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Effects of NMDA receptor antagonists on morphine tolerance: a c-Fos study in the lumbar spinal cord of the rat

Stéphanie Le Guen *, Gwénaëlle Catheline, Jean-Marie Besson

Unité de Recherche de Physiopharmacologie du Système Nerveux, INSERM U161 and EPHE, 2 rue d'Alésia, 75014 Paris, France

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Abstract

This study investigated the contribution of NMDA receptors to the development of tolerance to the antinociceptive properties of morphine at the level of the spinal cord dorsal horn. The expression of c-Fos protein following intraplantar (i.pl.) injection of carrageenin (6 mg/150 μl of saline) was used. In naive rats, acute intravenous (i.v.) administration of morphine (3 mg/kg) decreased the total number per section of Fos-Like-Immunoreactive (Fos-LI) neurons by 51%, observed at 2 h after injection of carrageenin. In tolerant rats, acute morphine did not significantly modify the total number of Fos-like immunoreactive neurons/section. In rats receiving chronic morphine and chronic injections of the non-competitive ((+)-MK 801 maleate: (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) or the competitive (LY 235959: [3*S*-(3α,4aα,6β,8aα)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid) NMDA receptor antagonists, only partial tolerance to the acute effects of morphine were observed (decrease of 42% and 38%, respectively). Administration of an antagonist at the strychnine-insensitive glycine site of the NMDA receptor ((+)-HA-966: *R*(+)-3-Amino-1-hydroxypyrrolidin-2-one) did not affect the development of morphine tolerance. These findings suggest that compounds attenuating the actions of the NMDA receptor via blockade of the glycine modulatory site may be substantially different from those acting at the ion channel of the NMDA receptor complex. This in vivo experiment in freely moving animals demonstrates for the first time an attenuation of tolerance at the cellular level.

Keywords: c-Fos; Spinal cord; Inflammation; NMDA receptor antagonist; Tolerance; Morphine

1. Introduction

Although opioid drugs such as morphine are widely used for the management of pain, their clinical usefulness is limited by the development of tolerance and dependence that occurs after repeated treatment. Tolerance is indicated by a decreased efficacy of the drug with repeated administration and results in a need to increase the morphine dose in order to achieve the desired analgesic effect. However, the possibility of using increasing doses of morphine is limited by the occurrence of adverse side effects such as respiratory depression and constipation. Thus, there is considerable interest in the development of novel drugs that delay, inhibit, or reverse the development of morphine tolerance.

Recent studies have implicated the excitatory amino acids and their receptors in the chronic actions of opioids (for reviews, see Bhargava, 1994; Herman et al., 1995; Inturrisi, 1997) and nociception (Dickenson, 1994; Dickenson et al., 1997; Wiesenfeld-Hallin, 1998). Many behavioral studies have demonstrated that competitive or non-competitive NMDA receptor antagonists attenuate or reverse the development of tolerance to the analgesic effects of morphine in rodents (for reviews, see Trujillo, 1995; Elliott et al., 1995; Herman et al., 1995; Inturrisi, 1997). The non-competitive NMDA receptor antagonist, MK-801 ((5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine/Dizocilpine) has been shown to attenuate the development of morphine tolerance as gauged by the tail-flick analgesic assay in mice (Elliott et al., 1994b; Bilsky et al., 1996) and in rats (Trujillo and Akil, 1991; Bhargava and Matwyshyn, 1993; Gutstein and Trujillo, 1993; Trujillo and Akil, 1994; Manning et al., 1996; Mao et al., 1998), hot-plate analgesic assays in mice (Gonzalez et al., 1997) and in rats (Ben-Eliyahu et al., 1992; Dunbar and Yaksh, 1996) and the formalin test in mice (Lutfy et al., 1996). In addition, the competitive

* Corresponding author. Tel.: +33-1-40-78-93-50; Fax: +33-1-45-88-13-04; E-mail: leguen@broca.inserm.fr

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NMDA receptor antagonist, LY 235959 ([3*S*-(3α,4aα,6β, 8aα)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid) prevents/reverses the development of antinociceptive tolerance as determined by the tail-flick test in mice (Bilsky et al., 1996; Bhargava, 1997); other competitive NMDA receptor antagonists have also been shown to attenuate the development of morphine tolerance (hot-plate in rats: Tiseo et al., 1994; tail-flick in mice: Elliott et al., 1994a). However, there have been few studies of the effects of glycine/NMDA receptor antagonists on morphine tolerance; ACEA-1328 (5-nitro-6,7-dimethyl-1,4-dihydro-2,3-quinoxalinedione) has been shown to block morphine tolerance in the formalin test in mice (Lutfy et al., 1996) and ACPC (1-Aminocyclopropane carboxylic acid), a partial agonist of the glycine site associated with the NMDA receptor, has been shown to block tolerance to mu and delta opioid receptors agonists (Kolesnikov et al., 1994). Overall, there is considerable behavioral evidence, but to our knowledge, no electrophysiological studies in vivo of the reversal or attenuation of morphine tolerance by NMDA receptor antagonists. In vitro experiments have mainly been devoted to the dependence phenomenon (Bell and Beglan, 1995a; Feng and Kendig, 1996; see however Bell and Beglan, 1995b; Su et al., 1998).

There remains considerable interest in involvement of NMDA receptor in the development of tolerance to the antinociceptive effects of morphine. In this study, we have used the c-Fos technique, in awake animals, as a marker of neuronal activity at the level of the dorsal horn of the spinal cord, the first relay site for the transmission and integration of nociceptive messages (Besson and Chaouch, 1987). This approach provides additional information on spinal nociceptive processing to that, which can be obtained by behavioral and electrophysiological methods. This technique can be considered as a simultaneous high-resolution photographic image of the level of neuronal activity of different populations of neurons (superficial vs. deep laminae neurons) at a given time point. There is considerable evidence that the expression of the nuclear protein c-Fos encoded by the immediate-early gene c-*fos* (see references in Martin and Magistretti, 1998) reflects the long term intracellular changes associated with sustained nociceptive processing, such as arthritis, at the spinal cord level (Abbadie et al., 1994a). In addition, numerous studies have demonstrated, especially in the dorsal horn, that c-Fos protein expression provides an indirect marker of neurons involved in spinal nociceptive transmission (for review, see Chapman and Besson, 1997).

