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Intrathecal *N*-Methyl-D-aspartate (NMDA) Induces Paradoxical Analgesia in the Tail-Flick Test in Rats

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ÁLVAREZ-VEGA, M., A. BAAMONDE, M. GUTIÉRREZ, A. HIDALGO AND L. MENÉNDEZ. *Intrathecal* N-*methyl-D-aspartate (NMDA) induces paradoxical analgesia in the tail-flick test in rats.* PHARMACOL BIOCHEM BEHAV **65**(4) 621–625, 2000.—The intrathecal (IT) administration of NMDA in rodents has usually been reported to produce hyperalgesic reactions, although some articles describe that spinal NMDA can lead to analgesia. We show here that the nociceptive behavior (biting, scratching, licking; BSL) observed after NMDA injection (1–8 μg/rat; IT) is followed by a long period of increased tail-flick latencies, not longer detected 24 h after NMDA administration. The NMDA-receptor antagonist CPP (10–100 ng/rat; IT) blocked the BSL behavior induced by NMDA. In the tail-flick test, this antagonist induced analgesia by itself, and was able, at 30 ng/rat, to prevent the NMDA-mediated analgesia. The implication of opiate mechanisms was discarded since naloxone (3 and 10 mg/kg; IP) did not antagonize NMDA-induced analgesia. Finally, the involvement of the intracellular calcium binding protein calmodulin was assessed. The calmodulin inhibitor, calmidazolium (30–300 μg/rat; IT) only blocked the excitatory effect (BSL) without modifying the tail-flick analgesia produced by NMDA (4 μg). These results show that a single intrathecal administration of NMDA sequentially induces both nociceptive and antinociceptive, nonopiate responses in rats. © 2000 Elsevier Science Inc.

N-Methyl-D-aspartate Spinal cord Nociception Pain Analgesia CPP Calmidazolium Naloxone BSL behavior Tail flick Rat

THE antinociceptive effects of the spinal administration of *N*-methyl-D-aspartate (NMDA) antagonists seen both by electrophysiological (7) and behavioral assays (4) strongly support that NMDA receptors are involved in nociceptive spinal transmission. Specifically, the activation of NMDA receptors by excitatory amino acids seems to play an important role in the spinal sensitization that occurs in nociceptive neurons after intense painful stimulation (8,17). In accordance, the spinal injection of NMDA in rodents has usually been reported to induce either hyperalgesic reactions (6) or a direct aversive behavior consisting in biting, scratching, and/or licking (BSL) of the corresponding dermatomes (1,13).

In contrast with these general observations, a few reports describe that intrathecal administration of NMDA in rodents can also induce some types of analgesic responses (2,11,14,16). Although among these reports, there are important differences related both to the experimental protocols used (doses of NMDA, testing procedure) and to the analgesic reactions observed (intensity of the analgesia, time course of the effect, etc.) these results seem to indicate that spinal injection of NMDA can activate antinociceptive mechanisms. In particular, two of these cited reports have proposed different mechanistic interpretations for this phenomenon. In one case, the analgesia induced by the NMDA was attributed to

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the release of analgesic mediators, such as opiates (14), while the other study suggested that it could derivate from toxic, irreversible effects produced by NMDA (2).

In a previous work from our laboratory, we have studied the pharmacological modulation of the nociceptive reaction (BSL) induced by the intrathecal injection of NMDA in rats (3). Searching the expected hyperalgesia subsequent to this NMDA receptor activation, we found that when the tail-flick latencies are measured after the initial period of excitation they were paradoxically augmented. In this study we aimed to characterize this increase of the tail-flick latencies after NMDA by studying the relationship with dose and time of the effects induced by NMDA both in the BSL (a nociceptive behavioral reaction) and the tail-flick test (where the analgesic reaction was observed) in rats. In addition, because antinociception is a rather unexpected consequence of NMDA receptor activation, we have tested if a NMDA antagonist could prevent this NMDA-evoked analgesia. Finally, we have also studied the possible involvement opioid mechanisms and of calmodulin, a calcium-binding protein probably involved as an intracellular mediator of the excitatory effects induced by spinal NMDA (13).

METHOD

Animals

Male Wistar rats, weighing 250–350 g, (Animalario de la Universidad de Oviedo; Reg. 33044 13A) exposed to a light–dark cycle of 12 h and with water and food ad lib, were used. The experiments were performed between 15:00 and 20:00 h. Animals were used only once, and killed at the end of the experiment by overexposure to CO_2.

Drugs

NMDA (*N*-methyl-D-aspartate; Sigma) and CPP (3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid; Tocris Cookson) were solved in 50 μl of distilled water. Calmidazolium (Compound R24571; Sigma), was solved in 50 μl of 10% DMSO (dimethyl sulfoxide, Sigma). Naloxone (Naloxone hydrochloride, Sigma) was dissoved in saline. Control groups received their corresponding solvent, that is, either distilled water, DMSO, or saline. For intrathecal injections, rats were lightly anesthetized with ether and a direct lumbar puncture with a hypodermic syringe was made between the L4 and L5 vertebrae (12). Intraperitoneal administrations of naloxone or saline were performed 15 min before intrathecal injections in a final volume of 1 ml/kg.

