Mechanism of Attenuation of Morphine Antinociception by Chronic Treatment with L-Arginine

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ABSTRACT
The effects of twice-daily injections of L-arginine or D-arginine (200 mg/kg i.p.) for 4 days on morphine-induced antinociception, brain nitric oxide synthase activity and brain and serum distribution of morphine and brain μ-opioid receptors labeled with [3H][D-Ala2,MePhe4,Gly5-ol]enkephalin were determined in male Swiss-Webster mice. Chronic treatment with L-arginine, but not D-arginine, decreased the antinociceptive response to morphine in mice, increased the activity of nitric oxide synthase in the midbrain and decreased brain levels of morphine, compared with vehicle-injected controls. Significant decreases in morphine levels were observed in midbrain, pons and medulla, hippocampus, striatum and spinal cord of L-arginine-treated mice, in comparison with vehicle-injected mice. However, the levels of morphine in cortex, amygdala and hypothalamus of L-arginine- or D-arginine-treated mice did not differ from those of vehicle-injected controls. Acute treatment with L-arginine (200 mg/kg i.p.) or D-arginine (200 mg/kg i.p.) did not modify either morphine antinociception or morphine distribution in brain regions or the spinal cord. Chronic administration of L-arginine or D-arginine did not alter the Bmax or kV values of [3H][D-Ala2,MePhe4,Gly5-ol]enkephalin binding to the mouse brain membranes. These results suggest that chronic treatment with L-arginine reduces the antinociceptive effect of morphine by decreasing brain nitric oxide synthase activity and by decreasing the concentration of morphine in certain brain regions and spinal cord.

Considerable evidence suggests that NMDA/NO pathways may be involved in the acute and chronic actions of opioid drugs that are used primarily for the relief of moderate- to severe-intensity pain. Opioid drugs produce their actions by interacting with three major types of opioid receptors, namely μ, δ and κ, with morphine or DAMGO, [d-Pen²,d-Pen⁵]enkephalin and U-50,488H, respectively, being the prototypical agonists. NO, a second messenger involved in the regulation of cell function, is formed enzymatically from L-arginine by NOS after the activation of the NMDA receptor (Moncada et al., 1991). NOS is inhibited by several L-arginine derivatives, such as N⁴-nitro-L-arginine and L-NMMA (Rees et al., 1990; Thorat et al., 1994). Drugs that modify the concentration of NO in the central nervous system appear to modify opioid-induced antinociception. Furthermore, NO is involved in nociceptive processes, particularly in the spinal cord (Haley et al., 1992). Synaptic transmission in the central and peripheral nervous system seems to be modulated by NO (Meller and Gebhart, 1993). NO donors such as 3-morpholino-sydnonimine, sodium nitroprusside and hydroxylamine given i.t. induced hyperalgesia in rats, as measured by the tail-flick test, whereas NOS inhibitors such as L-NAME given i.t. or i.c.v. elicited a slight antinociception. However, the effects of other routes of administration of NO donors or NOS inhibitors were not determined (Przewlocka et al., 1994). In the abdominal constriction test, L-arginine had no effect but L-NAME and L-NMMA by themselves produced marked antinociception, as evidenced by inhibition of the abdominal constriction response in mice (Moore et al., 1991; Mustafa, 1992). In the tail-flick test, L-NAME, L-NMMA and L-arginine had no effect (Dambisya and Lee, 1995). In another study, i.c.v. administration of L-arginine (30 mg/mouse) elicited antinociception in mice, as assessed by the tail-flick test. The antinociceptive action of L-arginine was attributed to the formation of kytorphin (L-Tyr-L-Arg) in the brain (Kawabata et al., 1994, 1996). Thus, L-arginine appears to produce nociception and antinociception in tests that are sensitive to weak analgesic agents. On the other hand, acute administration of L-arginine, the precursor of NO, p.o. or i.p. but not i.c.v. reduced morphine antinociception in mice. These effects were observed at 300 and 1000 mg/kg doses of

ABBREVIATIONS: AUC, area under the time-response curve; DAMGO, [d-Ala²,MePhe⁴,Gly⁵-ol]enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; i.t., intrathecal(ly); L-NAME, N⁴-nitro-L-arginine methyl ester; NMDA, N-methyl-D-aspartate; L-NMMA, N⁴-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; RIA, radioimmunoassay.