In these studies, we have evaluated the effects of three different types of NMDA receptor antagonists on the development of tolerance to the antinociceptive effects of morphine on lumbar spinal cord neurons involved in nociceptive processes. The nociceptive stimulation used was the intraplantar injection of lambda-carrageenin (Winter et al., 1962) in freely moving rats, which produces an acute restricted inflammation associated with spinal c-Fos expression (references in Chapman et al., 1995; Honoré et al., 1995a,b). A preliminary account of this study has been presented at the annual meeting of the American Society for Neuroscience in New Orleans, November 1997 (Le Guen et al., 1997).

2. Materials and methods

2.1. Animals

Experiments were performed on 104 adult male albino Sprague–Dawley rats (Charles River, France), weighing 200–225 g. They were housed six per cage in a room with controlled temperature (22 ± 1°C) and a 12-h alternating light–dark cycle. Food and water were made available continuously. The ethical guidelines of the International Association for the Study of Pain, for investigations of experimental pain in conscious animals were followed (Zimmermann, 1983).

2.2. Experimental design

To induce morphine tolerance, morphine chlorhydrate, powder, was incorporated into a sustained-release preparation and was injected subcutaneously (s.c.), once a day in a volume of 2 ml (10:00 a.m.), during 4 days (10 mg/kg on day 1 and 2, 20 mg/kg on days 3 and 4). The vehicle of the sustained-release preparation was made by mixing 50% (v/v) of saline, 42.5% (v/v) paraffin oil (Sigma) and 7.5% (v/v) of an emulsifying agent, mannide monooleate (Arlacel A, Sigma), forming a thick white emulsion (Collier et al., 1972; Frederickson and Smits, 1973). Tolerance to the antinociceptive effects of morphine was evaluated on day 5 by the acute intravenous administration of morphine (3 mg/kg) with the method of c-Fos protein immunoreactivity in the carrageenin model of inflammatory nociception. Morphine hydrochloride (injectable solution, 10 mg/ml, Meram) diluted in saline (0.9% NaCl) was injected intravenously (i.v.) in the tail, in a volume of 0.25 ml, 10 min before carrageenin administration.

In the first experimental series ($n = 23$), in order to guarantee morphine tolerance in our model, four groups of rats were considered.

Two groups of naive rats received the vehicle of the sustained-release preparation for 4 days and then received either acute morphine (3 mg/kg, i.v., $n = 6$) or saline ($n = 6$) on day 5.

Two groups of rats received chronic morphine over 4 days (10 mg/kg on day 1 and 2, 20 mg/kg on days 3 and 4) to render tolerance and then received either acute morphine (3 mg/kg, i.v., $n = 5$) or saline ($n = 6$) on day 5.

In the second ($n = 31$ rats for MK 801 and LY 235959) and third ($n = 50$ rats for HA-966) experimental series, the effects of treatment with NMDA receptor antagonists on

morphine tolerance were tested. In each series, one group of control-tolerant rats (receiving acute saline on day 5) was performed in order to test that chronic treatment with NMDA receptor antagonists by itself did not influence the nociceptive responses. The three NMDA receptor antagonists studied, *R*-(+)-HA-966, (+)-MK 801 maleate and LY 235959 were purchased from Tocris Cookson, Bristol, UK and dissolved in distilled water. They were administered chronically, s.c., during 4 days, in a volume of 0.25 ml, twice a day (30 min before injection of sustained-released morphine preparation and 8 h later). Since the aim of these experiments was focused on tolerance, we did not consider the effect of these antagonists in naive rats. Thus, for each compound, three groups of rats were performed.

One group of control rats received chronic morphine (10 mg/kg on day 1 and 2, 20 mg/kg on days 3 and 4) during 4 days, thus rendering tolerance and then received acute saline (i.v.) on day 5 ($n = 5$ and $n = 6$ rats for the second and third experimental series, respectively).

Two groups of rats received chronic morphine (10 mg/kg on day 1 and 2, 20 mg/kg on days 3 and 4) and chronic treatment with NMDA receptor antagonists during 4 days as described above, and then received either acute morphine (3 mg/kg, i.v.) or saline on day 5.

Single doses of (+)-MK 801 (0.1 mg/kg, s.c., twice a day, during 4 days) and LY 235959 (2.5 mg/kg, s.c., twice a day, during 4 days) were used for the chronic treatment regime. The dose of (+)-MK 801 was chosen since previous studies have shown that higher dose of (+)-MK 801, in combination with morphine, is toxic (Trujillo and Akil, 1991; Bhargava and Matwyshyn, 1993). However, even with this dose two rats died. The dose of LY 235959 was chosen based on previous studies (Bilsky et al., 1996; Bhargava, 1997; Bhargava and Thorat, 1997). The effects of three doses of (+)-HA-966 (2.5 or 5 or 10 mg/kg, s.c., twice a day, during 4 days) were considered, since data from previous studies were not available.

2.3. Sub-chronic pain model

All of the rats used in these studies were unanaesthetized and received an intraplantar (i.pl.) injection of lambda-carrageenin (type IV, Sigma, 6 mg/150 μl of saline) on day 5 in the right hindpaw. Rats were perfused 2 h after this injection, a delay that has been shown to evoke c-Fos expression in numerous neurons in the dorsal horn of the spinal cord (Honoré et al., 1995a) and correspond to the peak effects of i.v. morphine on spinal carrageenin-evoked c-Fos expression (Honoré et al., 1995b). In these studies, non-stimulated rats receiving i.v. saline, i.v. morphine or chronic vehicle of sustained-released preparation were not included since numerous studies performed in our laboratory have previously shown that, in these rats, there is almost no c-Fos labelling (less than five Fos-positive neurons per section) at the level of the spinal cord (Honoré et al., 1995a, 1997).