Biting, Scratching, and Licking (BSL) Behavior

Immediately after the IT injection of NMDA, the rats were placed in a transparent plastic cage (45 × 25 × 20 cm), in which they were observed during a period of 15 or 30 min, divided in 5-min periods. Because the initial study of the time course of the BSL behavior observed after the administration of NMDA indicated that BSL is already almost absent 15 min after injection, further measures were limited to the first 15 min following the intrathecal injection. The intrathecal injections of 50 μl of water or 10% DMSO did not produce, by themselves, any nociceptive or antinociceptive effect.

Tail-Flick Assay

The mean of three consecutive measures made at 1-min intervals was calculated (basal latency). At different times after drug or solvent administration, similar tail-flick measures were taken (experimental latencies). To avoid tissue damage, a cutoff time of 10 s was set. Trials were automatically terminated if a response did not occur within 10 s. Experimental tail-flick measures started 15 min after the injection of NMDA, a time at which nociceptive behavior diminishes. Initially, the time course assays were performed at 15, 30, 60, and 90 min after injection. Afterwards, because the analgesic effect was maximal 30 min after the intrathecal injection, two experimental measures were taken 15 and 30 min after drug administration. In these groups of animals, in which tail-flick measures were only taken 15 and 30 min after injection, the BSL reaction was always measured during the previous 0–15-min period.

All the experiments were conducted according to ethical guidelines (18) and approved by the Comisión de Ensayos Clínicos y Bioética del Principado de Asturias (Spain).

Statistics

The means of the time spent in nociceptive behaviour (BSL) and their standard errors were calculated for each group of rats for three successive intervals of 5 min after the intrathecal injection. In the tail flick test, the means of the latencies to flick the tail at each time and their standard errors were calculated. In both tests, overall comparisons were made using an initial one-way analysis of variance (ANOVA). This was followed by the Newman–Keuls test to calculate the significance of individual differences among groups at each time studied. When only two groups were compared, the Student's *t*-test for unpaired data was used. The level of significance was set at $p < 0.05$.

RESULTS

Nociceptive Behavior and Tail-Flick Analgesia Produced by NMDA (1–8 μg; IT)

The intrathecal injection of NMDA (1–8 μg) induces a nociceptive behavior (BSL) that was continuously measured during 30 min (Fig. 1A). An increase in the time spent in BSL

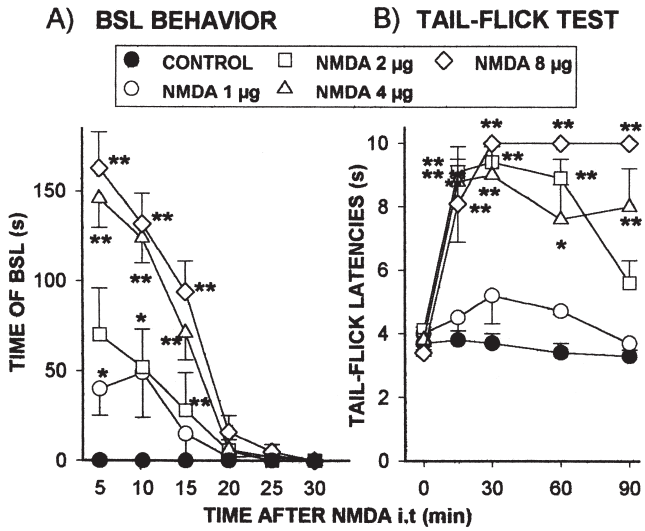

FIG. 1. Effects induced by the IT administration of NMDA (1, 2, 4, and 8 μg) or solvent on BSL behavior (A) and tail-flick test (B). The mean and standard errors of the seconds spent in BSL during 5-min periods are represented in A, and the tail-flick latencies measured before and at several times after injection are shown in B (n = 6–8). Comparisons between the NMDA-treated groups and the solvent-treated group were made at each time using an ANOVA followed by a post hoc Newman–Keuls test. *$p < 0.05$; **$p < 0.01$.

was observed with increasing doses, being almost maximal in the presence of 4 μg of NMDA, because the injection of 8 μg did not induce a further increase in this behavior. For this reason, the dose of 4 μg was selected for further studies. As Fig. 1A shows, BSL behavior progressively decreases with time in such a way that no significant BSL was evoked from the 10–15-min period after injection.

In other groups of animals treated with the same doses of NMDA, tail-flick measures were taken 15, 30, 60, and 90 min after intrathecal injections (Fig. 1B). Except for 1 μg of NMDA, the other doses assayed (2, 4, and 8 μg) produced significant increases in tail-flick latencies that were maximal in the presence of 8 μg, and that persisted during the time that lasted the assay (90 min) when 4 or 8 μg was injected.

