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Further evidence that nitric oxide modifies acute and chronic morphine actions in mice

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Abstract

The effects of the nitric oxide (NO) synthase inhibitor, N^{ω}-nitro-L-arginine (L-NNA, 2.5–10 μg i.c.v.), and the NO synthesis precursor, L-arginine (L-Arg, 2.5–10 μg i.c.v.), on morphine-induced analgesia, and on morphine-induced tolerance and dependence were examined in mice. Administration of L-NNA diminished the morphine-induced analgesia. L-Arg pretreatment increased the analgesic effect of morphine. Repeated pretreatment (three times, at 24-h intervals) with L-NNA diminished both acute and chronic tolerance to morphine, whereas both the acute and the chronic morphine-induced tolerance increased after the repeated (three times, at 24-h intervals) administration of L-Arg. Neither L-NNA nor L-Arg affected the signs of morphine dependence, as assessed by naloxone (1 mg/kg, s.c.)-precipitated withdrawal. Our data suggest that increased NO synthesis potentiates morphine analgesia and enhances the development of morphine tolerance in mice.

Keywords: N^{ω}-nitro-L-arginine; L-Arginine; Nitric oxide (NO); Morphine; Tolerance; Dependence

1. Introduction

Nitric oxide (NO) may play an important role in mammalian vital functions, since it has proved to be identical with endothelium-derived relaxing factor (EDRF). This compound may prove to be the first member of a new neurotransmitter group which differs considerably from known ones as to both structure and action; it may also have a role in the intracellular transfer of information. The synthesis of NO from L-arginine (L-Arg) allows the regulation of cell functions and of communication between cells. In all cell types inspected to date, NO originates from oxidation and splitting-off of the terminal N-atom of L-Arg, which is catalysed by NO synthase. This enzyme is competitively inhibited by certain analogues of L-Arg, among them, the N^{G}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA) (Rees et al., 1990). Histochemical studies with antibodies to constitutive (Ca^{2+}-calmodulin-dependent) NO synthase revealed that NO synthase is found in both the brain and the peripheral nerves (Bredt et al., 1990).

It is widely accepted that nitric oxide may occupy a key position in the antinociceptive and tolerance-inducing action of opiates and in the endogenous mediation of pain. In vitro investigations demonstrated that morphine increases cGMP production (Minneman and Iversen, 1976). Duarte and Ferreira (1992) reported that the NO → cGMP pathway may be involved in the antinociception induced by morphine in the central nervous system. Evidence exists that NO modulates the synaptic transfer of signals in both the central and the peripheral nervous system, and it is presumed that NO plays a role in the mediation of nociceptive events in the periphery and in the spinal chord (Haley et al., 1992). There have been several attempts to clarify the role of NO in pain sensitivity and the interaction of NO with opiates but, particularly concerning the role of NO in opiate analgesia and withdrawal, the results remain inconsistent (Cappendijk et al., 1993; Przewlocki et al., 1993; Majeed et al., 1994; Pasternak et al., 1995; Vaupel et al., 1995).

Accordingly, the present experiments were carried out in order to elucidate how central inhibition (by L-NNA, an

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NO synthesis inhibitor) and stimulation (by L-Arg) of NO synthesis modify morphine effects in mice.

2. Materials and methods

2.1. Animals

Male CFLP mice (30 ± 5 g) of an outbred strain (LATI, Gödöllö, Hungary) were used at the age of 6 weeks. They were kept under a standard light–dark cycle (lights on between 0600 and 1800 h), with food and water available ad libitum. At least a week of habituation was allowed before the beginning of experiments. The animals were kept and treated according to the rules of the Ethical Committee for the Protection of Animals in Research (Albert Szent-Györgyi Medical University).