2.4. Immunohistochemistry for Fos-like immunoreactivity (Fos-LI)

At 2 h after the carrageenin injection, the animals were deeply anaesthetised with 55 mg/kg intraperitoneal sodium pentobarbital (Sanofi, Libourne, France) and underwent intracardiac perfusion with 200 ml phosphate-buffered saline 0.1 M (PBS) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord were then removed, post fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen sections of the lumbar spinal cord, L4–L5 segment, 40 μm thick, were cut and collected in PB to be processed immunohistochemically as free floating sections. They were immunostained for c-Fos-like protein according to the avidin–biotin–peroxidase method (Hsu et al., 1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton X100 (Sigma), and were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein. The c-Fos antibody (Tebu, SC.− 52, 0.1 mg/ml diluted at 1:3000) is a rabbit polyclonal antibody raised against a peptide corresponding to amino acids 3–16, mapping at the amino-terminus of human c-Fos p62. The incubated sections were washed three times in 1% Normal Goat Serum in PBS with 0.3% Triton X100 (NGST) and incubated in biotinylated goat anti-rabbit immune globulin G for 1 h at room temperature, then washed twice in NGST and incubated for 1 h in avidin–biotin–peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed three times in PB and developed in 1-naphtol ammonium carbonate solution (89.5 ml 0.1 M PB, 10 ml ammonium carbonate—1% in distilled water-, 0.5 ml 1-naphtol (N-199-2 Aldrich, 10% in absolute alcohol) and 0.1 ml hydrogen peroxide 30% (w/w) solution) for 5 min and were washed three times in PB to stop the staining reaction. The sections were mounted on gelatin-subbed slides and air-dried. The stain was intensified and made alcohol resistant through basic dye enhancement in 0.025% crystal violet (42555 Aldrich, St. Quentin Fallavier, France) in bidistilled water for 3 min. After two short distilled water rinses to take off the excess stain, sections were differentiated in 70% and absolute alcohol and the differentiation time was evaluated under the microscope. After being air dried, the slides were cover slipped. As immunochemistry of different experiments might vary, the spinal cord sections of rats from the same experiment were immunoreacted at the same time to justify the use of statistical tests and, in each set of experiments, the different experimental groups were compared with the respective control group.

2.5. Counting of c-Fos labelled neurons

Tissue sections were first examined using dark field microscopy to determine the segmental level according to

Molander et al. (1984), as well as the gray matter landmarks. The sections were then examined under light field microscopy at ×125 to localize Fos positive cells. Labelled nuclei were counted using a camera lucida attachment. To study the laminar distribution, four regions were defined: superficial dorsal horn (laminae I–II, superficial), nucleus proprius (laminae III–IV), neck of the dorsal horn (laminae V–VI), and the ventral gray (laminae VII–X). We have previously shown that the most numerous c-Fos positive neurons were localized in the L4–L5 segment after intraplantar carrageenin (Honoré et al., 1995a). For all the pharmacological studies, for each rat, two sets of analyses were made: (1) the total number of Fos-Like Immunoreactive (Fos-LI) neurons in the gray matter for the 10 most labelled sections through L4–L5 segment, and (2) the number of Fos-LI neurons per specific laminar region of the spinal gray matter in these 10 sections. All Fos-LI neurons were analyzed without considering the intensity of the staining (i.e., lightly- to darkly-stained nuclei were included). The investigator responsible for plotting and counting the Fos-LI neurons was blind to the experimental situation of each animal.

2.6. Statistical tests

Statistical analysis was performed to compare the total number of spinal Fos-like immunoreactive neurons, using one-way analysis of variance for the different groups of animals, and two-way analysis of variance for the different groups of animals and the laminar regions (ANOVA, Statview for Macintosh). To compare the ankle or the paw edema, we used one-way analysis of variance for the different groups of animals. For multiple comparisons, the Fisher's PLSD (Protected Least Significant Difference) test was used. Effects were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Carrageenin evoked spinal c-fos expression

Two hours after intraplantar (i.pl.) carrageenin, Fos-like immunoreactive (Fos-LI) neurons, which were stained to variable degrees, were located in the ipsilateral dorsal horn of the spinal cord. The number of Fos-LI neurons in the contralateral dorsal horn was not significantly different to the extremely low number of spinal Fos-LI neurons previously described in non-stimulated rats. The Fos-LI neurons were preferentially located in the superficial laminae (I–II, 60%) of the dorsal horn and in the deep laminae (V–VI, 25%), while restricted number of neurons were observed in the nucleus proprius (III–IV, 5%) and in the ventral horn (10%) in all pharmacological groups studied. We observed the development of an unilateral peripheral edema following i.pl. carrageenin; both the paw and ankle diameters of the injected hind paw were increased, whereas contralateral hind-paw was not significantly affected (data not shown).

