dose-response data in study 1 were analyzed with the BLISS-21 computer program. This program maximized the log-likelihood function to fit a gaussian normal sigmoid curve to the dose-response data and provided the ED₅₀ value and a 95% confidence interval (CI) (Umans and Inturrisi, 1981). The formalin test data in studies 3 and 4 were analyzed by a one-way analysis of variance and the Student’s t test, respectively. Statistical significance was accepted at P < .05.

**Results**

**Study 1: Effects of d- and l-methadone on the tail-flick test.** Figure 1 compares the antinociceptive activity of l- and d-methadone as a function of the spinal dose. l-Methadone produced dose-dependent antinociception and the analysis yielded an ED₅₀ value for spinal l-methadone of 15.6 µg/rat (7.0–29.8 µg rat, 95% CI). None of the rats that received d-methadone became an analgesic responder at a cumulative spinal dose of 460 µg/rat, which was the highest dose administered.

**Study 2: Naloxone prevents the effects of l-methadone on tail-flick latency.** Spinal naloxone at 30 µg/rat did

![Fig. 1. Dose-response curves for intrathecal (IT) l- and d- methadone in the rat tail-flick test. l-Methadone produced dose-dependent antinociception (analgesia) with a ED₅₀ value of 15.6 µg/rat (7.0–29.8 µg, 95% CI). d-Methadone produced no antinociceptive effects at doses that ranged from 20 to 460 µg/rat.](image-url)
not affect base-line tail-flick latencies (data not shown) or produce an antinociceptive (analgesic) response (fig. 2). However, this dose of spinal naloxone completely blocked the antinociceptive effects of an 80 μg/rat dose of d-methadone from 15 to 75 min after drug administration (fig. 2).

Study 3: Effects of d-methadone on the formalin test. Spinal d-methadone at 32 μg/rat did not produce any overt central nervous system effects and each rat given this dose was able to negotiate the 60-degree mesh immediately before the injection of formalin. Spinal d-methadone at 160 and 320 μg/rat produced transient motor paralysis of the hind limbs in 44 and 100% of the rats, respectively. The onset of the paralysis was approximately 1 min after the administration of d-methadone and lasted 30 sec to 7 min. However, by the initiation of the formalin test, each rat had recovered from the paralysis and was able to negotiate the 60-degree mesh. Similar motor effects have been observed after the administration of a large spinal dose of the NMDA receptor antagonist, ketamine, to rats (Chaplan et al., 1997). These effects were very rapid in onset, which resembled the motor effects of a local anesthetic, and resolved rapidly, probably as a result of the dilution of the drug in spinal CSF.

Spinal d-methadone did not affect the number of flinches during phase 1 (fig. 3A), but dose-dependently reduced the phase 2 flinching behavior, with the 320 μg/rat dose producing a 66% decrease in flinching (fig. 3B).

Study 4: Effects of naloxone on the antinociceptive effects of d-methadone in the formalin test. The coadministration of spinal naloxone at 30 μg/rat did not affect the ability of spinal d-methadone at 250 μg/rat to significantly reduce phase 2 flinching compared with spinal saline in the formalin test (fig. 4). There was no statistical difference in the number of phase 2 flinches between the two drug-treated groups (fig. 4).

Study 5: Antagonism by d-methadone of the nociceptive behavioral effects of NMDA. Pretreatment with d-methadone at a dose of 250 μg (809 nmol/rat) completely blocked an ED₉₀ dose (2.4 nmol/rat) of NMDA. This dose of d-methadone shifted the NMDA dose-response curve to the right, so that the ED₉₀ value for NMDA was increased more than 3-fold (table 1).