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L-arginine and were reversed by the NOS inhibitors L-NAME and L-NMMA (Brignola et al., 1994). Intrathecal administration of L-NAME has been shown to enhance morphine antinociception in rats (Przewlocki et al., 1993), suggesting involvement of the spinal NO system.

The effect of chronic administration of L-arginine on morphine antinociception has not been determined. Although, in the aforementioned studies, NO was implicated in the acute and chronic actions of opioid drugs, other factors, such as the activity of NOS and the distribution of opioid agents in the central nervous system, have received little attention. In the present studies, the effects of chronic administration of L-arginine or D-arginine on the antinociceptive activity of morphine have been determined. The effects of such treatments on NOS activity and the distribution of morphine in certain brain regions, spinal cord and serum were evaluated. As controls, the effect of acute administration of L-arginine on morphine antinociception and its distribution in brain regions and spinal cord have also been determined. Finally, the effect of chronic administration of L-arginine or D-arginine on the binding of [3H]DAMGO to brain membranes was also assessed.

Methods

Animals. Male Swiss-Webster mice weighing 25 to 30 g (Sasco King Animal Co., Oregon, WI) were acclimated to a room with controlled ambient temperature (23 ± 1°C) and humidity (50 ± 10%) and a 12-hr dark/light cycle (6:00 A.M. to 6:00 P.M.). The animals were housed under these conditions for at least 4 days before being used. The animals were given food and water continuously.

Chemicals. Morphine sulfate was purchased from Mallinckrodt Chemical Co. (St. Louis, MO). L-Arginine and D-arginine were purchased from Sigma Chemical Co. (St. Louis, MO). The drugs were dissolved in physiological saline and injected i.p. or s.c. in a volume of 10 ml/kg body weight. The RIA kit for morphine was obtained from Diagnostic Products Corp. (Los Angeles, CA). [3H]Arginine (specific activity, 64.0 Ci/mmol) was purchased from Amersham International (Arlington Heights, IL). [3H]DAMGO (specific activity, 50.0 Ci/mmol) was obtained from the National Institute on Drug Abuse (Rockville, MD). Unlabeled levorphanol was purchased from Research Biochemicals Inc. (Natick, MA).

Chronic treatment with L-arginine or D-arginine. Mice were injected i.p. with vehicle, L-arginine (200 mg/kg) or D-arginine (200 mg/kg) twice each day for 4 days. On day 5, the antinociceptive response to morphine (2.5, 5.0 or 10 mg/kg s.c.) was determined as described below.

Measurement of the antinociceptive response. The antinociceptive response to morphine was measured by the tail-flick test as described earlier (D’Amour and Smith, 1941; Bhargava and Zhao, 1996; Zhao and Bhargava, 1996). At the beginning of the study, the light intensity in the tail-flick apparatus was adjusted so that the mean basal latencies for the tail-flick response were approximately 2 sec. To minimize tail skin tissue damage, the cut-off time was set at 10 sec. The tail-flick latencies were determined before and 30 min after the injection of morphine (2.5, 5.0 or 10 mg/kg s.c.). The basal tail-flick latencies were subtracted from the effect induced by the drug for each mouse. The antinociceptive response for each mouse was converted into AUC. Data were expressed as AUC (mean ± S.E.M.). Ten mice were used for each treatment group. The differences in the antinociceptive responses in chronic vehicle- and chronic L-arginine- or D-arginine-treated mice were determined by using Student’s t test. A value of P < .05 was considered to be significant.