2.2. Surgery

For intracerebroventricular (i.c.v.) cannulation, mice were anesthetized with sodium pentobarbital (Nembutal, CEVA, France; 50 mg/kg intraperitoneally (i.p.)), and a polyethylene cannula was inserted into the right lateral cerebral ventricle. The experiments started 4 days after i.c.v. cannulation. Upon conclusion of the experiments, 10 μl methylene blue was injected into the ventricle of the decapitated animals through the i.c.v. cannula in order to detect the tip of the cannula. Animals with improper cannula placement were excluded from the statistical analysis.

For chronic tolerance and withdrawal studies, the animals were lightly anesthetized with ether (Lek-Chinoin, Budapest), and a morphine pellet was implanted subcutaneously (s.c.) into the sacral area through a small section in the neck area 4 days after i.c.v. cannulation. The pellet consisted of morphine–HCl (35 mg), microcrystalline cellulose (Avicel Ph 101) (37.5 mg), silicon dioxide (Aerosil) (0.75 mg) and Ca^{2+}-stearate (1.5 mg). The diameter of the pellet was 6 mm and its hardness was 27 ± 1 Strong–Cobb units.

2.3. Treatments

For i.c.v. treatment, the drugs (L-NNA and L-Arg) were dissolved in 0.9% saline and injected in a volume of 2 μl. The control groups were treated with saline for the acute and chronic studies. In all experiments involving measurement of the antinociceptive effect, morphine–HCl (Alkaloida, Tiszavasvári, Hungary; 5 mg/kg, s.c.) was used. In acute tolerance studies, 100 mg/kg of morphine–HCl (s.c.) was used as a tolerance-inducing dose. Animals showing respiratory complications were eliminated from the experiments. In chronic tolerance and withdrawal studies, morphine pellets were implanted. The control mice received placebo pellets for the chronic study. The precipitated withdrawal syndrome was induced with naloxone–HCl, 1 mg/kg s.c. (Narcanti, Dupont Pharma).

2.4. Procedures

All experiments were started with an initial tail-flick latency measurement, pain sensitivity being measured immediately before, and 30 and 60 min after the test morphine challenge. The radiant heat tail-flick method of D'Amour and Smith (1941) was used. The antinociceptive effect was expressed according to the equation:

$$\text{analgesic effect}\,[\%] = \frac{TF_n - TF_0}{TF_{max} - TF_0} \times 100$$

where TF_0 is the tail-flick latency in the preliminary test mentioned above, or (in all tolerance studies) before the injection of the test dose of morphine, TF_n is the value of a repeated corresponding measurement n (30, 60 or 120) min after morphine injection, and TF_{max} indicates the cut-off time (20 s). The control tail-flick latencies (TF_0) were 3.1 ± 0.9 s in all groups.

The following experiments were performed.

(1) Effects of L-NNA and L-Arg on pain sensitivity.

(2) Effects of L-NNA and L-Arg on the acute antinociceptive effect of a single challenging dose of morphine. L-NNA or L-Arg was given i.c.v. once, 60 min prior to the challenging dose of morphine (5 mg/kg, s.c. in studies with L-NNA and 2.5 mg/kg, s.c. in studies with L-Arg), and pain sensitivity was assessed 30 and 60 min later.

(3) Acute tolerance studies in which animals were pretreated three times with L-NNA or L-Arg at 24-h intervals. The saline and tolerant animals received i.c.v. saline injections. A tolerance-inducing dose of morphine (100 mg/kg, s.c.) was injected 60 min later; 6 h later, a challenging dose of morphine (5 mg/kg) was used to assess the antinociceptive effect.

(4) Chronic tolerance and withdrawal studies in which L-NNA or L-Arg was given i.c.v. 60 min before the morphine-containing pellets were implanted. The saline and tolerant animals received i.c.v. saline injection. The pretreatment was repeated three times at 24-h intervals. On day 4, a challenging dose of morphine (5 mg/kg) was given and the antinociceptive effect was determined. Three hours later, the animals received naloxone (1 mg/kg, s.c.) and the precipitated withdrawal signs were assessed. The precipitated abstinence syndrome was measured by scoring the latency of the appearance of stereotyped jumping from a circular platform 35 cm in diameter and 70 cm high. A cut-off time of 900 s was used. The weights and temperatures of all animals were recorded just before and 30 and 60 min after the injection of naloxone, and the changes in both parameters were calculated.

