Concurrent Spinal Infusion of MK801 Blocks Spinal Tolerance and Dependence Induced by Chronic Intrathecal Morphine in the Rat

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Background: MK801, an N-methyl-D-aspartate receptor antagonist, has recently been reported to attenuate tolerance to, and withdrawal from morphine. This study analyzes tolerance and withdrawal in a chronic intrathecal infusion model of morphine and MK801.

Methods: Intrathecal catheters, attached to 7-day miniosmotic infusion pumps, were implanted in rats and infused with saline, 20 nmoh/morphine, MK801 (10 and 3 nmoh/morphine), and 10 nmoh/MK801. Analgesia was measured on the hot plate daily. On the day 7, groups received 3 mg/kig intraperitoneal naloxone and six signs of withdrawal were assessed: vocalization to air motion or light touch, abnormal posture, spontaneous vocalization, escape attempts, “wet dog shakes,” and ejaculation. Similar groups were tested only on days 1 and 7. Intrathecal morphine dose-response curves were obtained on day 8. A separate morphine-tolerant group received 10 nmoh MK801 on day 7. Rats from each group received 10 nmoh intrathecal morphine 1 week later.

Results: Infusion of MK801 with morphine resulted in a dose-dependent preservation of effect, and attenuated three of six signs of withdrawal. Infusion of MK801 (10 and 3 nmoh/morphine) prevented the reduction of potency observed with morphine alone. ED50 values (maximum percent effect, nm morphine) were: saline (16), morphine (496), MK801 (10 nmoh/morphine) (4), and 10 nmoh/MK801 (0.3). Acute administration of MK801 was ineffective in restoring sensitivity to morphine. One week after cessation of infusion, there was no significant difference between groups.

Conclusions: Chronic spinal MK801 attenuates tolerance to, and withdrawal from morphine in a dose-dependent fashion, supporting the hypothesis that N-methyl-D-aspartate receptor activity plays a role in the reorganization of spinal function produced by chronic opioid receptor activation. Chronic intrathecal MK801 appears to sensitize the spinal cord to intrathecal morphine. (Key words: Analgesics; narcotic; dependence; morphine; spinal; tolerance. Receptors: N-methyl-D-aspartate.)

RECENT studies in rats using the hot plate or tail flick tests to assess antinociception, have shown that antagonism at the N-methyl-D-aspartate (NMDA) receptor site attenuates tolerance and withdrawal otherwise observed after chronic morphine administration. These initial systemic studies found that: (1) MK801 given alone, was without significant antinociceptive effect, (2) MK801 administered to rats already rendered morphine-tolerant was largely ineffective in restoring sensitivity to morphine, (3) MK801 need not be present at the time of testing to demonstrate attenuation of tolerance to morphine, and (4) MK801 could prevent tolerance for periods as long as 12 days when coadministered with morphine. While without effects by itself, it is not clear whether MK801 augments antinociception when administered simultaneously with morphine. Some studies indeed report an additive effect during the initial period of tolerance induction, whereas other investigators have not reported this interaction when MK801 was administered after an initial period of opioid exposure.

The ability of MK801 to prevent morphine tolerance also has been demonstrated at spinal sites when administered to spinalized rats, and in intrathecal models of daily coadministration of MK801 and morphine. While these provide strong support for the role of an NMDA receptor in opioid tolerance, these models used daily intrathecal injections to investigate the ability of MK801 to prevent tolerance, and thus involve daily handling, a factor that may be implicated in associative learning and tolerance. In addition, it is possible that daily injections can result in a state of intermittent withdrawal in the period preceding each successive injection of morphine; a factor we believe may be responsible for what has been described as “hyperalgesia” observed during the course of induction of tolerance, where rats have been reported to develop hypersensitivity to a thermal noxious stimulus during daily bolus delivery of morphine.

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In previous work, we characterized the loss of antinociceptive activity of opioids as a consequence of continuous spinal infusion using chronically implanted spinal catheters coupled to subcutaneous osmotic pumps.\textsuperscript{10-13} In these studies, the initiation of a chronic infusion resulted in an initial concentration-dependent increase in escape latency, which declined daily, returning to baseline by 4–6 days. This loss of effect occurred in the absence of intervening testing and, in spite of a continued and measured presence of spinal drug. Importantly, consistent with the inactivation of opioid-sensitive sites, examination of the animals’ responses to a bolus “probe” dose of morphine after chronic exposure revealed a right shift in the probe drug dose-response curve, with the magnitude of the right shift being proportional to the infusion concentration of the toleragen. That this right shift is not a consequence of a general dysfunction of the animal, is indicated by the absence of a similar change in sensitivity to probe drugs acting on a separate receptor (e.g., \( \alpha_2 \) agonists).\textsuperscript{10}

The specific aims of the current study were: (1) to attempt to reproduce previous findings and assess the effects of NMDA receptor antagonism on this model of continuous spinal opioid exposure, as well as the effects of daily testing on this model; (2) to quantify the magnitude of the tolerance development induced by chronic opioid exposure by assessing the degree of shift in the bolus dose-response curves of each group after a week of infusion (“probe dose-response curve”); (3) to assess the duration of effect of MK801 on morphine tolerance after its discontinuation; and (4) to assess naloxone-precipitated withdrawal in MK801-treated morphine-tolerant rats in an intrathecal model.