Measurement of NOS activity. NOS activity was determined in the brain regions (cerebellum, midbrain, cortex and remainder of the brain) and spinal cord of mice treated chronically with vehicle, L-arginine or D-arginine as described above. NOS activity was determined as described earlier (Barjavel and Bhargava, 1994a,b), as the rate of conversion of [14C]arginine to [3H]citrulline. Briefly, the appropriate tissue was homogenized in HEPES buffer (20 mM, pH 7.4) containing 0.5 mM EDTA and 2 mM β-mercaptoethanol, using an ultrasonic dismembrator, for 10 sec at setting 8. The homogenate was centrifuged at 15,000 × g for 30 min at 4°C. A 50-μl volume of supernatant sample (150–250 μg protein) was added to the incubation medium containing 50 mM HEPES buffer, pH 7.4, 0.5 mM β-mercaptoethanol, 1 mM dithiothreitol, 2 mM NADPH, 0.5 mM CaCl2 and 5 μM L-arginine plus 1 μCi/ml [3H]arginine. The incubation was carried out in duplicate for 15 min at 37°C. The blanks were run similarly but without NADPH and CaCl2. The reaction was stopped by the addition of 500 μl of stop buffer (20 mM HEPES, pH 5.5, containing 2 mM EDTA). The contents of the incubation tubes were transferred to 0.6 ml of Dowex AG 50W-X8 (Na+ form) resin. [3H]Citrulline formed was eluted from the column using 2 ml of distilled water. A 250-μl aliquot of the eluate was added to a scintillation vial containing 5 ml of Scint-AFX scintillation fluid (Packard Instrument Co., Meriden, CT). The radioactivity in the samples was determined in a Packard liquid scintillation counter with 60% efficiency. The protein concentration in the samples was measured by the method of Lowry et al. (1951). NOS activity was expressed as picomoles of [3H]citrulline formed per minute per milligram of protein. The difference in NOS activity in treated and control tissues was determined by analysis of variance followed by unpaired Student’s t test. A value of P < .05 was considered to be statistically significant.

Measurement of morphine concentrations in brain regions, spinal cord and serum of L-arginine- and D-arginine-treated mice. Mice were treated with L-arginine or D-arginine for 4 days, as described above. On day 5, mice from each treatment group were injected with morphine (10 mg/kg s.c.) and sacrificed 60 min later. The brain, spinal cord and trunk blood were collected. The brain was dissected into seven regions, namely hypothalamus, pons and medulla, amygdala, hippocampus, corpus striatum, midbrain and cortex. The blood samples were centrifuged at 3000 rpm for 10 min at 4°C. Serum was separated and stored in deep freeze at −80°C. The concentration of morphine in serum was determined by RIA using 25 μl of the sample in duplicate, as described previously (Bhargava et al., 1991, 1992; Bhargava and Villar, 1991a,b, 1992). This detection method used 125I-labeled morphine that competed with morphine in the sample for antibody sites. The antibody adhered to the wall of a polypyrrolene tube. The reaction of morphine and antibody was terminated by decanting the supernatant. The antibody-bound radioiodinated morphine was counted in a gamma counter. The brain regions and spinal cord were weighed and homogenized in water (3–10 times the tissue volume), using a Polytron homogenizer (PT 10, setting 6 for 15 sec). The final homogenate contained approximately 60 mg of tissue/ml. The concentration of morphine in the homogenate was expressed as nanograms per gram of tissue. The recovery of morphine from brain regions was found to be quantitative. The limit of detection was 0.8 ng/ml of homogenate. The specificity and cross-reactivity of the antibody with morphine metabolites and other analogs have been described previously (Bhargava et al., 1991). The antibody showed cross-reactivity with the two morphine glucuronides of only 0.2% and with normorphine of 10%. Eight mice were used for each treatment group.

The differences in the morphine concentrations in various tissues and serum from vehicle-, L-arginine- and D-arginine-treated mice were determined by using analysis of variance followed by the Newman-Keuls test. A value of P < .05 was considered to be significant.