2.5. Statistical analysis

Statistical analysis of the data was made by one-way analysis of variance (ANOVA). For significant ANOVA values, groups were compared by Tukey's test for multiple comparisons with unequal cell size. A probability level of less than 0.05 was accepted as indicating a significant difference.

3. Results

3.1. Effects of L-NNA and L-Arg on tail-flick latency

Graded doses (1.25, 2.5, 5 and 10 μg) of either L-NNA or L-Arg 30, 60 or 120 min after i.c.v. administration had no analgesic effect themselves (data not shown).

3.2. Effects of L-NNA on a challenge dose of morphine

A single dose of morphine (5 mg/kg, s.c.) caused a near-maximal analgesic effect. Single doses of L-NNA (10, 5 or 2.5 μg, i.c.v.) diminished the analgesic effect of morphine at both 30 ($F(4,60) = 44.52$; $P < 0.05$) and 60 min ($F(4,60) = 16.27$; $P < 0.05$). Slight but non-significant differences were observed between the three L-NNA-treated groups (Fig. 1).

3.3. Effects of L-Arg on morphine analgesia

A subcutaneous injection of 2.5 mg/kg morphine lengthened the initial tail-flick latency to approximately 50% of the maximal value, by 30 min. A single dose of L-Arg (10 μg, i.c.v.) enhanced the analgesic effect of morphine, increasing the tail-flick latency to nearly 20 s at both 30 ($F(4,43) = 54.36$; $P < 0.05$) and 60 min ($F(4,43) = 58.74$; $P < 0.05$). Lower doses of L-Arg were ineffective (Fig. 2).

3.4. Effects of L-NNA and L-Arg on acute tolerance to morphine

A tolerance-inducing dose (100 mg/kg, s.c.) of morphine resulted in a significant decrease in the analgesic effect of morphine as compared with the morphine-naive control group. This decrease in tail-flick latency demonstrates the development of tolerance to morphine in morphine-treated animals. Repeated administration of L-NNA (10 or 5 μg, i.c.v.) before the tolerance-inducing morphine injection reduced morphine tolerance at both time points checked (30 and 60 min). L-Arg in a dose of 5 or 10 μg (i.c.v.) induced a significant decrease in tail-flick latency, producing a lower antinociceptive effect than in the tolerant group, enhancing the development of acute tolerance to morphine. L-NNA and L-Arg displayed significant contrary tolerance-modifying effects at 30 min ($F(7,76) = 57.70$; $P < 0.05$) and 60 min ($F(7,76) = 33.11$; $P < 0.05$). Neither L-NNA nor L-Arg affected acute morphine tolerance when administered in a dose of 2.5 μg (i.c.v.) (Fig. 3).