**Methods**

**Animals**

Approval for this study was obtained from the University of California San Diego Animal Care Committee. Male Sprague-Dawley rats (weighing 350–400 g) were implanted and thereafter housed in individual standard cages at room temperature on a 12 h/12 h light/dark cycle (lights on 7:00 AM). Testing was performed during the light cycle at 12:00 noon. Animals had free access to food and water. Each rat was implanted as described later with a subarachnoid catheter attached to a subcutaneous osmotic pump filled with saline or drug(s). Rats were assigned to one of the other group randomly. All rats received a 7-day infusion and after testing on the last day were killed by an overdose of barbiturate.

**Preparation of the Catheter with Infusion Pump and Implantation**

The preparation of the catheter has been described previously.\textsuperscript{13} In brief, a 16-cm length of PE-10 tubing is connected by heat fusion with a hot air jet to a 2-cm length of PE-60 tubing. A 1-cm piece of Silastic tubing (Tuzik, Norwalk, MA) previously soaked in chloroform to increase its internal diameter, is then passed over both ends of the PE-10 tubing so as to form a loop at a distance of 3 cm from the end of the PE-10 tubing fused to the PE-60. The long end of the catheter is stretched to reduce diameter, soaked in alcohol (70%) overnight, and cut to a length of 9 cm from the silastic tubing. Alzet osmotic minipumps (model 2001 delivering 1 \( \mu \)l/h; Alza, Palo Alto, CA) were filled with drug(s) or saline and attached to the saline-flushed catheter. This pump is designed to deliver a constant infusion of 1 \( \mu \)l/h for 7 days after an initial activation period in the animal of 4 h. In vitro pilot studies at 37°C confirmed the accuracy of the pump with catheter attached. The catheter and pump were implanted between the hours of 9:00 AM and 12:00 PM, according to the procedure originally described for chronic catheterization of the rat spinal cord,\textsuperscript{14} with the additional modification of the subcutaneous osmotic pump. In brief, animals were anesthetized with halothane and placed in a stereotaxic head holder. A midline incision is made to expose the atlantooccipital membrane. The membrane is pierced and the PE-10 end of the catheter is placed intrathecal to a distance of 8.5 cm, that is, caudal to the level of the thoracolumbar junction. The pump is then attached to the PE-60 end of the catheter and implanted subcutaneously in a pouch to lie just behind one or the other shoulder. A 14-G needle is used to make a small hole in the forehead. The loop end of the catheter is passed through this hole. This PE-10 loop can then be cut and used to administer external doses of drug at the end of the 7-day infusion period. The wound is sutured, including a loose ligature at the base of the loop to prevent it from moving. Animals fully recovered 15–30 min after implantation. Those showing any signs of motor impairment were killed with an overdose of barbiturate.

**Drugs and Injection**

The following drugs were used for continuous spinal infusion: morphine sulfate (Merck, Sharp and Dohme, Philadelphia, PA), and (+)-naloxone (Research Biochemicals, Natick, MA). Drugs were dissolved in sterile normal saline (0.9% NaCl). The drug free base, were expressed in units of \( \mu \)g/ml for the infusion calculations. A constant infusion concentration of drugs was used in previous experiments to achieve a consistent increase in hot plate latency.

**Experimental Paradigm**

The study was divided into two phases. Phase I consisted of a 6-day exposure to chronic morphine, and was used to assess the effect of morphine on the rodent’s nociceptive threshold after administration of a single acute dose of morphine. The animals received morphine subcutaneously 30 min before testing on the first day, and then daily for 6 days. On day 7, the animals were killed and the brain examined for evidence of morphine toxicity. Animals were divided into two groups: (1) saline treated, and (2) saline treated, plus naloxone. The animals were placed into individual plastic cages at room temperature on a 12 h/12 h light/dark cycle. Testing was performed during the light cycle at 12:00 noon. Animals had free access to food and water. Each rat was implanted as described later with a subarachnoid catheter attached to a subcutaneous osmotic pump filled with saline or drug(s). Rats were assigned to one of the other group randomly. All rats received a 7-day infusion and after testing on the last day were killed by an overdose of barbiturate.

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SPINAL OPIOID TOLERANCE AND NMDA RECEPTOR

West Point, PA), and (+)-MK801 hydrogen maleate (Research Biochemicals, Natick, MA). Drugs were dissolved in sterile normal saline. Drug doses, calculated as the free base, were expressed in nanomoles per hour (nm/h) for the infusion concentrations, or nanomoles per rat for the postinfusion dose-response studies. Morphine infusion concentration was 20 nm/µl/h in all animals receiving morphine because this dose was found in previous experiments to have yielded a maximal increase in hot plate latency on day 7 after implantation.

Experimental Paradigms

The study was divided into two phases. Phase 1 was directed toward defining the change in response over time during chronic morphine infusion and the magnitude of the withdrawal response. Phase 2 was designed to assess the effects of daily handling on the nociceptive threshold at 7 days and to assess the subsequent right shift in probe dose-response curve as a function of treatment group.

