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Effects of NMDA receptor antagonists on inhibition of morphine tolerance in rats: binding at μ-opioid receptors

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Abstract

Past studies have shown antagonists of excitatory amino acid receptors, both *N*-methyl-D-aspartate (NMDA) and non-NMDA, to produce an antinociceptive effect in vitro and in vivo. Additionally, NMDA receptor antagonists have been demonstrated to prevent morphine tolerance. We had found that one NMDA receptor antagonist, ketamine, potentiates morphine's analgesic effect in post-operative patients. Our latest experiment was performed to examine the modulatory effect of competitive and non-competitive NMDA receptor antagonists on morphine antinociception and tolerance. A PE_{10} catheter was intrathecally (i.t.) implanted in male Sprague-Dawley rats for drug administration. The antinociceptive effect of morphine, D-(−)-2-amino-5-phosphonovaleric acid (D-AP5) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cyclohepten-5,10-imine maleate (MK-801) was measured using the hot-water tail immersion test. Neither competitive nor non-competitive NMDA receptor antagonists had an antinociceptive effect by themselves, but they did potentiate the antinociceptive effect of morphine. Both D-AP5 (AD_{50} = 0.18 μg) and MK-801 (AD_{50} = 0.57 μg) shifted the antinociceptive dose-response curve of morphine (AD_{50} = 4.2 μg) to the left. Both D-AP5 (4 μg/h) and MK-801 (10 μg/h) when co-administered with i.t. morphine infusions (10 μg/h) also inhibited the development of tolerance. In [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin ([^{3}H]DAMGO) binding assays, MK-801 (B_{max} = 32.90 ± 3.33 fmol/mg) treatment prevented the down-regulation of μ-opioid receptor high-affinity sites induced by continuous morphine infusions alone (B_{max} = 13.97 ± 1.47 fmol/mg). D-AP5 (B_{max} = 20.78 ± 3.36 fmol/mg) did not prevent the reduction of μ-opioid receptor high-affinity sites. However, high-affinity sites in rats treated with D-AP5 and morphine displayed a higher affinity (K_D = 0.45 ± 0.09 nM) than those of control animals (K_D = 0.95 ± 0.08 nM). Results of this study indicate that competitive as well as non-competitive NMDA receptor antagonists enhance morphine's antinociceptive effect, and prevent the development of morphine tolerance. Thus, in our opinion, there opens a new frontier in clinical pain management, especially for those patients who require long-term opioid treatment for pain relief.

Keywords: NMDA receptor; Opioid receptor; Morphine tolerance; (Intrathecal administration)

1. Introduction

Opioids are the therapeutic mainstay of clinical pain management. However, administration of larger doses is often accompanied by narcotic-associated side-effects. In addition, analgesic tolerance will develop if opioids are used for a long time. This phenomenon further limits their therapeutic efficacy. Two possible mechanisms for this tolerance development have been suggested. On the cell membrane, receptor uncoupling and receptor down-regulation are found after tolerance development (Wong et al., 1992a, b). There is accumulating evidence that opioid tolerance can be inhibited by antagonizing the NMDA receptor system (Trujillo and Akil, 1991, 1994; Kest et al., 1993; Gutstein and Trujillo, 1993). Additionally, our previous study demonstrated that the non-competitive NMDA receptor antagonist, ketamine, on epidural administration to postoperative patients potentiated morphine's analgesic effect (Wong et al., 1995). Therefore, understanding the underlying tolerance mechanisms of the

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opioid receptor system and its interaction with the NMDA receptor system might enable us to prolong and enhance the effectiveness of opioids in clinical pain management.