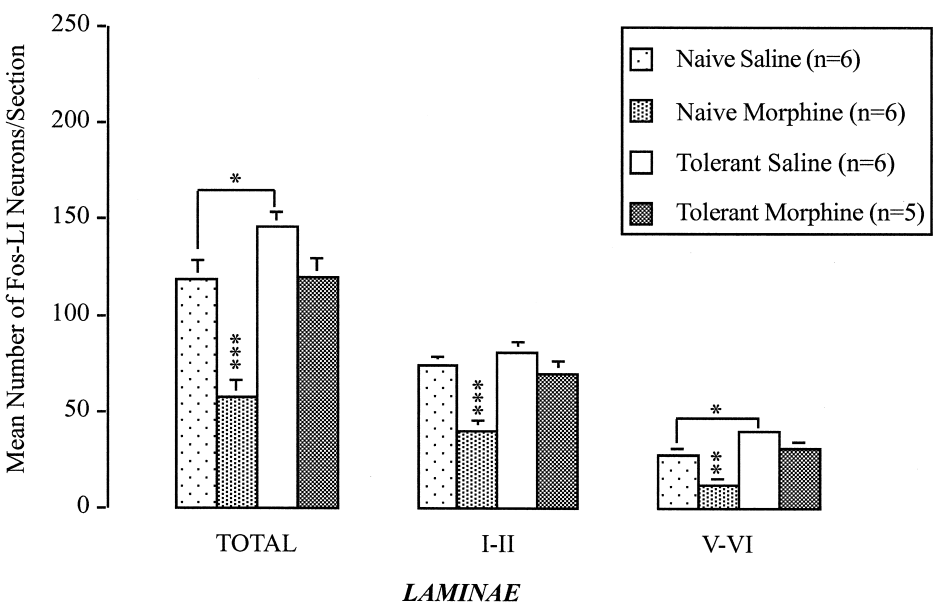

Fig. 1. Spinal c-Fos expression and morphine tolerance. Rats were pretreated daily with increasing doses of morphine (10 mg/kg on days 1 and 2, 20 mg/kg on days 3 and 4) before the acute effects of morphine (3 mg/kg, i.v.) were tested on day 5 on carrageenin-evoked spinal c-Fos expression. Acute morphine was injected 10 min before intraplantar carrageenin (6 mg/150 μl of saline). Results are expressed as mean number of Fos-like immunoreactive (Fos-LI) neurons ± S.E.M. for the L4–L5 segments. Laminar distribution illustrating mean number of Fos-LI neurons in laminae I–II or V–VI. Note the slight increase in tolerant rats that received acute saline compared to naive rats. Statistical analysis were performed with ANOVA and Fisher's protected least-square differences test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.2. Control of tolerance state

As shown in Fig. 1, the chronic morphine treatment regime was effective in the production of morphine tolerance. Indeed, acute morphine (3 mg/kg, i.v., on day 5) was unable to significantly reduce the total number of Fos-like immunoreactive (Fos-LI) neurons in tolerant rats (120 ± 9 and 145 ± 8 Fos-LI neurons in tolerant rats receiving either acute morphine or saline, respectively, $P > 0.05$, Fig. 1 and Fig. 2 panels C and D). In contrast, acute morphine significantly decreased the total number of Fos-LI neurons in drug-naive rats (57 ± 8 and 118 ± 9 Fos-LI neurons in naive rats receiving either acute morphine or saline, respectively, $P < 0.0001$, Fig. 1 and Fig. 2 panels

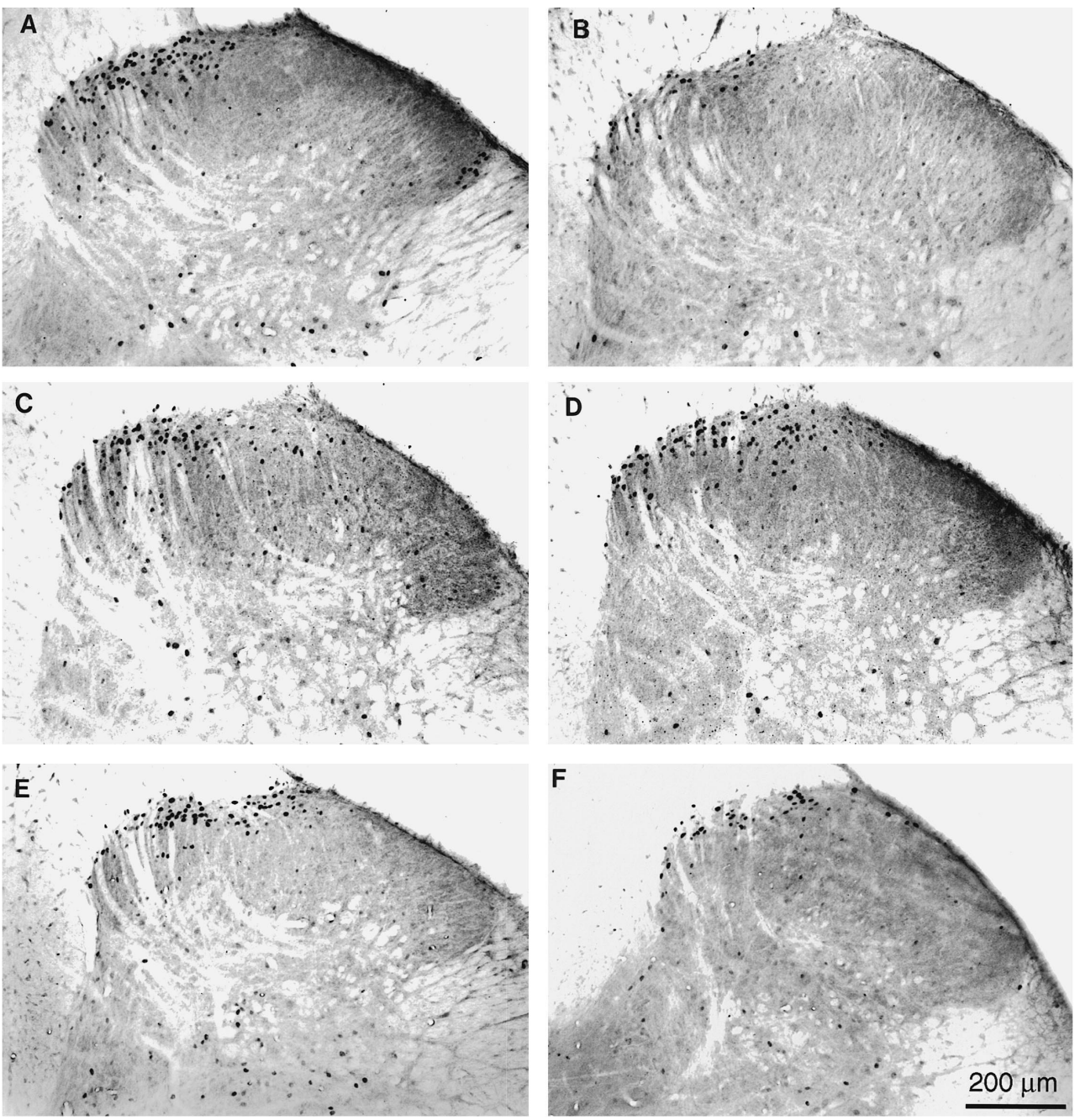

Fig. 2. Photomicrographs of examples of Fos-positive neurons in 40-μm sections of the dorsal horn of lumbar L4–L5 segment 2 h after intraplantar carrageenin. Naive rats receiving acute saline (A), acute morphine, 3 mg/kg, i.v. (B). Tolerant rats receiving acute saline (C), acute morphine, 3 mg/kg, i.v. (D). Chronic morphine and MK 801 treated rats with acute saline (E), acute morphine, 3 mg/kg, i.v. (F). Tolerant rats were pretreated with increasing dose-regimen of morphine for 4 days (10 mg/kg on days 1 and 2, 20 mg/kg on days 3 and 4). Naive rats received the same volume of vehicle for 4 days. Scale bar: 200 μm.