Determination of the effects of acute administration of L-arginine or D-arginine on the antinociceptive activity of morphine and the distribution of morphine in brain regions and spinal cord of mice. Mice were injected with L-arginine (200 mg/kg i.p.), D-arginine (200 mg/kg i.p.) or the vehicle. They were then injected with morphine (10 mg/kg s.c.) 10 min later. The antinocip-
The binding was carried out in a total volume of 0.25 ml, which contained 0.05 M Tris-HCl buffer and 0.1 ml of the homogenate (200–250 µg of protein). The binding of [³H]DAMGO was performed according to the procedure of Magnan et al. (1982), using [³H]DAMGO in the concentration range of 1.25 to 40 nM. All binding assays were carried out in duplicate at 25°C for 60 min. The specific binding was defined as the difference in binding observed in the absence and presence of 10 µM levorphanol. Binding was terminated by rapidly filtering the contents of the incubation tubes under reduced pressure, using a Brandel M-24R cell harvester and Whatman GF/B glass fiber filters. The filters were washed twice with 5 ml of the same ice-cold buffer used for the assay. The filters were transferred to liquid scintillation vials containing 5 ml of Scint-A XF scintillation fluid (Packard). After an overnight equilibration period, the radioactivity in the samples was determined using a Packard liquid scintillation counter (model 4640) with 60% counting efficiency. The concentration of protein in the samples was determined by the method of Lowry et al. (1951).

The receptor densities (B_max values) and apparent dissociation constants (K_d values) were determined from the saturation curves and the Scatchard plots using the program LIGAND (Munson and Rodbard, 1980). The data were expressed as mean values ± S.E.M. Six mice were used for each treatment group. The data were analyzed by using Student’s t test. A value of P < .05 was considered to be significant.

**Results**

**Effects of chronic administration of L-arginine or D-arginine on the antinociceptive actions of morphine in mice.** Administration of morphine produced dose-dependent antinociception in mice, as evidenced by the increasing AUC_{0–210min} values. Chronic administration of L-arginine (200 mg/kg i.p.) twice each day for 4 days decreased the antinociceptive action of morphine. As shown in figure 1, the antinociceptive response to morphine (2.5 mg/kg) was decreased by 51%, but this change was not significant. However, the responses to 5.0 and 10.0 mg/kg doses of morphine were decreased by 75 and 76% and were significant at the P < .05 and P < .001 levels, respectively. The same treatment regimen with D-arginine did not affect morphine-induced antinociception.

**Effects of acute administration of L-arginine or D-arginine on the antinociceptive activity of morphine in mice.** Acute administration of a 200 mg/kg dose of L-arginine or D-arginine did not modify either the basal tail-flick latency (table 1) or the antinociception induced by morphine (10 mg/kg). As shown in figure 2, the AUC_{0–210min} values for morphine in vehicle-, L-arginine- or D-arginine-treated mice were virtually identical.

**Effects of chronic administration of L-arginine or D-arginine on the activity of NOS in brain regions and spinal cord of mice.** The activity of NOS was found to be the highest in cerebellum, followed in decreasing order by midbrain, cortex, remainder of the brain and spinal cord. Chronic administration of L-arginine (200 mg/kg i.p.) twice each day for 4 days increased the NOS activity in midbrain by 15.2% (P < .05), but in other brain regions and spinal cord the NOS activity remained unchanged. A similar treatment regimen with D-arginine, on the other hand, did not modify NOS activity in any brain region or the spinal cord of the mouse (fig. 3).

**Effects of chronic administration of L-arginine or D-arginine on the distribution of morphine in brain regions, spinal cord and serum of mice.** The distribution of morphine in brain regions and spinal cord of mice given vehicle, L-arginine (200 mg/kg i.p.) or D-arginine (200 mg/kg i.p.) for 4 days and then given morphine (10 mg/kg s.c.) is shown in figure 4. Chronic administration of L-arginine decreased the concentration of morphine in midbrain (40%, P <

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal Tail-Flick Latency a (sec)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>1.89 ± 0.07</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.89 ± 0.05</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>2.07 ± 0.07</td>
</tr>
</tbody>
</table>

a Mean ± S.E.M. (n = 30).
The concentration of morphine in cortex, amygdala and hypothalamus of L-arginine- and vehicle-injected controls did not differ. Chronic treatment with D-arginine, however, did not alter the concentration of morphine in any brain regions or spinal cord of the mice (fig. 4). Chronic administration of L-arginine or D-arginine treatment did not affect serum concentration of morphine (table 2).