Lines of evidence from past studies demonstrated an interaction between excitatory amino acid receptors and opioid receptor systems. The excitatory amino acids, glutamate and aspartate, have already been demonstrated to be involved in nociception transmission in the spinal cord (Headley and Grillner, 1990; Aanonsen et al., 1990). Intense concentration of glutamate binding sites in the substantia gelatinosa (where opioid receptors are known to exist) of rat spinal cord has also been demonstrated (Greenamyre et al., 1984). Excitatory amino acid receptors, in particular the NMDA receptor, have also been found to be involved in neuroplasticity (Collingridge and Singer, 1990) and opioid gene expression (Summers et al., 1993). Trujillo and Akil (1991) demonstrated that the non-competitive NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cyclohepten-5,10-imine maleate (MK-801), when administered systemically, attenuated opioid tolerance and dependence without affecting the antinociceptive effect of morphine. They thus further confirmed that the antagonism of morphine-induced tolerance was at the spinal sites in a spinalized rat model (Gutstein and Trujillo, 1993). Also, an increase in release of excitatory amino acids following opioid withdrawal has been demonstrated (Akaola and Aston-Jones, 1992). Intracerebroventricular administration of the excitatory amino acid receptor antagonist, kynurenic acid, further attenuates such opioid withdrawal-induced hyperactivity in locus coeruleus neurons. Recently, we used microdialysis to demonstrate that the release of excitatory amino acids increased in nucleus accumbens, locus coeruleus neurons and the striatal system of chronic morphine-tolerant rats experiencing naloxone-induced withdrawal (submitted). Moreover, we found that epidurally administered ketamine potentiated morphine's analgesic effect in postoperative patients (Wong et al., 1995).

In the present study, a rat spinal model with intrathecal (i.t.) catheter inserted via the lumbar intervertebral space was used to examine the effect of NMDA receptor antagonists, both competitive (D-(−)-2-amino-5-phosphonovaleric acid; D-AP5) and non-competitive (MK-801) on the antinociceptive effect of morphine, and the development of tolerance to morphine.

2. Materials and methods

2.1. Animal model

With institutional approval of the National Defense Medical Center, male Sprague-Dawley rats weighing 300–350 g were anesthetized with intraperitoneal chloral hydrate 400 mg/kg. Lumbar subarachnoid catheterization was performed as described by Wang et al. (1991). The injection port, covered by a removable PE_{50} cap, was brought out of the skin via a separate small opening lateral to the main skin incision. After the animals had recovered from anesthesia, 30–50 μl of 2% lidocaine was injected through the subarachnoid space catheter. The correct positioning of the catheter was evidenced by the prompt sensory and motor block of the hind limbs, developing in 1–5 min and lasting 20–30 min. These animals were then housed in the Animal Facility of the National Defense Medical Center and allowed to recover. 5 days after catheterization, a mini-osmotic pump was implanted under chloral hydrate anesthesia (400 mg/kg, intraperitoneal). The pump (Model 2001, Alzet, Palo Alto, CA with pump rate = 1 μl/h for 7 days) was first filled with morphine or other test drugs, then connected to the i.t. catheter with PE_{50} tubing, and finally placed under the skin, between the scapulas. Rats that experienced gross neurological injury or had fresh blood in their cerebrospinal fluid were excluded from the study. The animals were housed individually and were kept on a 12-h light/dark cycle. Food and water were freely available. All tests were performed during the light phase of the cycle. The treatment of the study animals conformed to the 'Guiding Principle in the Care and Use of Animals' as approved by the Council of the American Physiological Society, and National Defense Medical Center Animal Care and Use Committee.

2.2. Tolerance induction

Morphine is the prototypic non-opioid agonist at the μ-opioid receptor. Spinal opioid tolerance at the μ-sites was induced by continuous i.t. infusions of morphine (10 μg/h) via the osmotic pump for 6 days. We defined evidence of tolerance as a loss of the antinociceptive effect of morphine infusions as shown by hot water tail immersion test. The antinociceptive effect of MK-801 (10 μg/h) and D-AP5 (4 μg/h) administered by continuous i.t. infusion for 6 days were examined. The effect of these drugs on morphine tolerance was examined by co-administering morphine with i.t. infusions of either MK-801 (10 μg/h) or D-AP5 (4 μg/h) for 6 days. Control animals were given equal amounts of saline.

2.3. Antinociceptive test

The hot-water tail immersion test was used to measure the drugs' antinociceptive effect. The heat intensity was set at 50 ± 0.2°C and the cut-off time was set at 10 s to allow for multiple tests without excessive tissue injury. The rat was placed in a plastic restrainer for drug injection and antinociception assay. The

antinociceptive effect of the NMDA receptor antagonists, D-AP5 and MK-801, were examined. All drugs were dissolved in normal saline. 10 μl of each drug was flushed with 10 μl of saline. All the antinociceptive tests were performed 30 min after acute administration of the drug. Heat stimuli were applied to rats at 90-s intervals: 4 times over a 6-min testing period. The tail flick responses to the hot water tail immersion test were converted from latencies (s) into MPE (Maximum Percent Effect).