Phase 1. In the first phase, animals were first tested on the hot plate and then implanted. Testing was carried out daily on the hot plate between the hours of 10:00 AM and 12:00 noon from day 0, the day of implantation, to day 7; i.e., at the end of 7 days of infusion. In this phase of the experiment, two doses of MK801 were chosen, 3 nm/h and 10 nm/h as a coin infusion with morphine. The higher MK801 dose of 10 nm/h was found to have the maximal effect in attenuating tolerance with the least number of animals expressing side effects when administered in a pilot study. In preliminary studies, we observed that MK801 doses exceeding 15 nm/h resulted in weight loss and motor weakness, whereas doses of 1 nm/h or less were found to have little effect on morphine tolerance. Groups were thus examined with 3 nm/h MK801 + morphine sulfate, 10 nm/h MK801 + morphine sulfate, 10 nm/h MK801 + morphine sulfate, and saline. A separate group of each treatment group of rats were tested only on days 0, 1, and 7 to assess if there was any effect on latencies from daily testing itself.

Finally, a group infused with morphine sulfate alone was given an intrathecal bolus dose of 10 nm MK801 on day 7 to determine whether acute intrathecal MK801 would restore morphine sensitivity in morphine-tolerant rats. Bolus doses higher than 10 nm produced side effects and thus were not studied.

To assess dependency in these animals, all rats in those groups tested daily on the hot plate were then entered into a withdrawal study as follows. At the end of the 7-day infusion period; i.e., on day 7 at 12:00 noon, rats from each group were given 0.3 mg/kg intraperitoneal naloxone and observed for a period of 1 h, in a circular transparent observation chamber, for the presence or absence of signs of withdrawal. The observer was blinded as to what group to which each rat belonged. Signs of withdrawal chosen for assessment had been previously determined to be prevalent in animals infused for 7 days with intrathecal morphine that received the same dose of naloxone. These signs were: (1) vocalization in response to air motion produced by blowing gently on the rat through a straw three times, or in response to light touch with a 3-cm piece of PE-10 tubing; (2) Spontaneous vocalization; (3) abnormal posture indicated by hind paw lift; 4. ejaculation; 5. had shaking, also often called ‘‘wet doghead shakes’’; 6. escape attempts—one or more attempts to escape from the chamber.

Animals were killed and dissected to assess the integrity of the catheter and the infusion system. Any animal found to have a blocked or misplaced catheter was eliminated from the study.

Phase 2. In phase 2, all groups were tested on the hot plate only on days 1, 2, and 7. Implanted rats were then entered into the following study. On day 7, after testing on the hot plate at approximately 12:00 noon, the external loop of catheter was cut, and the intrathecal part of the catheter flushed with 10 µl sterile normal saline. On day 8, 24 h after stopping the infusion, a single probe dose of intrathecal morphine was administered. Based on preliminary studies, rats were given one of the following intrathecal probe doses of morphine; those infused with saline were given either 1 nm, 10 nm, or 100 nm/10 µl morphine; those infused with morphine alone were given 1 nm, 10 nm, 30 nm and 100 nm/10 µl morphine; those infused with MK801 10 nm/h + morphine were given either 0.01 nm, 0.1 nm, 1 nm, or 10 nm/µl morphine; those infused with MK801 alone were given either 0.01 nm, 0.1 nm, 1 nm, or 10 nm/µl morphine. Hot plate latencies were measured at 0, 30, 60, and 120 min. This assignment was continued until at least four or more rats in each group were obtained for each intrathecal probe dose, generating a dose-response curve for each group. Each rat was used only once.

Finally, rats from each of these groups were retested 1 week later, after cessation of infusion, to assess their response to an intrathecal probe dose of 10 nm of morphine on the hot plate. Hot plate latencies were measured at 0, 15, 30, and 60 min.
administration of the probe dose of morphine, testing was performed at 30, 60, and 120 min.

Hot-plate data are expressed either as mean latencies for each group, or as maximum percent effect (MPE). Maximum percent effect is calculated as follows:

$$\text{MPE} = \frac{\text{post-drug latency} - \text{baseline}}{\text{cutoff time} - \text{baseline}} \times 100$$

where postdrug latency is the response measured at the particular time after initiation of infusion or after intrathecal dose of probe drug. Baseline is the preinjection or preprobe latency, and the cutoff time is 60 s.

**Statistics**

Analysis of the dose-response curves and statistics were obtained with computer software programs (Abacus Concepts, StatView, Abacus Concepts, Berkeley, CA). Where applicable, data from hot-plate testing, i.e., absolute latencies or calculated %MPE, were analyzed using one- or two-way analysis of variance (ANOVA) to detect differences between groups. When differences were found, these findings were subjected to Scheffe’s F test (significant at 95%). Unless stated otherwise, single points of comparison were made using a standard paired or unpaired t-test. Using linear regression, calculation of the ED$_{50}$ (95% confidence intervals) test for relative potency was carried out where applicable. The tolerance ratio (the ratio of ED$_{50}$ in drug-infused animals to ED$_{50}$ of saline-infused animals), and 95% confidence intervals were calculated. Differences yielding critical values corresponding to $P < 0.05$ were considered statistically significant.

**Results**

**Daily Hot Plate Response Latencies in Chronically Infused Rats**

**Saline-infused Rats.** There was no significant difference between latencies on days 1 and 7 in rats that were tested daily, i.e., on days 0 through 7 (phase 1 rats: $15 \pm 1$ vs. $15 \pm 1$ s, $n = 5$, $P > 0.1$; fig. 1A), or in those that were tested only on days 0, 1, and 7 (phase 2 rats) ($15 \pm 1$ vs. $17 \pm 1$ s, $n = 11$, $P > 0.1$; fig. 1B).