A and B). More precise analysis revealed that similar modifications were observed for both superficial (I–II) and deep dorsal horn neurons (V–VI, Fig. 1).

Moreover, there was an increase (23%) in the total number of Fos-LI neurons in control-tolerant rats (145 ± 8 Fos-LI neurons) compared to control-naive rats (118 ± 9 Fos-LI neurons, $P < 0.05$), significant was only reached when considering the deep dorsal horn of the spinal cord (laminae V–VI, 40 ± 3 and 28 ± 4 Fos-LI neurons in control-tolerant and control-naive rats, respectively, Fig. 1). No behavioral signs of withdrawal were exhibited in rats tolerant to morphine.

3.3. The effect of (+)-MK 801 maleate, a non-competitive NMDA receptor antagonist, on morphine tolerance

Rats simultaneously treated with (+)-MK 801 (0.1 mg/kg, s.c., twice daily during 4 days) and chronic morphine were sensitive to acute morphine on day 5 (3 mg/kg, i.v.), which significantly reduced the mean total number of Fos-like immunoreactive (Fos-LI) neurons evoked by carrageenin injection. The mean total numbers of Fos-LI neurons were 84 ± 11 and 144 ± 10 in chronic morphine and MK 801 treated rats receiving either acute morphine and saline, respectively ($P < 0.01$, Fig. 3). Individual examples of Fos immunoreactivity in these experimental groups are represented in Fig. 2 (panels E and F). Further analysis revealed that acute morphine in chronic morphine and MK 801 treated rats reduced the number of Fos-LI neurons in both the deep laminae (19 ± 3 and 43 ± 5 Fos-LI neurons, respectively, $P < 0.01$) and superficial laminae (52 ± 5 and 77 ± 4 Fos-LI neurons, respectively, $P < 0.01$, Fig. 3).

Chronic treatment with MK 801 itself did not influence the spinal Fos expression in chronic morphine treated rats receiving acute saline. This is clearly shown when comparing the number of Fos-LI neurons in tolerant rats and in chronic morphine and MK 801 treated rats (157 ± 27 and 144 ± 10 Fos-LI neurons, respectively, $P > 0.05$, Fig. 3).

3.4. The effects of LY 235959, a competitive NMDA receptor antagonist, on morphine tolerance

Rats simultaneously treated with LY 235959 (2.5 mg/kg, s.c., twice daily during 4 days) and chronic morphine were sensitive to acute morphine on day 5 (3 mg/kg, i.v.), which significantly reduced the mean total number of Fos-like immunoreactive (Fos-LI) neurons evoked by carrageenin injection. The mean total numbers of Fos-LI neurons were 79 ± 4 and 127 ± 7 in chronic morphine and LY 235959 treated rats receiving acute morphine and saline, respectively ($P < 0.05$, Fig. 4). Further analysis revealed that acute morphine in chronic morphine and LY 235959 treated rats reduced the number of Fos-LI neurons in both the deep laminae (20 ± 3 and

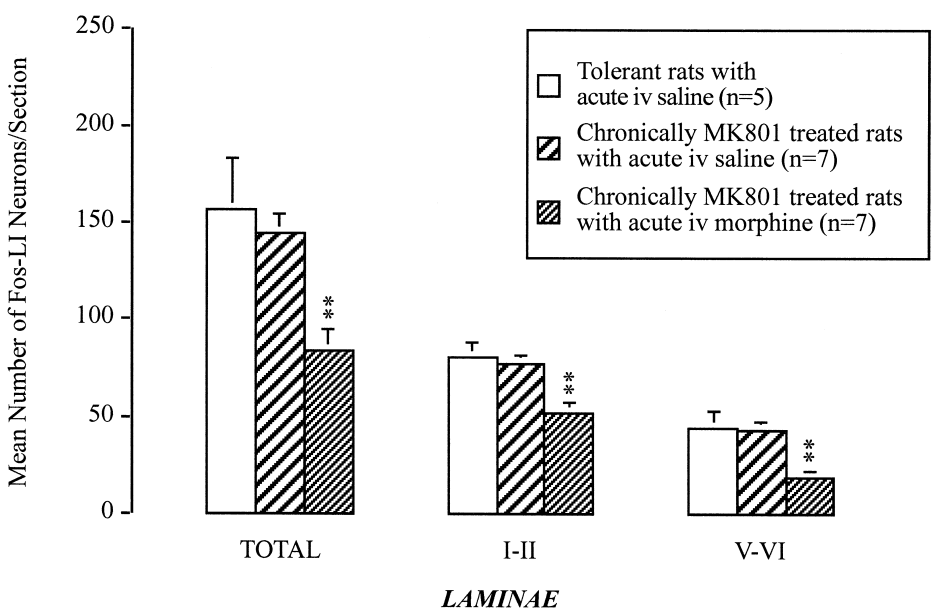

Fig. 3. Effects of (+)-MK 801 maleate, a non-competitive NMDA receptor antagonist, on morphine tolerance. Rats were pretreated daily with increasing doses of morphine (10 mg/kg on days 1 and 2, 20 mg/kg on days 3 and 4) and MK 801 (0.1 mg/kg s.c., twice a day for 4 days), before the acute effects of morphine (3 mg/kg, i.v.) were tested on day 5 on carrageenin-evoked spinal c-Fos expression. Acute morphine was injected 10 min before intraplantar carrageenin (6 mg/150 μl of saline). Results are expressed as mean number of Fos-like immunoreactive (Fos-LI) neurons ± S.E.M. for the L4–L5 segments (n = number of rats/group). Laminar distribution illustrating mean number of Fos-LI neurons in laminae I–II or V–VI. Statistical analysis were performed with ANOVA and Fisher's protected least-square differences test (**$P < 0.01$).