Discussion

Abundant evidence suggests that NMDA receptors are involved in the nociceptive responses to formalin. Pretreatment with a competitive NMDA receptor antagonist [e.g., APV(3-amino-5-phosphonovaleric acid) or a noncompetitive NMDA receptor antagonist [e.g., MK-801, (±)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen malate], dextromethorphan or ketamine] reduces nociceptive behavioral and/or electrophysiological responses induced by formalin (Coderre and Melzack, 1992; Haley et al., 1990; Yamamoto and Yaksh, 1992; Vaccarino et al., 1993; Hunter and Singh, 1994; Elliott et al., 1995; Shimoyama et al., 1996). The effects of NMDA receptor antagonists are primarily on phase 2 behaviors of the formalin response (Coderre and Melzack, 1992). Phase 2 of the formalin test appears to reflect central sensitization. The barrage of C-fiber inputs produced by formalin most likely activates spinal cord NMDA receptors, which results in the sensitization of dorsal horn neurons. This results in the amplification of the response of the dorsal horn neuron to the C-fiber inputs. These C-fiber inputs continue throughout the period of behavioral nociceptive responses (McCall et al., 1996). NMDA antagonists, by blocking the activation of the NMDA receptors, prevent sensitization of dorsal horn neurons and thereby reduce the behavioral nociceptive responses to formalin. NMDA receptor antagonists alter tail-flick latencies only at doses significantly higher than those required to affect the formalin test (Nasstrom et al., 1992; Elliott et al., 1995).

A more direct assessment of the NMDA receptor antagonist activity of d-methadone is provided by its ability to antagonize NMDA-induced nociceptive behaviors. NMDA, when localized to the spinal cord of the rat, produces dose-dependent, nociceptive behaviors that are antagonized by APV, an NMDA receptor antagonist, but not by a non-NMDA or an NK-1 receptor antagonist (Okano et al., 1993). Table 1 demonstrates that the same dose of d-methadone that is effective in the formalin test (fig. 4) is also able to antagonize the nociceptive effects of NMDA.

The tail-flick test is an opioid-sensitive test and has been used extensively to evaluate the analgesic effects of opioids (Szekely, 1982). Opioid agonists such as morphine are effective in suppressing acute nociceptive responses such as those produced in the tail-flick assay as well as the nociceptive responses produced during phases 1 and 2 of the formalin test (Yaksh and Malmberg, 1994). The activity or lack of activity of a drug, as a function of dose, in the tail-flick test (fig. 1) and in the formalin test (fig. 3, A and B) as well as the ability of the opioid antagonist, naloxone, to block (fig. 2) or fail to block (fig. 4) an antinociceptive effect can be used to determine whether a drug is acting primarily by an opioid or a nonopioid mechanism. Clearly d-methadone appears to act...
As a nonopiod in the assays conducted in this study. Furthermore, the ability of a nonopiod drug such as d-methadone to affect phase 2 but not phase 1 of the formalin test (fig. 3, A and B) and to antagonize NMDA-induced nociceptive behaviors (table 1), when taken together with the demonstration that d-methadone is a noncompetitive NMDA receptor antagonist in vitro (Gorman et al., 1997), strongly suggests that d-methadone is antinociceptive in vivo by virtue of its NMDA receptor antagonist activity.

The present study suggests that the clinically available racemic methadone may possess in vivo NMDA receptor antagonist activity in addition to its well-established opioid agonist activity. NMDA receptor antagonists have potentiated the antinociceptive effects of morphine (Chapman and Dickenson, 1992; Mao et al., 1996). Thus, it is possible that the NMDA receptor antagonist activity of the d-isomer of methadone may potentiate the opioid antinociceptive effects of l-methadone. In addition, NMDA receptor antagonists attenuate the development of morphine tolerance (Tiseo and Inturrisi, 1993; Elliott et al., 1995; Shimoyama, N. et al., 1996). Therefore, the NMDA receptor antagonist activity of d-methadone may act to attenuate the development of tolerance to the opioid component of racemic methadone, but this needs to be demonstrated directly. Clinically, NMDA receptor antagonists are effective in the treatment of neuropathic pain syndromes (Backonja et al., 1994; Eide et al., 1994; Max et al., 1995) which may often be less responsive to opioid such as morphine. Thus, as a result of its NMDA receptor antagonist activity, racemic methadone may have antinociceptive actions that are different from other mu opioids such as morphine or hydromorphone which do not bind to NMDA receptors (Gorman et al., 1997). Anecdotal case reports have suggested the successful management with methadone of pain syndromes that were unresponsive to morphine (Leng and Finnegan, 1994; Gardner-Nix, 1996).

In conclusion, spinal d-methadone is antinociceptive in the rat formalin test and antagonizes NMDA-induced nociceptive behaviors. This in vivo activity appears to be the result of NMDA receptor antagonist activity. The extent to which this activity affects the pharmacology of racemic methadone remains to be determined.

### References


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