Neither was there a significant difference between these two saline-infused groups (ANOVA, $P > 0.1$), demonstrating no significant effect after day 1, from implantation, infusion of the vehicle saline, or from daily

**Morphine-infused Rats.** No significant difference between groups (ANOVA, $P > 0.1$; fig. 1C).

On days 1, 2, and 7, saline-infused rats received saline $10 \text{nmol/h}$, and MK801-infused rats received saline $10 \text{nmol/h}$, with no difference in any group (ANOVA, $P > 0.1$; fig. 1D).
testing. All saline-infused rats (n = 16), however, did show a small, but statistically significant rise in latencies on day 1 compared to baseline values on the day 0; (12 ± 1 vs. 15 ± 1 s, P < 0.05).

**Morphine-infused Rats.** There was no significant difference between morphine-infused rats tested daily (n = 15), and those tested only on days 0, 1, and 7 (n = 15; ANOVA P > 0.1; fig. 1). All morphine-infused rats, i.e., those included in phases 1 and 2 of the study, showed a maximal increase in HP latencies on day 1 over baseline on day 0 (45 ± 3 vs. 13 ± 1 s), returning to near baseline values on day 7 (day 7: 16 ± 1 vs. day 0: 13 ± 1 s). There was no significant difference between latencies of all saline-infused rats (n = 16) and all morphine-infused rats (n = 30) on day 7 (16 ± 1 vs. 16 ± 1 s, ANOVA P > 0.1).

On day 8, 24 h after stopping the infusion, latencies of morphine-infused rats, tested only on days 0, 1, and 7, were significantly less than those on day 7 (day 8, 11 ± 1 ms. day 7, 15 ± 1 s, n = 15, P < 0.05, Scheffe) and significantly less than those of the group of similarly tested rats receiving saline, on day 8 (16 ± 2 s, n = 11, P < 0.05, Scheffe; fig. 1B), demonstrating an increased thermal sensitivity in the morphine-infused rats only after discontinuation of the morphine infusion.

**Rats infused with 10 nm/h MK801.** Although 10 nm/h MK801-infused rats tested daily showed a small but significant increase in latencies on days 0, 1, 4, and 6, as compared to saline-infused rats tested daily (ANOVA, P < 0.05, Scheffe), there was no significant difference in calculated % MPE between these two groups (ANOVA P > 0.1; fig. 1A). Also, there was no significant difference between the same group of MK801-infused rats on day 0, as compared to those on day 7 (day 0, 16 ± 2 vs. day 7, 20 ± 3 s, P > 0.1), or compared to those of saline-infused rats on day 7 (15 ± 1 s, P > 0.1). In addition, there was no significant difference between rats infused with MK801 alone and tested daily (n = 6), as compared to a separate group of rats infused with MK801 alone, but only tested on days 0, 1, and 7 (n = 17, ANOVA, P > 0.1, fig. 1B).

**MK801 + Morphine-coinfused Rats.** Rats coinfused with 10 nm/h MK801 + 20 nm/h morphine that were tested daily showed a comparable increase in latencies on day 1 (37 ± 5 s, n = 11) that was not significantly different from morphine-infused rats that were also tested daily (40 ± 4 s, n = 15, P > 0.1, fig. 2). Latencies remained elevated in 10 nm/h MK801 + morphine-coinfused rats tested daily from days 1 through 7, and there was no significant difference between days 1 and 7 (37 ± 5, 37 ± 4 s, P > 0.1, n = 11). On day 8, 24 h after the infusion was stopped, latencies in rats tested only on days 0, 1, and 7, declined to near baseline values with only a small, but significant increase as compared to baseline on day 0 (18 ± 1 vs. 15 ± 1 s, n = 19, P < 0.05). However, these latencies were not significantly different from saline-infused rats tested similarly (16 ± 2, n = 11, P > 0.1), but they were significantly higher than morphine-infused rats tested similarly (11 ± 1 s, n = 15; fig. 1B).

Thus, coinfusion of MK801 at 10 nm/h with morphine resulted in maintenance of elevated latencies during infusion, which, on cessation of infusion, returned toward baseline but did not go below baseline values, as seen in morphine-infused rats. Latencies in rats tested daily and infused with 3 nm/h MK801 and 20 nm/h morphine (n = 9), were significantly increased over saline-infused rats, except on days 3 and 7 (ANOVA P < 0.05, Scheffe; fig. 2). These latencies also were significantly increased over those of morphine-infused rats from days 4 through 7 (ANOVA P < 0.05, Scheffe). Latencies observed in this group were numerically less but not significantly different than those of rats coinfused with the larger dose of 10 nm/h MK801 + morphine, except on day 1 (ANOVA P > 0.05).

**Fig. 2.** Time course of the antinoceptive effect expressed as percent maximum effect, observed with chronic infusion for 7 days of intrathecal morphine, MK801 10 nm/h, MK801 10 nm/h with morphine, and saline. Each line represents the mean ± SE of five or more rats tested only on days 1, 7, and one day after discontinuation of infusion.
Morphine-infused Rats Given Intrathecal MK801 Acutely on Day 7. Rats infused with morphine alone that were given a 10-nmol bolus of intrathecal MK801 on day 7 (n = 4), failed to show a significant increase in latencies when measured at 10, 30, 60, and 120 min; (17 ± 0.4 vs. 20 ± 1 vs. 19.5 ± 1, P > 0.1), thus demonstrating a lack of acute effect of MK801 in restoring sensitivity to morphine (fig. 3).