$$\text{MPE}(\%) = \frac{(\text{post-injection latency} - \text{baseline latency})}{(\text{cut-off latency} - \text{baseline latency})} \times 100$$

2.4. Effect of NMDA receptor antagonists on morphine dose-response effect on antinociception

Morphine's dose-response effect on antinociception (i.t.) was generated 10 min after D-AP5 (5 μg) or MK-801 (10 μg) i.t. bolus injection. The antinociceptive dose-response effect of morphine, D-AP5 and MK-801 alone (i.t.) was also evaluated in the hot water immersion tail test. The tail-flick latencies were measured 30 min after drug injection.

2.5. Membrane preparation

All rats were killed by decapitation after antinociceptive tests at day 6. Spinal cord tissue was removed quickly and placed in a freezer. Spinal cords were divided into two halves: the cervicothoracic and the lumbosacral segments. Control spinal cord tissue was also obtained by decapitation and stored at −70°C. The tissue was homogenized with a PT20 polytron (setting 6) in 50 mM Tris · HCl (pH 7.4) containing 50 μg/ml of soybean trypsin inhibitor, 1 mM EDTA, 10 μl/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. This standard buffer (buffer A) was also used as the standard binding assay buffer. The total membrane particulate fraction was obtained by centrifugation at 40000 × g for 20 min and stored in a −70°C freezer before use for membrane binding assays.

2.6. Membrane pretreatment and opioid receptor binding

The membrane pellets were thawed at room temperature. Before their use for binding assays, the membranes had been preincubated for 30 min at room temperature in buffer B, or buffer B containing additional Na^+ (100 mM) or Na^+ (100 mM) + GDP (100 μM). Buffer B was made of 5 mM Tris · HCl buffer (pH 7.4) containing 50 μg/ml soybean trypsin inhibitor, 1 mM EDTA, 10 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride. After preincubation, the membranes were washed 3 times with buffer A by centrifuging at 40000 × g for 20 min to remove excess Na^+ and GDP from membranes.

All binding assays were performed in buffer A. Mg^{2+} (5 mM) was routinely added to the binding buffer except when indicated in the figure legends. All binding incubations were carried out at room temperature for 2 h, and a rapid filtration method was used with a Skatron semi-automatic cell harvester (model

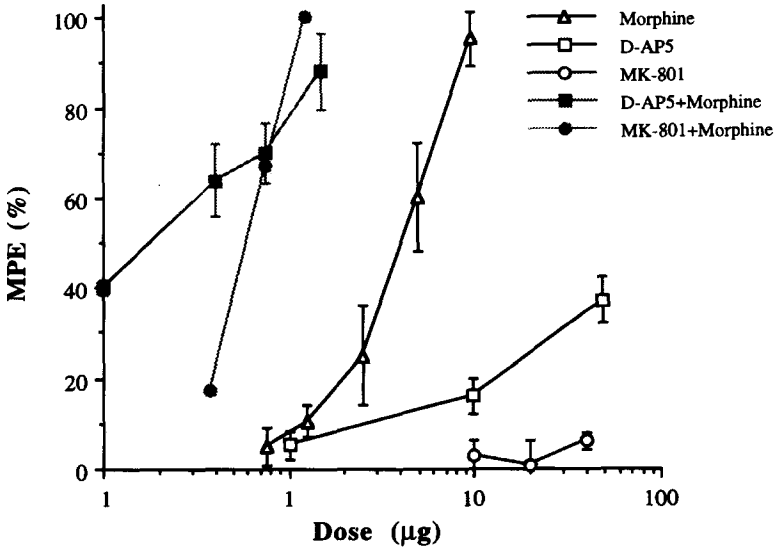

Fig. 1. The Antinociceptive effect of various drugs on rats. The antinociceptive effect of i.t. injections of D-AP5 (5 μg) and MK-801 (10 μg) and their influence on the antinociceptive effect of morphine were examined. These drugs, when co-administered with morphine, were given 10 min before morphine injection. Hot water tail immersion latencies were measured 30 min after drug injection. All data points are averages for no fewer than six rats, and the results are expressed as means ± S.E.M..