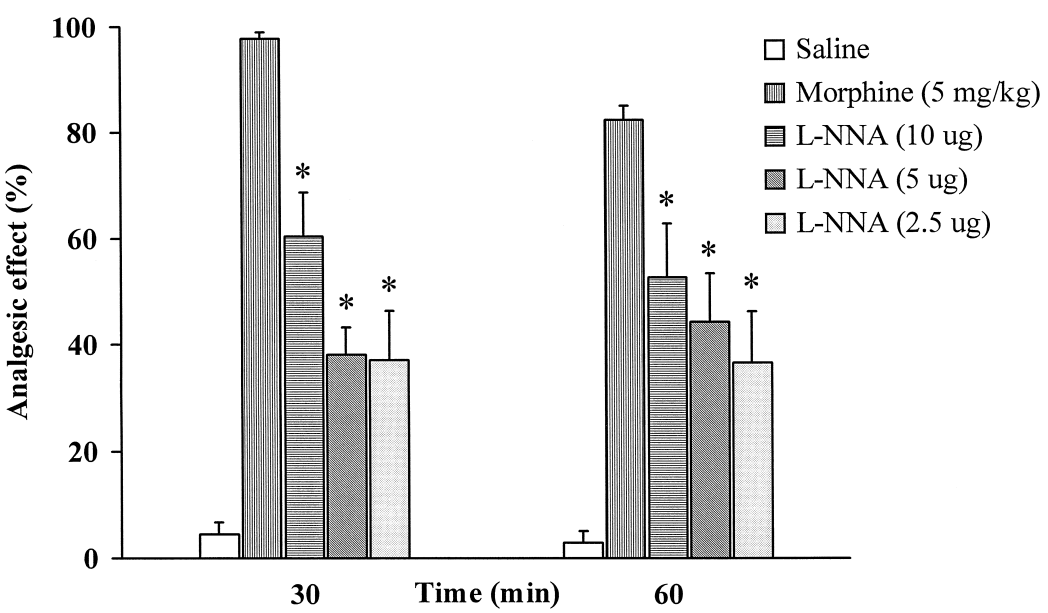

Fig. 1. Effect of L-NNA on morphine analgesia. Groups of mice ($n \geq 9$) received a s.c. injection of morphine alone (5 mg/kg) or 60 min after L-NNA (2.5, 5 or 10 μg, i.c.v.) pre-treatment. The saline group received a s.c. injection of saline 60 min after i.c.v. saline pre-treatment. The vertical lines at the top of the bars denote the S.E.M. * $P < 0.05$, L-NNA (all doses) vs. morphine.

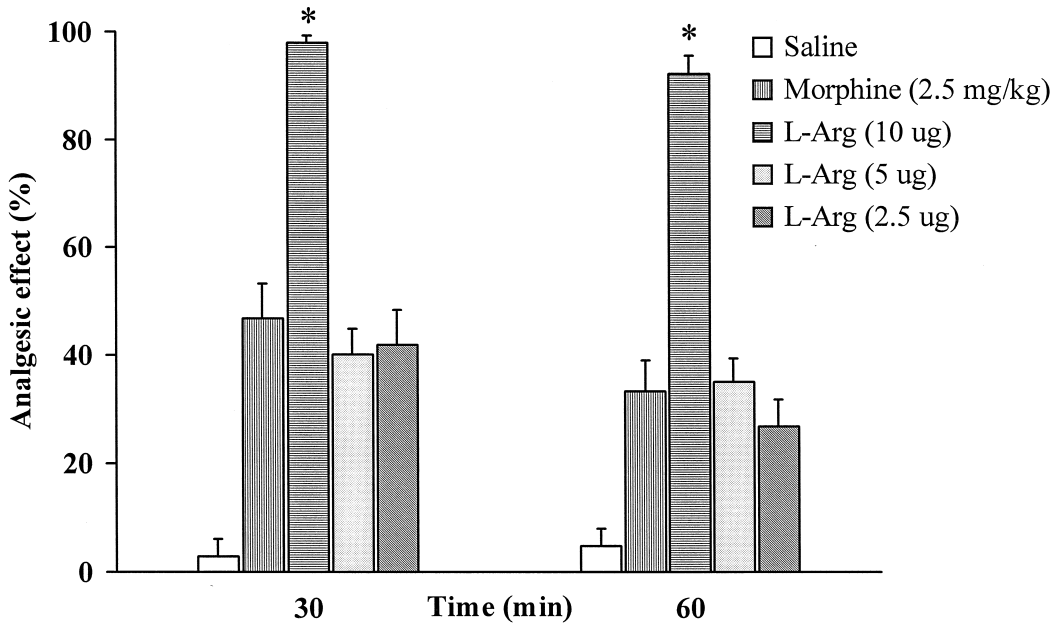

Fig. 2. Effect of L-Arg on morphine analgesia. Groups of mice ($n \geq 9$) received a s.c. injection of morphine alone (2.5 mg/kg) or 60 min after L-Arg (2.5, 5 or 10 μg, i.c.v.) pretreatment. The saline group received a s.c. injection of saline 60 min after i.c.v. saline pretreatment. The vertical lines at the top of the bars denote the S.E.M. $*$ $P < 0.05$, L-Arg (10 μg) vs. morphine.