Withdrawal Studies. All groups tested daily on the hot plate were entered into a withdrawal study receiving 0.3 mg/kg naloxone intraperitoneally on day 8 (fig. 4). None of the rats infused with saline (n = 5) showed any of the six withdrawal signs examined. Two rats in the group that received MK801 alone (n = 6) showed one sign of withdrawal each. In contrast, morphine-infused rats (n = 7), all exhibited three or more of the six signs of withdrawal assessed. MK801 and morphine-infused rats (n = 11) showed a significant reduction in three of the six signs of withdrawal, i.e., vocalization to air motion or light touch, spontaneous vocalization, and paw lift when compared to morphine-infused rats (P < 0.05). MK801 (3 nm/h) and morphine-infused rats (n = 8) showed only a significant reduction in one sign of withdrawal, i.e., ejaculation when compared to morphine-infused rats.

Probe Dose-response curves

After severing the pump catheter connection on day 7 and flushing the catheter with 10 µl saline, 24 h was allowed to elapse and the rat was then given one of several doses of intrathecal morphine to generate the probe dose response curve.

Saline and MK801-infused Rats and Probe Dose Response. Twenty-four hours after terminating saline infusion, bolus doses of intrathecal morphine revealed a mononic dose response curve with an ED₅₀ of %MPE of 11 nm. In rats receiving MK801, the intrathecal morphine %MPE probe dose-response curve showed a 36-fold parallel left shift, as compared to saline-infused rats. The ED₅₀ as measured by percent area under the curve (%AUC) also was decreased by 26-fold showing an increased duration of effect, as well as peak effect (fig. 5A and table 1).

Morphine-infused Rats and Probe Dose Response. The intrathecal morphine %MPE probe dose-response curve in rats treated previously with 20 nm/h intrathecal morphine infusions displayed a significant right shift, such that the ED₅₀ was increased by a factor of 14 as compared to the dose-response curve of saline-infused rats (fig. 5A and table 1). Probe doses greater than 150 nm of morphine began to produce a hyperalgesic response (e.g., spontaneous vocalization) as previously described. Thus, it was not possible to test even a half log dose increase of intrathecal morphine probe over that of the highest dose given, i.e. 100 nm. The ED₅₀ as measured by %AUC also was increased by a factor of 200 as compared to saline-infused rats (fig. 5B and table 1). This indicates that in morphine-tolerant animals, the peak effect as well as the duration of effect of the probe dose of spinal morphine is considerably reduced.

Morphine + MK801-infused Rats and Probe Dose Response.

Fig. 3. Time course of the antinociceptive effect expressed as percent maximum effect of a single bolus of intrathecal MK801 10 nm injected after 7 days of chronic intrathecal morphine and saline infusion. Each bar represents the mean ± SE of four rats.

Fig. 4. Time course of percent maximum effect (± SE) in rats given saline, morphine, or morphine + MK801 7 days after cessation of infusion. Each bar represents the mean ± SE of five or more rats per group.
Spinal Opioid Tolerance and NMDA Receptor

Fig. 4. Time course of the antinociceptive effect expressed as percent maximum effect of a single bolus of intrathecal morphine 1 week after discontinuation of a chronic infusion for 7 days of intrathecal morphine, 10 nmol/h MK801, 10 nmol/h MK801 with morphine, and saline. Each line presents the mean ± SE of five or more rats.

or more rats in each group infused with saline, morphine, MK801 + morphine, and MK801 alone, and tested on days 0, 1, 7, and 8, were given a 200-μM intrathecal probe dose of morphine 7 days after discontinuation of their infusion, i.e., on day 15. There was no significant difference in HP latencies as measured by %MPE or %AUC between saline-infused rats (n = 4) and MK801 + morphine-infused rats (n = 4), MK801-infused rats (n = 5), or morphine-infused rats (n = 8) (P > 0.1, ANOVA, fig. 6). Thus, there was no residual evidence of hypersensitivity to morphine 7 days after termination of MK801 infusion, or of tolerance in morphine-infused rats when compared to saline control rats.

Discussion

Previous work has shown that the administration of MK801 can attenuate tolerance to, and withdrawal