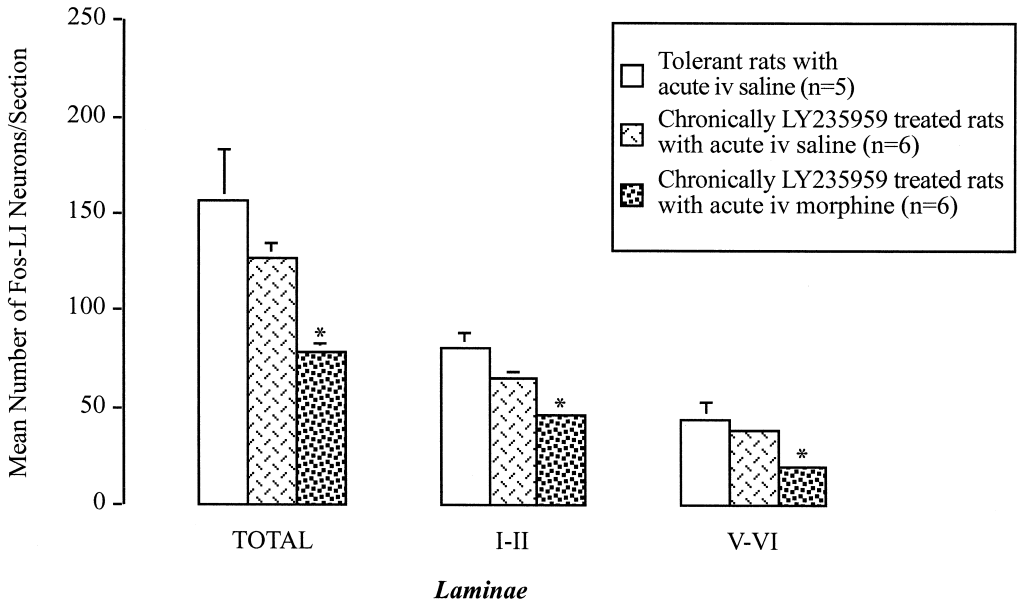

Fig. 4. Effects of LY 235959, a competitive NMDA receptor antagonist, on morphine tolerance. Rats were pretreated daily with increasing doses of morphine (10 mg/kg on day 1 and 2, 20 mg/kg on days 3 and 4) and LY 235959 (2.5 mg/kg s.c., twice a day for 4 days), before the acute effects of morphine (3 mg/kg, i.v.) were tested on day 5 on carrageenin-evoked spinal c-Fos expression. Acute morphine was injected 10 min before intraplantar carrageenin (6 mg/150 μl of saline). Results are expressed as mean number of Fos-like immunoreactive (Fos-LI) neurons ± S.E.M. for the L4–L5 segments (n = number of rats/group). Laminar distribution illustrating mean number of Fos-LI neurons in laminae I–II or V–VI. Statistical analysis were performed with ANOVA and Fisher's protected least-square differences test (*$P < 0.05$).

39 ± 3 Fos-LI neurons, respectively, $P < 0.05$) and superficial laminae (47 ± 3 and 65 ± 3 Fos-LI neurons, respectively, $P < 0.05$, Fig. 4).

Chronic treatment with LY 235959 itself did not influence the spinal Fos expression in chronic morphine treated rats receiving acute saline. This is clearly shown when comparing the number of Fos-LI neurons in tolerant rats and in chronic morphine and LY 235959 treated rats (157 ± 27 and 127 ± 7 Fos-LI neurons, respectively, $P > 0.05$, Fig. 4).

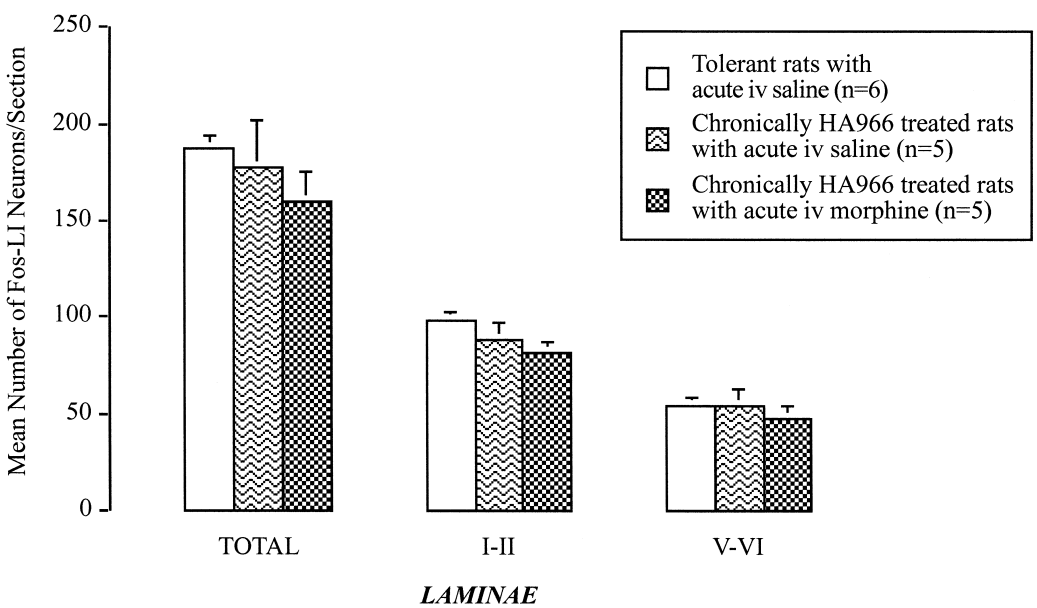

Fig. 5. Effects of (+)-HA-966, an antagonist at the glycine site of the NMDA receptor, on morphine tolerance. Rats were pretreated daily with increasing doses of morphine (10 mg/kg on days 1 and 2, 20 mg/kg on days 3 and 4) and HA-966 (5 mg/kg s.c., twice a day for 4 days), before the acute effects of morphine (3 mg/kg, i.v.) were tested on day 5 on carrageenin-evoked spinal c-Fos expression. Acute morphine was injected 10 min before intraplantar carrageenin (6 mg/150 μl of saline). Results are expressed as mean number of Fos-like immunoreactive (Fos-LI) neurons ± S.E.M. for the L4–L5 segments (n = number of rats/group). Laminar distribution illustrating mean number of Fos-LI neurons in laminae I–II or V–VI. Statistical analyses were performed with ANOVA and Fisher's protected least-square differences test.