11021, Skatron Instruments, Sterling, VA) to terminate the binding. In our previous study (Wong et al., 1992a), we showed that continuous μ-opioid agonist i.t. infusion induced tolerance and μ-opioid receptor high-affinity site reduction. Therefore, [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin ([^{3}H]DAMGO) (~ 0.1–10 nM), which only measures μ-receptor high-affinity sites in the present of Mg^{2+}, was used as the radiolabeled ligand to examine the μ-opioid receptor binding characteristics. Non-specific binding was determined in the presence of 1 μM of unlabeled naloxone. The protein concentration was approximately 500 μg/tube and the incubation volume was 1 ml. All binding assays were run in triplicate. Scatchard analysis was carried out with EBDA (McPherson, 1983) and LIGAND (Munson and Rodbard, 1980) programs. Protein concentration was determined by the method of Lowry et al. (1951); crystalline bovine serum albumin was used as the standard.

2.7. Drugs

Morphine sulfate was obtained from Narcotics Control Bureau of the Health Department of Taiwan. [^{3}H]DAMGO was purchased from NEN Co. (Wilmington, DE, USA). All other chemicals were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared with preservative-free sterile normal saline and kept frozen until used. Sterility was maintained at all steps of the study. Osmotic minipumps were purchased from Alzet Corporation (Palo Alto, CA, USA).

2.8. Statistical analysis

All data were presented as means ± S.E.M. for the given number of rats or experiments. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis as appropriate. A significant difference was defined as $P < 0.05$.

3. Results

3.1. Antinociceptive effects of various drugs on rats

As shown in Fig. 1, i.t. morphine administration produced an antinociceptive effect: the AD_{50} was 3.45 μg. Neither the competitive (D-AP5) nor the non-competitive (MK-801) NMDA receptor antagonist produced significant antinociception in the hot water immersion tail test. However, both the competitive (D-AP5) and non-competitive (MK-801) NMDA receptor antagonists potentiated morphine's antinociceptive effect. They shifted the dose-response curve to the left to resultant AD_{50} values of 0.6 and 0.19 μg, respectively.

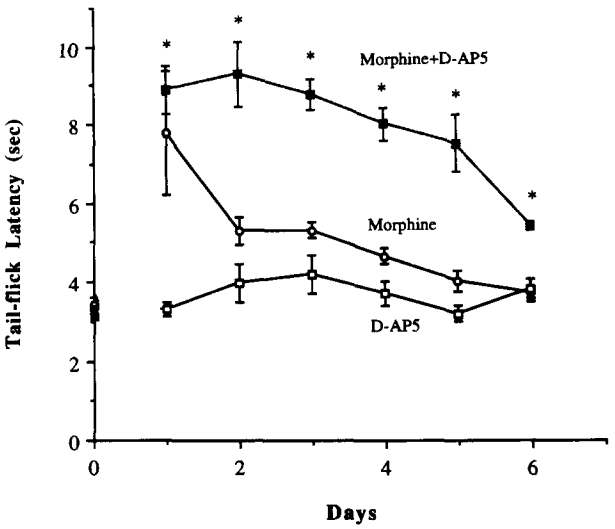

Fig. 2. The effect of D-AP5 on morphine tolerance. The effect of D-AP5 on morphine tolerance was examined by co-administering D-AP5 (4 μg/h, i.t.) with morphine (10 μg/h, i.t.) for 6 days. Morphine tolerance was induced by continued i.t. infusion (10 μg/h). The antinociceptive effect of continuous D-AP5 infusion (4 μg/h, i.t.) was also examined. Tail-flick latencies were measured daily for 6 days. All data points are averages for no fewer than six rats except as mentioned in Results, and the results are expressed as means ± S.E.M. * $P < 0.01$ (compared with the morphine-infused group).