Fig. 5. Withdrawal signs after chronic infusion for 7 days of intrathecal morphine, 10 nmol/h MK801, 10 nmol/h MK801 + morphine, 3 nmol/h MK801 + morphine, or saline. Each column presents the percent of each group of five or more rats ± SE, showing one of the following six signs of withdrawal: (1) vocalization in response to air motion or light touch, (2) spontaneous vocalization, (3) abnormal posture, (4) ejaculation, (5) "wet dog head shakes," (6) escape attempts.
from, chronic systemic and spinal morphine administration.\textsuperscript{13,16} These previous studies, while insightful, typically employ bolus drug delivery or pellet systems that produce widely changing drug concentrations over a brief period of time. To define the properties of the reported effects of NMDA antagonism on opioid tolerance and dependence, we initiated studies employing the well-characterized model of continuous spinal delivery in the rat. Continued exposure of the spinal cord to a fixed concentration of morphine will result in (1) a progressive loss of spinal opioid receptor-mediated antinociception, with a reliable right shift in the dose-response curve for bolus doses of spinal morphine,\textsuperscript{10} and (2) signs of withdrawal after precipitation with naloxone, including vocalization to touch, spontaneous vocalization, abnormal posturing, ejaculation, head shaking, or escape attempts. Intrathecal MK801, a use-dependent blocker of the NMDA ionophore, has no effect on the hot plate response,\textsuperscript{7} and will not acutely increase the response latency when given in a rat that has been rendered tolerant by chronic exposure to spinal morphine (current study). However, as demonstrated in the current study, when delivered concurrently with morphine, but not as a bolus after loss of opioid activity, it preserves, in a dose-dependent fashion, the antinociception produced by morphine. These results suggest that during continued opioid exposure there is a change in spinal function leading to a reduction in opioid responsiveness and this reorganization is mediated in an essential fashion by the activation of the NMDA glutamate receptor.

### Spinal Opioid Tolerance

In the current model, loss of effect, and a right shift in the dose-response curve to intrathecal morphine, was observed in morphine-infused animals that were tested daily or tested only at the beginning and end of the infusion.\textsuperscript{12,15} Thus, it appears that the significant effects are not attributable to behavioral conditioning, where pairing of drug administration and environmental cues may play a role in the apparent progressive loss of drug effect.\textsuperscript{17} Changes in spinal morphine kinetics and metabolism also appear unlikely to account for the progressive reduction.\textsuperscript{10} Similarly, the ability to induce antinociception when agonists for other receptors are delivered in a morphine-tolerant rat appears to exclude a “nonspecific” change in nociceptive processing. Pharmacologically, chronic opioid exposure results in a right shift of the dose-response curve and frequently a reduction in the maximum achievable effect. Importantly, the magnitude of the right shift is inversely proportional to the intrinsic efficacy of the agonist as determined in experiments with spinal delivered μ-opioid and α\textsubscript{2} adrenergic agonists.\textsuperscript{18–20} Such changes are consistent with a reduction in the number of receptors, or in coupling of the receptors to the intracellular second messengers that mediate the respective actions of these agonists. Binding studies often have shown a modest reduction in binding with chronic opioid exposures, but these results have not been consistent.\textsuperscript{21–24}

**Mechanisms of N-methyl-D-aspartate Antagonist-Morphine Interaction**

The current studies were carried out only with MK801. However, results with systemic delivery also have been observed with a structurally distinct competitive NMDA antagonist, LY-274614.\textsuperscript{16} This joint action supports the likely role for the role of the NMDA ionophore with which these two agents are known to interact with high affinity.\textsuperscript{25} Hot plate latencies in morphine + MK801- and MK801-infused rats returned to baseline after discontinuation of infusion. This argues against any permanent neurologic effect that might have accounted for the observed persistence of antinociception. Any synergistic action between MK801 and morphine would appear unlikely because: (1) MK801 given alone had no observable effect, (2) MK801 when administered with morphine as a coinfusion had no effect on latencies on the second day of infusion (3) MK801 when delivered as a bolus failed to increase hot plate latencies in the morphine-tolerant rat (4) Investigations by other previous studies have shown no simple additive effect of MK801 and morphine in intrathecal studies,\textsuperscript{7} in systemic studies,\textsuperscript{1} or in spinalized rats.\textsuperscript{6} In the formalin test in which MK801 is active, bolus spinal injections of MK801 and morphine will not reinforce saline

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**Table 1. ED\textsubscript{50} and 95% Confidence Interval for Probe Intrathecal Morphine Response Curves (% MPE) Assessed after 7-day Infusions of Saline, Morphine, MK-801 10 nmol/h + Morphine, and MK-801 Alone-infused Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>ED\textsubscript{50} % MPE (95% CI)</th>
<th>Tolerance Ratio (ED\textsubscript{50} drug/saline ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline (nmol)</td>
<td>11 (6–20)</td>
<td>1.0</td>
</tr>
<tr>
<td>Morphine (nmol)</td>
<td>159 (42–594)</td>
<td>15 ± 12</td>
</tr>
<tr>
<td>MK-801 + morphine (nmol)</td>
<td>4 (1–15)</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>MK-801 (nmol)</td>
<td>0.3 (0.1–0.5)</td>
<td>0.27 ± 0.01</td>
</tr>
</tbody>
</table>

Anesthesiology, V 84, No 5, May 1996
SPINAL OPIOID TOLERANCE AND NMDA RECEPTOR

![Graph showing dose response and percent area under concentration](attachment:image.png)

Fig. 6. Dose response expressed as percent maximum effect (top) and percent area under concentration (bottom) of intrathecal morphine after chronic infusion for 7 days of intrathecal morphine, 10 mg/hr MK801, 10 mg/hr MK801 with morphine, or saline. Each dose-effect point represents the mean ± SE with four or more rats.