**Effects of acute administration of L-arginine or D-arginine on the distribution of morphine in brain regions and spinal cord of mice.** Acute administration of a 200 mg/kg dose of L-arginine or D-arginine did not alter the concentration of morphine determined 60 min after the administration of a 10 mg/kg dose of morphine. As can be seen in figure 5, the morphine concentrations in specific brain regions or the spinal cords of mice treated acutely with L-arginine or D-arginine did not differ from those in animals treated with the vehicle. Similarly, the serum concentration of morphine was not modified by acute treatment with L-arginine or D-arginine (table 2).

**Effects of chronic administration of L-arginine or D-arginine on the binding of [3H]DAMGO to mouse brain μ-opioid receptors.** [3H]DAMGO bound to mouse brain homogenate with a $B_{max}$ value of $165.6 \pm 10.3$ fmol/mg protein and a $K_d$ value of $3.06 \pm 0.52$ nM. Chronic administration of L-arginine or D-arginine to mice did not alter the binding characteristics of [3H]DAMGO in the brain (table 3).

### Discussion

The present studies clearly demonstrate that chronic administration of L-arginine but not of D-arginine causes a reduction in the antinociceptive action of morphine, a μ-opioid receptor agonist, in mice. Thus, the effect of arginine was stereospecific. To our knowledge, this is the first study on the effect of chronic administration of L-arginine on morphine antinociception. In an earlier study, the effect of acute treatment with L-arginine or D-arginine on morphine antinociception was studied in mice. The doses of L-arginine that produced inhibition were 300 and 1000 mg/kg and the degree of inhibition was rather small (Brignola et al., 1994). However, in the present study, chronic treatment with L-arginine produced a robust decrease in the antinociceptive action of morphine in mice. Because in our studies chronic administration of L-arginine (200 mg/kg) produced an 80% reduction in morphine antinociception, the effect of the same dose of L-arginine given acutely on morphine antinociception was deter-
Effects of chronic administration of L-arginine or D-arginine on the parameters of [3H]DAMGO binding to mouse brain membranes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Binding Parametersa</th>
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<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$</td>
<td>$K_d$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>165.6 ± 10.3</td>
<td>3.06 ± 0.52</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>162.5 ± 8.0</td>
<td>2.28 ± 0.16</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>169.1 ± 9.5</td>
<td>2.85 ± 0.34</td>
</tr>
</tbody>
</table>

a Mice were injected i.p. with vehicle, L-arginine (200 mg/kg) or D-arginine (200 mg/kg), twice per day, for 4 days and were sacrificed on day 5 for the binding studies.

b Mean ± S.E.M. (n = 6).

mined in mice. Acute L-arginine treatment did not alter morphine antinociception in mice. It is clear that chronic L-arginine treatment induces changes that result in decreased morphine antinociception.

Attempts were also made to determine the potential mechanisms by which L-arginine modified morphine antinociception. In an earlier study, where acute administration of high doses of L-arginine p.o. or i.p. but not i.c.v. decreased morphine antinociception, the effect on NOS activity in brain or spinal cord was not determined. It was assumed that L-arginine affected the formation of NO (Brignola et al., 1994). In the present study, chronic treatment with L-arginine produced a 15% increase in the activity of NOS only in the midbrain. The midbrain is an important brain region involved in pain perception and contains the periaqueductal gray matter, with a high density of endogenous opioid systems. The increase in NOS activity would enhance the production of NO. Previous studies have implicated NO in nociceptive processes. Production of NO by i.t. administration of L-arginine resulted in a rapid, transient and dose-dependent facilitation of the nociceptive tail-flick reflex (Meller et al., 1992a). Similarly, NO of the spinal cord mediates the NMDA-induced facilitation of the tail-flick nociceptive reflex (Meller et al., 1992a; Kitto et al., 1992) and thermal hyperalgesia in a model of neuropathic pain in rats (Meller et al., 1992b). As indicated above, the majority of studies show NO to have an algesic action. Therefore, increased production of NO as a result of chronic administration of L-arginine would result in antagonism of the antinociceptive response to morphine, as was observed in the present studies. NO in the spinal cord has been shown to modulate the descending pain control system for antinociception activated by supraspinally applied morphine. Thus, activation of NO system in the spinal cord attenuates morphine antinociception (Xu and Tseng, 1995). However, the effect of i.t. administration of activators of NO on the antinociception induced by peripheral administration of morphine is not known. How L-arginine activates NO in the spinal cord and modifies morphine antinociception is not clear, because in our studies the activity of NOS was modified by chronic treatment with L-arginine.