3.5. The effect of (+)-HA-966, an antagonist at the glycine site of the NMDA receptor, on morphine tolerance

In rats simultaneously treated with (+)-HA-966 (5 mg/kg, s.c., twice a day during 4 days) and chronic morphine, acute morphine administration on day 5 (3 mg/kg, i.v.) did not modify the mean total number of Fos-like immunoreactive (Fos-LI) neurons induced 2 h after intraplantar carrageenin. In this experimental series, the mean total numbers of Fos-LI neurons were 160 ± 14 and 177 ± 24 in chronic morphine and HA-966 treated rats receiving acute morphine and saline, respectively ($P > 0.05$, Fig. 5). No modifications were detectable when considering the laminar distribution (Fig. 5). Similar results were obtained after chronic treatment with lower (2.5 mg/kg) and higher (10 mg/kg) doses of HA-966 (data not shown).

Chronic treatment with (+)-HA-966 itself did not influence the spinal Fos expression in chronic morphine treated rats receiving acute saline. This is clearly shown when comparing the number of Fos-LI neurons in tolerant rats and in chronic morphine and HA-966 treated rats (193 ± 13 and 177 ± 24 Fos-LI neurons, respectively, $P > 0.05$, Fig. 5).

4. Discussion

In this article, we present evidence that competitive (LY 235959) or non-competitive (MK 801) NMDA receptor antagonists administered simultaneously with morphine during 4 days attenuates the development of tolerance to the antinociceptive effects of morphine observed on day 5. These data confirm and extend previous behavioral studies indicating that the development of tolerance to the antinociceptive effects of morphine can be prevented by a concurrent treatment with these same NMDA receptor antagonists (Marek et al., 1991; Trujillo and Akil, 1991; Ben-Eliyahu et al., 1992; Bhargava and Matwyshyn, 1993; Gutstein and Trujillo, 1993; Tiseo and Inturrisi, 1993; Tiseo et al., 1994; Elliott et al., 1994b; Trujillo and Akil, 1994; Bilsky et al., 1996; Dunbar and Yaksh, 1996; Manning et al., 1996; Bhargava, 1997; for reviews, see Elliott et al., 1995; Herman et al., 1995; Trujillo, 1995; Inturrisi, 1997) or by others, such as ketamine (Trujillo and Akil, 1994; Shimoyama et al., 1996) and dextromethorphan (Elliott et al., 1994a; Trujillo and Akil, 1994; Manning et al., 1996; Mao et al., 1996; see however Hoffmann and Wiesenfeld-Hallin, 1996). In contrast, even with three different doses of chronic treatment, the glycine site NMDA receptor antagonist (HA-966) failed to alter the development of tolerance following chronic morphine administration.

Our approach based on the expression of the c-Fos protein at the spinal cord level is not only complementary to previous behavioral observations, but presents several advantages: (1) to focus the studies of tolerance at the level of the dorsal horn of the spinal cord which plays a major role in the integration of nociceptive messages before they reach the brain (references in Besson and Chaouch, 1987), (2) to visualize numerous spinal cord neurons, many of which receive noxious inputs (references in Chapman and Besson, 1997), (3) this technique which is used in non-anaesthetised animals allows the observation of drug-effects on different neuronal populations simultaneously (superficial vs. deep laminae dorsal horn neurons) and has been shown in our hands, to be particularly useful for the study of the pharmacology of nociceptive processing (Chapman and Besson, 1997).

In agreement with previous studies on carrageenin-evoked spinal c-Fos expression (Draisci and Iadarola, 1989; Noguchi et al., 1991, 1992; Honoré et al., 1995a,b), 2 h after intraplantar injection of carrageenin, 85% of the total number of Fos-like immunoreactive neurons were observed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn, which contain numerous neurons receiving noxious inputs (Besson and Chaouch, 1987). At 2 h after carrageenin injection, Fos-like immunoreactive neurons were predominantly located in the superficial laminae (60%), thus in good agreement with numerous previous studies following various types of peripheral noxious stimuli (see references in Chapman and Besson, 1997). It is known that with a longer delay (3–4 h), there is an increase in the number of Fos-like immunoreactive neurons in deeper laminae (Williams et al., 1989; Bullitt et al., 1992; Honoré et al., 1995a). However, to gauge the effects of acute morphine, we selected a delay of 2 h, which has been shown, under similar conditions, to correspond to the maximum effect of intravenously administered morphine (Honoré et al., 1995b).

In naive rats, acute systemic administration of morphine (3 mg/kg, i.v.), 10 min before carrageenin, strongly reduced the number of Fos-like immunoreactive neurons in the superficial laminae and deep dorsal horn. These results are in good agreement with numerous previous studies that have shown that systemic morphine reduces spinal c-Fos expression evoked by various modalities of peripheral nociceptive stimulation (see references in Chapman and Besson, 1997).