Anesthesiology, V 84, No 5, May 1996

years that the significant behavioral conditioning and environmental stimuli appear to progress in spinal morphine is unlikely to account for the apparent progressive increase in spinal morphine tolerance. Similarly, the ability of opioid antagonists for other receptor systems to treat morphine-tolerant rats appears to be indicative of nociceptive pain. The endogenous opioid system in chronic opioid exposure produces an analgesic effect, but the role of the right shift is still unclear. The present studies demonstrate that the efficiency of the systems with spinally deafferented receptors is not reduced by the opioid agonists, and that a reduction in the activity of the neuronal system is associated with these results. Binding studies have demonstrated that the reduction in binding with opioids may be due to the opioid antagonist coupling in spinal cord. The present study indicates that the opioid antagonists do not interact in the development of spinal morphine tolerance.

NMDA Receptor

Glutamate is a principal excitatory amino acid acting on the NMDA receptor. If glutamate receptor activity is necessary for, or facilitates development of the tolerant state, then delivery of an opioid should either increase spinal glutamate release or augment receptor effects of restoring glutamate levels. In the anesthetized rat, we have demonstrated that spinal infusion of morphine for 6 days does not affect resting release of spinal excitatory amino acids in dialysates. However, at the time of withdrawal, there is a large increase in glutamate release, which is blocked by clonidine and MK801. These results are consistent with the effects observed with opioid tolerance on spinal C-fos activity where acute delivery of systemic morphine has little effect on the expression of spinal C-fos, although it blocks stimulation-evoked increases, but during withdrawal there is a massive increase in C-fos activity. Alternatively, if spinal glutamate release is not augmented by morphine then it is possible that the postsynaptic effects are augmented. Activation of the NMDA receptor increases entry of extracellular calcium into the cell, which stimulates the production of nitric oxide. Inhibition of nitric oxide synthetase has been shown to attenuate some signs of withdrawal in morphine-tolerant rats, i.e., wet dog shakes and weight loss. Thus, it is possible that NMDA receptor antagonism could act to prevent withdrawal and by inhibiting nitric oxide synthetase activation of the NMDA receptor also evokes

of MK801 and morphine interact at most in an additive fashion. These data emphasize that the ability of MK801 to maintain an opioid effect as long as 7 days does not represent an acute reversal of possible changes initiated by spinal morphine, but reflects the prevention of the evolution of the opioid-tolerant state. This concept of a prevention of further development of the reduction in spinal function also is supported by the observation that co-treatment with MK801 prevented the appearance of a precipitated withdrawal. Importantly, however, naloxone-prefixed withdrawal signs in the rat chronically exposed to spinal morphine are attenuated by the delivery of intrathecal MK801. Thus, these studies suggest that chronic, but not acute antagonism of the NMDA receptor will prevent the evolution of tolerance and dependence, whereas acute antagonism will only prevent manifestations of the opioid withdrawal state, and not reverse an established tolerant state.

The apparent preservation of the effects of morphine in the presence of MK801 may reflect a concurrent increase in sensitivity to opioid receptor occupancy. Thus, in the current studies, we unexpectedly observed that the chronic delivery of MK801 alone resulted in a left shift of the morphine dose response that was present 24 h, but not 7 days after termination of chronic delivery. This has not, to our knowledge, been hitherto reported, and may have direct clinical implications should drugs of this class enter clinical practice. The persistence of this effect at a time in which MK801 was not present, emphasizes that the effect is unlikely to be due to altered kinetics, and most likely reflects a trophic change in spinal opioid action. We are not aware of any studies in which binding was examined with chronic NMDA antagonists exposure. Upregulation of receptor number with chronic antagonism has been reported for several systems, but not, to our knowledge, for NMDA sites. In any case, given the antagonistic effects we would expect from a more efficient NMDA receptor-linked system, increased NMDA sites or glutamate release would not likely account for the enhanced responsiveness to morphine, per se. These results thus raise the possibility that NMDA antagonists may not only prevent the processes that lead to a reduction in morphine sensitivity, but with chronic exposure result in a facilitation of the effects of receptor coupling.

Glutamate is a principal excitatory amino acid acting on the NMDA receptor. If glutamate receptor activity is necessary for, or facilitates development of the tolerant state, then delivery of an opioid should either increase spinal glutamate release or augment receptor effects of restoring glutamate levels. In the anesthetized rat, we have observed that spinal infusion of morphine for 6 days does not affect resting release of spinal excitatory amino acids in dialysates. However, at the time of withdrawal, there is a large increase in glutamate release, which is blocked by clonidine and MK801. These results are consistent with the effects observed with opioid tolerance on spinal C-fos activity where acute delivery of systemic morphine has little effect on the expression of spinal C-fos, although it blocks stimulation-evoked increases, but during withdrawal there is a massive increase in C-fos activity. Alternatively, if spinal glutamate release is not augmented by morphine then it is possible that the postsynaptic effects are augmented. Activation of the NMDA receptor increases entry of extracellular calcium into the cell, which stimulates the production of nitric oxide. Inhibition of nitric oxide synthetase has been shown to attenuate some signs of withdrawal in morphine-tolerant rats, i.e., wet dog shakes and weight loss. Thus, it is possible that NMDA receptor antagonism could act to prevent withdrawal and by inhibiting nitric oxide synthetase activation of the NMDA receptor also evokes
a spinal release of glutamate, which can be blocked by the nitric oxide synthetase inhibitor L-nitro-L-arginine methyl ester.\textsuperscript{33}