3.5. Effects of L-NNA and L-Arg on chronic morphine administration and withdrawal

The effects of L-NNA and L-Arg on chronic tolerance were investigated in animals with morphine pellets implanted. Repeated (three times, at 24-h intervals) L-NNA pretreatment (5 or 10 μg, i.c.v.) caused a significant increase in tail-flick latency (decrease in tolerance) as compared with the tolerant group at 30 min. A significant increase in chronic tolerance to morphine was observed in the L-Arg (5 or 10 μg, i.c.v.) pretreated animals at the same time ($F(7,95) = 76.38$; $P < 0.05$). Neither L-NNA nor L-Arg modified chronic morphine tolerance at 60 min (Fig. 4).

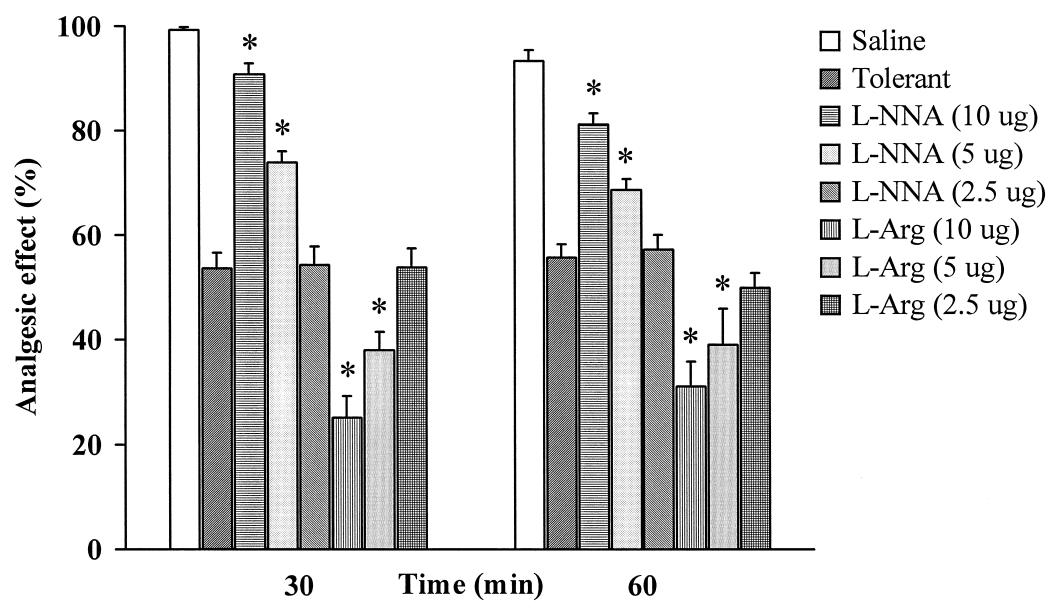

Fig. 3. Effect of L-NNA and L-Arg on acute tolerance to morphine. Groups of mice ($n \geq 9$) received daily injections of either L-NNA (2.5, 5 or 10 μg/day, i.c.v.) or L-Arg (2.5, 5 or 10 μg/day, i.c.v.) three times. The tolerant group received saline i.c.v., 60 min later a tolerance-inducing dose of morphine was injected (100 mg/kg, s.c.), and 6 h later a challenging dose of morphine (5 mg/kg, s.c.) was administered to assess the antinociceptive effect. The saline group received saline at all time points before the challenging dose of morphine. The vertical lines at the top of the bars denote the S.E.M. $*$ $P < 0.05$, compared with the tolerant group.