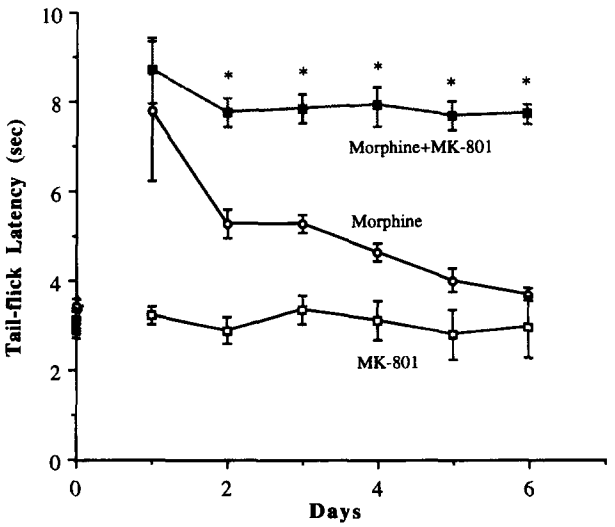

Fig. 3. The effect of MK-801 on morphine tolerance. The effect of MK-801 on morphine tolerance was examined by co-administering MK-801 (10 μg/h, i.t.) with morphine (10 μg/h, i.t.) for 6 days. Morphine tolerance was induced by continued i.t. infusion (10 μg/h). The antinociceptive effect of continuous MK-801 infusion (10 μg/h, i.t.) was also examined. Tail-flick latencies were measured daily for 6 days. All data points are averages for no fewer than six rats except as mentioned in the results section, and the Results are expressed as means ± S.E.M. * $P < 0.01$ (compared with the morphine-infused group).

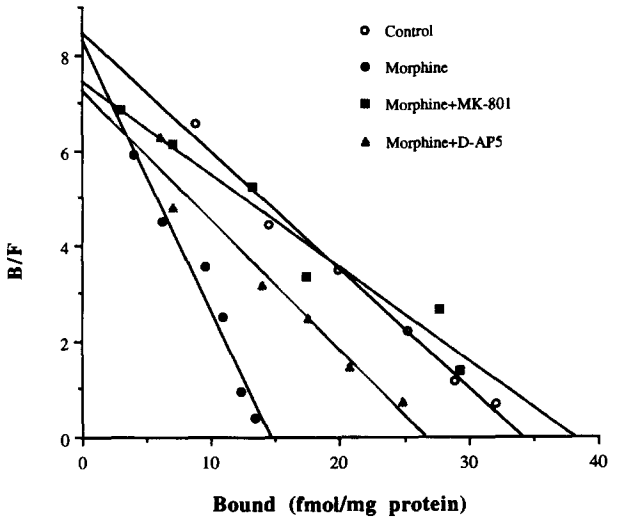

Fig. 4. Scatchard analysis of [^{3}H]DAMGO binding in rat spinal cord membranes with various i.t. drug infusions. Rats were continuously infused with either morphine (10 μg/h), or a combination of morphine and D-AP5 (4 μg/h) or MK-801 (10 μg/h) for 6 days. The rats were killed and their spinal cords were removed on the sixth day. Membranes were prepared for μ-opioid receptor saturation binding assays. [^{3}H]DAMGO was used as the radiolabeled ligand in a concentration range from 0.1 to 10 nM. Naloxone (10 μM) was used to determine the non-specific binding. Data points are from one of the six experiments, each with three pooled lumbosacral segments of spinal cords and performed in triplicate.

3.2. Effect of various drugs on morphine tolerance

As in our previous study, continuous morphine i.t. infusions induced antinociceptive tolerance at day 2. Continuous D-AP5 i.t. administration did not produce any antinociceptive effect and three of seven rats died after day 5 (Fig. 2). Co-administering D-AP5 (4 μg/h for 6 days) with morphine i.t. not only attenuated morphine tolerance but potentiated morphine's antinociceptive effect. The non-competitive NMDA receptor antagonist, MK-801 (10 μg/h for 6 days) i.t., produced the same results as D-AP5 (Fig. 3).

3.3. Effect of continuous i.t. infusion of various drugs on [^{3}H]DAMGO binding in rat spinal membranes

[^{3}H]DAMGO saturation binding assays, which only measure μ-opioid receptor high-affinity sites, yielded Hill coefficients around 1. As in our previous study (Wong et al., 1992a, b), Scatchard analysis showed a decrease (56%) of μ-opioid receptor high-affinity sites in tolerant rat spinal cord membranes (Table 1). MK-801 prevented both morphine tolerance development as well as the reduction of high-affinity sites. Additionally, the competitive NMDA receptor antagonist, D-AP5, also inhibited morphine-induced tolerance, but could not prevent the reduction (35%) of μ-opioid receptor high-affinity sites, and the affinity increased 2-fold (Table 1). The Scatchard plot is shown in Fig. 4.