Spinal N-methyl-D-aspartate Antagonism and Withdrawal

As previously reported with systemic drug delivery, after chronic exposure to morphine, rats displayed a clear hyperalgesia and signs of behavioral agitation directed at the caudal dermatomes of the body during withdrawal. This suggested a hyperalgesic component in the tolerant state. Such hyperparoxitic has been recently reported by other investigators.\textsuperscript{7} With systemic delivery,\textsuperscript{1,4} acutely delivered spinal MK801 prevented some (but not all) of these signs, suggesting that during withdrawal there is a particular increase in glutamate release. As noted earlier, we recently showed that, using spinal dialysis in unanesthetized rats, naloxone treatment results in a prominent release of glutamate in animals rendered tolerant to morphine concentrations and treatment schedules identical to those employed here. This occurs with a time course that precisely parallels the withdrawal signs associated with the precipitated abstinence syndrome. Importantly, the observed hyperalgesia during withdrawal and its sensitivity to spinal NMDA antagonism also is consistent with the ability of intrathecal NMDA to evoke hyperalgesia in the non tolerant rat.\textsuperscript{34} Importantly, in rats infused concurrently with NMDA antagonists, withdrawal was not observed. Whether this is because MK801 was present and acutely able to prevent the manifestations of withdrawal or because MK801 prevented the evolution of the dependent state and subsequently removed the circumstances leading to abstinence-evoked glutamate release is not known.

Intracellular Mechanisms of Opioid Tolerance and the N-methyl-D-aspartate Receptor

Several changes in the receptor-effector mechanism have been proposed as a cause of tolerance. Morphine by its action at the \( \mu \) opioid receptor produces an inhibition of adenylate cyclase activity and thus a resultant decrease in cyclic adenosine monophosphate formation.\textsuperscript{35} Adenylate cyclase is inactivated or activated respectively by \( G_{i} \) and \( G_{s} \) glutamyl transpeptidase membrane bound proteins. Decreased cyclic adenosine monophosphate levels activate protein kinase C (PKC), and activation of PKC phosphorylates the \( \mu \) receptor-coupled \( G \) inhibitory protein, thereby suppressing its ability to mediate receptor-evoked inhibition of adenylate cyclase,\textsuperscript{36,37} thus leading to a compensatory increase in adenylate cyclase activity and increased levels of cyclic adenosine monophosphate in turn.\textsuperscript{38} Thus, changes in PKC activity or coupling of the \( \mu \) receptor-effector \( G \) protein have been proposed as a mechanism of tolerance. Protein kinase C translocation by NMDA receptor activation has been shown.\textsuperscript{39} The functional importance of such translocation in tolerance has been suggested by observations that loss of spinal opioid effect (tolerance) has also been blocked by an agent that prevents this translocation of PKC, \textit{i.e.}, monosialoganglioside (GM1) ganglioside.\textsuperscript{40} Such increases in PKC activity could have a variety of effects apart from the interference of \( G \) protein coupling, including modulation of K channel activity,\textsuperscript{41} which would attenuate the ability of opioids to hyperpolarize membranes, and reduction of the magnesium block of the NMDA receptor.\textsuperscript{42} In addition, diacylglycerol \textit{via} PKC activation diminishes the inhibitory effects of the \( \mu \) agonist on calcium channel opening.\textsuperscript{43-45}

There also is evidence that a glutamate receptor is coupled to the enzyme phospholipase C. This catalyzes the hydrolysis of phosphatidyl inositides to two second messengers:inositol 1,4,5-triphosphate, which causes the mobilization of intracellular calcium, and diacylglycerol, which activates PKC.\textsuperscript{46} Although quisqualate has a similar effect, NMDA does not mobilize intracellular calcium and at most is only a weak stimulator of phosphatidyl inositide turnover. Thus, blockade of the NMDA receptor by MK801 would not be expected to prevent activation of PKC in this manner and this mechanism would not likely account for the prevention of tolerance development.

Spinal Rceptor Tolerance and Glutamate

The current studies demonstrate that in the face of stable morphine concentrations, the loss of effect produced by spinal delivery is prevented by antagonism of the NMDA ionophore. As reviewed earlier, this emphasizes the role of glutamate release in the tolerance process. This simple consideration leads to the speculation that increased glutamate release may exacerbate the loss of response to the opioid. Such a consideration has surprising theoretical implications regarding two clinically relevant issues. First, considerable evidence suggests that a variety of pain states, such as observed after the injection of formalin into the paw or carrageen into the knee, may lead to a hyperalgesia that is reversed by NMDA antagonism.\textsuperscript{20,47-49} Consistent with this pharmacology, such stimuli have been shown to
be associated with a marked stimulus-dependent increase in spinal glutamate, which is prevented by opioids. It thus seems appropriate to consider that such pain states might in fact exaggerate the evolution of tolerance. This hypothesis is in direct conflict with previous comments that pain may diminish tolerance development and that this may somehow account for the difference in apparent rates of tolerance development observed in preclinical studies (using experimental pain models) and chronic clinical pain states. Second, as noted, in the face of transient opioid withdrawal, glutamate may show prominent increases. We thus speculate that in the case of bolus opioid dosing, such peaks and valleys of effect may in itself be associated with an enhanced evolution of the tolerant state. Such consideration would suggest that steady-state drug concentrations may under certain circumstances be superior to bolus delivery. These considerations provide a testable hypothesis with relevance both to our understanding of the neurobiology of drug tolerance as well as to the clinical importance of drug delivery paradigms.

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References