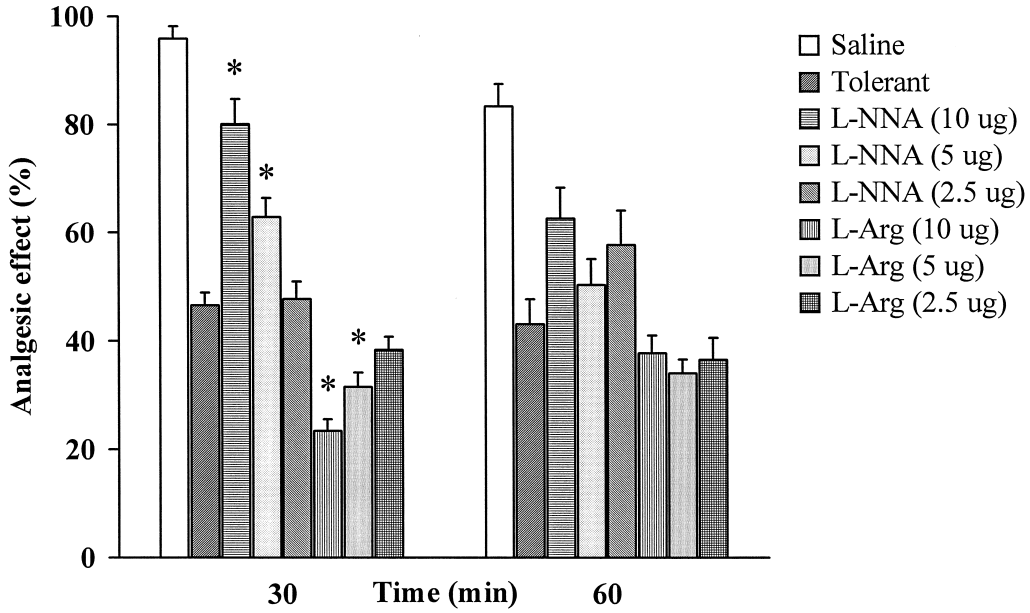

Fig. 4. Effects of L-NNA and L-Arg on chronic tolerance to morphine. Either morphine pellets or placebo pellets (in case of saline group) were implanted s.c. in groups of mice ($n \geq 9$). The mice received saline (in the saline and tolerant groups) or L-NNA or L-Arg i.c.v. injections on three consecutive days, and 60 min later a challenging dose of morphine (5 mg/kg) was administered to assess the antinociceptive effect. Each column denotes the mean ± S.E.M. (vertical bar). $* P < 0.05$, compared with the tolerant group.

No significant effect on the naloxone-induced decreases in rectal temperature, weight loss or jumping in morphine-tolerant animals was observed after pretreatment of the animals with graded (2.5–10 μg, i.c.v.) doses of either L-NNA or L-Arg (data not shown).

4. Discussion

The effects of L-Arg and L-NNA on morphine analgesia, on the acute and chronic tolerance to morphine and on morphine withdrawal in intact mice were examined in this study. L-NNA, an inhibitor of the NO synthase, was found to diminish the morphine-elicited analgesia at both single and repeated doses, while L-Arg, a substrate of NO synthase, enhanced the antinociceptive effect of morphine when administered similarly. In spite of numerous studies on the role of NO in morphine analgesia, the question remains controversial. Comparisons of results of the various studies has been hampered by the following factors.

- Differences in race or strain of the experimental animals.
- Different features of the various NO synthase inhibitors applied.
- Different procedures for the administration of L-Arg and NO synthase inhibitors.

It has been demonstrated that, in mice and rats, NO mediates the tail-flick reflex facilitated by *N*-methyl-D-aspartate (NMDA), this phenomenon depending on the neuronal activity of the spinal chord. Further, the analgesic effect of morphine in rats is increased by L-NAME (Przewlocki et al., 1993). Orally or i.p. administered L-Arg was reported to inhibit morphine analgesia in a dose-dependent manner, whereas i.c.v. administration was ineffective. L-NAME reversed the effect of L-Arg, and even exerted a slight antinociceptive effect itself, which was eliminated by L-Arg (Pasternak et al., 1995).