In rats rendered morphine tolerant, acute morphine was inefficient at decreasing the number of carrageenin-evoked Fos-like immunoreactive neurons for either the superficial and deep dorsal horn neurons (Fig. 1). It must be underlined that the number of Fos-like immunoreactive neurons induced during the development of carrageenin inflammation was slightly, but significantly increased in morphine tolerant rats as compared to naive rats. This increase was probably not due to withdrawal behaviors because we did not show any withdrawal signs in these rats. This increase was only significant when considering the number of Fos-like immunoreactive neurons in laminae V–VI. This finding is in keeping with a previous study (Rohde et al.,

1997) showing an increase in formalin-evoked Fos-like immunoreactive neurons, especially in the deep dorsal horn in morphine-tolerant rats as compared to naive rats. This increase was explained by a central sensitization of dorsal horn neurons during the development of tolerance. In keeping with this hypothesis, Mao et al. (1994, 1995) have shown that thermal hyperalgesia developed in animals rendered tolerant to the antinociceptive effects of morphine. However, this interpretation must be made with care, since according to our present results, the increase in Fos-like immunoreactive neurons was relatively minor and we were not able to detect such a modification in previous studies based on the same approach (Abbadie et al., 1994b; Honoré et al., 1997). These studies differed in the dosage used to induce tolerance, the first one employed four days of treatment with increasing doses of morphine (20, 40, 80, and 120 mg/kg); the second one used three days of treatment with a unique dose (80 mg/kg). The differences among these studies taken together, these discrepancies are difficult to explain and could relate to variations in the development of tolerance with different regimens; i.e., intermittent administration or continuous infiltration of morphine with implanted osmotic minipumps or pellets may lead to differential development of tolerance. In this respect, it has recently been demonstrated (Ibuki et al., 1997), that periodic reversal of opiate receptor occupancy resulted in an enhanced state of dependence and tolerance as compared to continuous exposure, suggesting an importance of steady opiate exposure for tolerance development. Thus, if there was a sensitization of spinal cord neurons, the magnitude of this effect may be related to the manner in which tolerance was induced.

In chronic morphine and MK 801 or LY 235959 treated rats, acute morphine decreased the carrageenin-induced spinal c-Fos expression by 42% and 38%, respectively. Thus, chronic administration of competitive (LY 235959) or non-competitive (MK 801) NMDA receptor antagonists in parallel with chronic morphine treatment attenuated tolerance in our model. In naive rats, morphine decreased spinal c-Fos expression by 51%. Interestingly, the effectiveness of this dose of morphine administered acutely to reduce carrageenin-induced spinal c-Fos expression in naive rats is very reproducible over several experiments (56%: Honoré et al., 1995b, 55%: Honoré et al., 1996, 64%: Honoré et al., 1997). These results are in agreement with many previous behavioral reports (Ben-Eliyahu et al., 1992; Bhargava and Matwyshyn, 1993; Gutstein and Trujillo, 1993; Elliott et al., 1994b; Tiseo et al., 1994; Trujillo and Akil, 1991, 1994; Bilsky et al., 1996; Dunbar and Yaksh, 1996; Feng and Kendig, 1996; Lutfy et al., 1996; Manning et al., 1996; Bhargava, 1997; Gonzalez et al., 1997; Mao et al., 1998) using various antinociceptive tests in mice or rats (see, however Haberny and Young, 1994; Bell and Beglan, 1995b). Furthermore, our results confirm that both competitive and non-competitive NMDA receptor antagonists can avert the development of morphine tolerance. This is in good agreement with a previous study showing that the attenuation of the development of mu-opioid analgesic tolerance was similar with a competitive NMDA receptor antagonist (LY 274614) and MK 801 in the tail-flick test in mice (Elliott et al., 1994b). The modifications we observed may reflect a direct spinal site of action of these drugs, since the spinal cord is rich in both opioid and NMDA receptors (for review see Coggeshall and Carlton, 1997). In this respect, NMDA receptors appear to be involved in the development of opiate tolerance at spinal sites, since MK 801 inhibited the development of tolerance in spinalized animals (Gutstein and Trujillo, 1993) and chronic spinal MK 801 attenuates tolerance to spinal morphine (Dunbar and Yaksh, 1996; Mao et al., 1998). Moreover, intrathecal injection of NMDA antisense have been shown to attenuate tolerance to intrathecal morphine in the rat, providing additional support for the critical role of spinal NMDA receptors in the development of tolerance to the antinociceptive effects of morphine (Inturrisi et al., 1996). However, we cannot exclude a supraspinal site of action of NMDA receptor antagonists, since we used systemic injections of NMDA receptor antagonists.

Surprisingly, in the present study, HA-966, a selective NMDA receptor antagonist acting at the glycine site of the receptor complex did not affect the development of tolerance to morphine. This lack of effect of HA-966 was confirmed by using three different chronic pretreatment doses (2.5, 5 or 10 mg/kg). Despite the fact that to our knowledge, there was no systematic studies upon effects of HA-966 on morphine tolerance (see, however Bristow et al., 1997 who reported attenuation of the symptoms of morphine-withdrawal in the rat treated with HA-966), our results contrast with previous studies. Indeed, another competitive NMDA receptor/glycine site antagonist (ACEA-1328) has been shown to block morphine tolerance in the tail-flick test (Lutfy et al., 1995) and in the formalin test (Lutfy et al., 1996) in mice; it was also demonstrated that ACPC, a partial agonist of the glycine site associated with the NMDA receptor, blocks tolerance to mu and delta opioid receptors agonists in the tail-flick test in mice (Kolesnikov et al., 1994). Differences may be due to the different species used in these studies (mice vs. rats), but this is unfeasible in regards to the results obtained by Bristow et al. (1997) on symptoms of withdrawal. Thus, the chemical structures of the drugs used may be of importance for the allosteric modulation of the glutamate site by the glycine co-agonist site (Reynolds, 1990; Robichon et al., 1997). Thus, there is a need for further investigations using others antagonists acting at the glycine site to confirm these results in our model.

This study clearly shows, for the first time, at the level of spinal neurons involved in nociception, that two NMDA receptor antagonists reduce the development of morphine tolerance. Thus, providing additional support to the numerous behavioral data for the critical role of NMDA recep-

tors in morphine tolerance. In regard to the importance of dorsal horn spinal cord in transmission and integration of nociceptive messages, our results suggested that spinal cord is an important site for the modulatory action of NMDA receptor antagonists on the development of tolerance to the antinociceptive effects of morphine.

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