4. Discussion

The present results demonstrated that neither competitive nor non-competitive NMDA receptor antagonists produced significant antinociception in the hot water immersion tail test – a phasic noxious stimulus. These results are thus consistent with previous findings that excitatory amino acid receptor antagonists cannot produce an antinociceptive effect in the case of thermal-induced phasic pain (Aanonsen and Wilcox, 1987). On the other hand, both the competitive and non-competitive NMDA receptor antagonists enhanced the antinociceptive effect of i.t. morphine on the same thermal phasic noxious stimulus. This is consistent with our earlier findings that epidural administration of ketamine, a non-competitive NMDA receptor antagonist used in clinical anesthesia, did not produce analgesia, but potentiated morphine's analgesic effect in postoperative patients (Wong et al., 1995). However, there are also reports that MK-801 neither produced an antinociceptive effect of its own nor altered that of morphine (Trujillo and Akil, 1991, 1994). This discrepancy is probably due to the different methods of drug

Table 1
Scatchard analysis of [^{3}H]DAMGO binding in the rat spinal cord lumbosacral segment of rats treated with various drugs

	Control	Morphine tolerance	Morphine + MK-801	Morphine + D-AP5
B_{max} (fmol/mg)	32.10 ± 2.65	13.97 ± 1.47 [a]	32.90 ± 3.33	20.78 ± 3.36 [b]
K_D (nM)	0.95 ± 0.08	0.75 ± 0.13	1.37 ± 0.24	0.45 ± 0.09 [b]
Hill coefficient	1.10 ± 0.03	0.94 ± 0.03	0.95 ± 0.03	1.02 ± 0.04

The rat spinal cord lumbosacral segments were removed on the sixth day after continuous i.t. drugs infusions and prepared for receptor binding assays. [^{3}H]DAMGO was used as the radiolabeled ligand for the saturation binding experiments (range from 0.5 to 10 nM). Unlabeled naloxone (10 μM) was used to determine the non-specific binding. The results are expressed as the means ± S.E.M.of at least four separate assays. Each assay was done with three pooled spinal cords and performed in triplicate. [a] $P < 0.01$ (compared with control group). [b] $P < 0.05$ (compared with control group).

administration. In fact, NMDA receptor antagonists play different roles in modulating nociception transmission, depending on whether they are introduced into spinal or supraspinal sites. Aanonsen et al. (1990) showed that NMDA injected into the subarachnoid space of mice produced a hyperalgesic state. This hyperalgesia could be depressed by the NMDA receptor antagonists, D-AP5 and MK-801. On the contrary, Jensen and Yaksh (1992) showed that NMDA injected into periaqueductal gray produced an antinociceptive effect.

A series of past experiments has shown that systemically administered NMDA receptor antagonists attenuate non-associative opioid tolerance in rats (Ben-Eliyahu et al., 1992; Trujillo and Akil, 1991, 1994). Gutstein and Trujillo (1993) have also demonstrated that systemic administration of the non-competitive NMDA receptor antagonist, MK-801, prevents morphine tolerance development in a spinalized rat model and suggested that opioid tolerance might be involved at the spinal site. Kest et al. (1993) showed that MK-801 co-administered with morphine inhibits the development of morphine tolerance, which is sufficient direct evidence for the fact that NMDA receptor antagonists prevent opioid tolerance at the spinal level. Consistently, our present study showed that the antinociceptive tolerance to morphine is prevented by the non-competitive NMDA receptor antagonist, MK-801, and the reduction of μ-opioid receptor high-affinity sites in tolerant rat spinal cord is not observed in rats that received morphine and MK-801 i.t. infusions. D-AP5 also inhibits morphine-induced antinociceptive tolerance, but prevents the decrease of μ-opioid receptor high-affinity sites to a lesser extent. Receptor affinity increases about 2-fold in the rat spinal cord.

In conclusion, the present results suggest that the NMDA receptors modulate morphine's analgesic effect. Antagonism of NMDA receptors by its antagonists not only enhances the antinociceptive effect of opioids but prevents the development of tolerance to them at the spinal cord level. These findings offer a promising therapeutic alternative in clinical pain management, in particular for patients who need long-term opioid administration for pain relief. NMDA receptors' inhibition of opioid tolerance might be due to their prevention of the reduction of receptor high-affinity sites, or their enhancement of the affinity of the μ-opioid receptor. The cellular mechanisms involved are worth exploring further.

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