In contrast, Majeed et al. (1994) found that, after either single or repeated (five times, daily) administration to mice, L-NAME did not produce an appreciable effect on the pain threshold and did not modify the analgesic effect of morphine.

In our study, we demonstrated that morphine analgesia increased after i.c.v. L-Arg and decreased after L-NNA pretreatment.

Others concluded that inhibition of the NO/cGMP system in the spinal chord by intrathecal (i.t.) L-NNA potentiated the antinociception induced by i.c.v. administered morphine, whereas activation of the NO/cGMP system by i.t. L-Arg pretreatment attenuated it. An increase of NO induced by L-Arg (i.c.v.) potentiated the β-endorphin but not the μ-, δ- or κ-opioid receptor agonist-induced antinociception in the mouse. L-Arg pretreatment (i.t.) did not potentiate the analgesia induced by i.c.v. administered β-endorphin, indicating that this potentiating effect is located at supraspinal, but not spinal sites (Xu and Tseng, 1993, 1995).

Chronic administration of morphine results in tolerance to analgesia; this can be caused by various mechanisms. Earlier studies were directed towards the opiate receptors themselves and their messengers. These studies emphasized the role of adenylate-cyclase in opiate tolerance. There is abundant data regarding the roles of other systems. NMDA receptor antagonists also block the tolerance to morphine (Babey et al., 1994). This is of importance

because it suggests that a direct action is exerted on tolerance itself. Some NMDA actions occur through initiation of NO synthesis, followed by continuous NO emission, which then acts on the accumulation of cGMP. Earlier experiments were carried out with non-competitive agents, such as MK-801. Similar results have been achieved with competitive antagonists, and with substances that act on the glycine regulatory site of the NMDA receptor (Babey et al., 1994). The i.p. administration of L-NAME blocked the development of tolerance (Majeed et al., 1994).

As regards acute and chronic tolerance to morphine, our findings support the view that tolerance disappears or diminishes after the central inhibition of NO synthase and increases after central NO synthase activation, confirming the role of NO synthase in morphine tolerance.

Functionally different neuronal NO synthase systems have been identified recently (Kolesnikov et al., 1997). The two isoforms of neuronal NO synthase were demonstrated to have opposite effects: one NO synthase isoform potentiated morphine analgesia and the other was reported to be important in morphine tolerance. These results support our findings concerning the role of NO in morphine analgesia and tolerance.

Varied and contradictory results have been published concerning opioid withdrawal. Some authors found that treatment with L-NAME, L-NNA, L-N^5-(1-iminoethyl)-ornithine (L-NIO) or 7-nitro indazole resulted in significant decreases in weight loss, diarrhea, hair licking and twitching, but did not affect the abnormal posture and relaxation. L-NNA, 7-nitro indazole and L-NIO increased the orientative-exploring activity linearly. The most varied effects were detected for escape jumps and salivation. Only L-NIO and 7-nitro indazole increased the number of jumps, but there was no such consistent effect on salivation (Vaupel et al., 1995).

Majeed et al. (1994) reported that L-NAME diminished weight loss in a dose-related manner in the mouse.

Cappendijk et al. (1993) found that L-NAME (30-200 mg/kg, i.p.) and L-NNA (7.5–100 mg, i.p.) exerted dose-related inhibitory effects on withdrawal jumping, but L-NMMA had no significant effect on withdrawal diarrhea.

These results reveal that the inhibition of NO synthesis diminishes some signs of morphine withdrawal, but many other symptoms are not diminished. It is not known at present which mechanism is responsible for this inconsistency.

In our investigations, we saw no differences in withdrawal signs between the L-NNA or L-Arg group and the tolerant control group, although the animals in the tolerant control group clearly showed the signs of morphine-elicited withdrawal. The results suggest that the possible roles of NO synthase inhibitors in clinical use, in connection with the development of dependence, need to be explored further.

Our data lend support to the presumed connection of endogenous NO with opiates, and with the role of NO in morphine analgesia, and in the acute and chronic tolerance to morphine.

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