NO of the spinal cord has been reported to cause hyperalgesia. The evidence was based on the fact that i.t. injection of L-arginine or NO donors facilitated the tail-flick response and decreased tail-flick latencies and this effect was reversed by N\textsuperscript{G}-nitro-L-arginine. However, the onset of such actions of L-arginine was rapid and the duration was short (2 min) (Kitto et al., 1992; Meller et al., 1992a). Other studies, however, failed to observe hyperalgesia after i.t. injections of L-arginine, because the tail-flick latencies were not modified (Xu and Tseng, 1995). Our studies also did not find a change in the basal tail-flick latencies after chronic administration of L-arginine, and thus the attenuation of morphine antinociception cannot be explained on the basis of any hyperalgesia of short duration (2 min) observed by Meller et al. (1992a).

Thus, our results on the lack of an effect on tail-flick latency by L-arginine are in agreement with those of Xu and Tseng (1995) and in contrast to those of Kitto et al. (1992) and Meller et al. (1992a).

Our results also show that chronic administration of D-arginine, which is not a substrate for NOS, neither modified morphine antinociception nor altered the concentration of morphine in brain regions and spinal cord, further supporting the view that chronic administration of L-arginine possibly modifies the transport of morphine across the blood-brain barrier.

Another possibility is that the decreased antinociceptive response to morphine with L-arginine may be related to alterations in the distribution of morphine in the central nervous system involved in the nociceptive response. The present studies indicated that arginine, in a stereospecific manner, decreased the concentration of morphine in midbrain, pons and medulla, hippocampus, corpus striatum and spinal cord. Thus, L-arginine, but not D-arginine, decreased the levels of morphine in spinal and supraspinal structures and could have been responsible for the decreased antinociceptive activity. Chronic treatment with L-arginine or D-arginine, however, did not affect the levels of morphine in serum. Acute treatment with L-arginine (200 mg/kg), which did not modify morphine antinociception, also failed to alter the concentration of morphine in brain regions or the spinal cord of mice. It is, therefore, clear that chronic administration of L-arginine induces certain changes in the brain and spinal cord, which result in decreased transport of morphine in the central nervous system. The decrease in morphine concentrations in the brain after chronic treatment with L-arginine might be the result of some change in the blood-brain barrier that decreases the movement of morphine to the brain. The fact that L-arginine treatment did not modify...
antinociception produced by i.c.v. administration of morphine (Brignola et al., 1994; J.-T. Bian and H. N. Bhargara, unpublished observations) further supports the view that NO/NOS systems may affect the blood-brain barrier to morphine. The exact mechanism by which l-arginine decreases the brain and spinal cord concentrations of morphine is not clear at present.

In addition to the altered concentrations of morphine in the central sites after chronic administration of l-arginine, the characteristics of central μ-opioid receptors labeled with [3H]DAMGO were also determined, in an effort to further determine the mechanism by which l-arginine decreases morphine antinociception. The present studies clearly demonstrated that the Bmax and Kd values for [3H]DAMGO in the brains of mice treated chronically with l-arginine or d-arginine did not differ from those in mice treated chronically with the vehicle. Thus, the decreased analgesic response to morphine after chronic l-arginine treatment is not related to changes in the brain μ-opioid receptors. Administration of l-arginine (i.c.v.) also failed to modify the antinociception induced by i.c.v. injections of morphine or DAMGO (Xu and Tseng, 1993). These studies further support the suggestion that l-arginine administered acutely or chronically does not modify the characteristics of the brain μ-opioid receptors.

In summary, the present studies demonstrate that chronic treatment with arginine stereospecifically increases central NOS activity and decreases morphine antinociception by decreasing the levels of morphine in both the spinal and supraspinal structures of the central nervous system. Further studies are warranted to understand the mechanisms involved in the attenuation of morphine antinociception by chronic treatment with l-arginine.

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References


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