Measure of the Tail-Flick Latencies 24 h After the Intrathecal Injection of NMDA (4 μg)

To establish if the increase in the tail-flick latencies is an irreversible consequence of the effect of NMDA at spinal neurons, tail-flick latencies were assessed 30 min and 24 h after the injection of 4 μg of NMDA. As can be seen in Fig. 2, the injection of NMDA increases the tail-flick latencies when measured 30 min later, but they returned to pretreatment levels when measured 24 h after injection in the same animals.

Effect of the NMDA Antagonist CPP on the Effects Induced by NMDA (4 μg) on BSL Behavior and the Tail-Flick Test

Intrathecal administration of the NMDA antagonist CPP (10–100 ng) induces a dose-dependent increase in tail-flick latencies 15 and 30 min after its administration (Fig. 3A). The effects produced by the coinjection of CPP (10–100 ng), together with NMDA (4 μg) in BSL behavior (from 0 to 15 min) and the tail-flick test (15 and 30 min after injection), were studied in the same group of animals. The BSL behavior evoked by 4 μg of NMDA was progressively inhibited by CPP, the inhibition reaching statistical significance when 100 ng of CPP were coadministered (Fig. 3C). In the tail-flick test, the coadministration of 30 ng of CPP prevented the NMDA-induced increase in the latencies 15 and 30 min after their administration, the latencies obtained in this group being similar to those of the control group. With lower (10 ng) or higher (100 ng) doses of CPP coadministered with NMDA, the tail-flick latencies were again significantly higher than controls (Fig. 3B).

Effect of the Opiate Antagonist Naloxone on the Effects Induced by NMDA (4 μg) on BSL Behavior and the Tail-Flick Test

Naloxone (3 and 10 mg/kg; IP) administered 15 min before spinal injection did not modify the basal tail-flick latencies or the analgesic effect induced by NMDA (4 μg/rat; IT) in this test (Fig. 4A). In the same way, the BSL behavior remained unaltered by these doses of naloxone (Fig. 4B).

Effects of the Calmodulin Inhibitor Calmidazolium on the Effects Induced by NMDA (4 μg) on BSL Behavior and the Tail-Flick Test

The intrathecal administration of calmidazolium (30–300 μg) increased tail-flick latencies 15 and 30 min after injection (Fig. 5A). When 100 or 300 μg of calmidazolium were coadministered with NMDA (4 μg), the BSL behavior was dose dependently reduced (Fig. 5C). In contrast, calmidazolium was unable to inhibit the effect of NMDA on the tail-flick latencies in these same groups of animals, and the latencies ob-

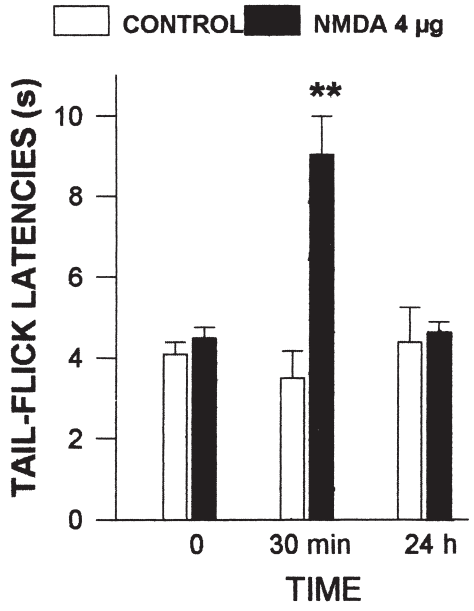

FIG. 2. Effects induced by a single injection of NMDA (4 μg) or solvent in the tail-flick test measured 30 min and 24 h later. The bars represent the means of the latencies obtained and their corresponding standard errors ($n = 6$). Comparisons are made at each time with the solvent group; $^{**}p < 0.01$, Student's t-test for unpaired data.

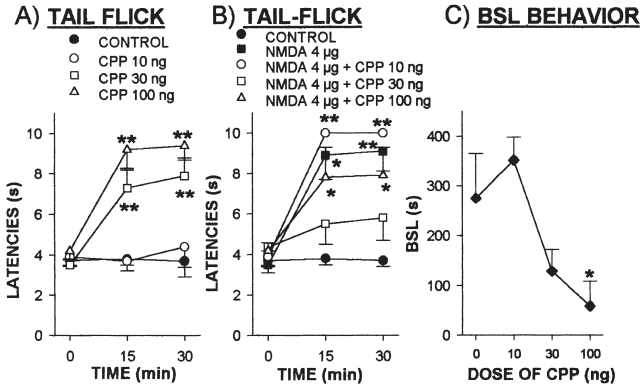

FIG. 3. (A) Effect of the intrathecal administration of CPP (10, 30, and 100 ng) or solvent measured 15 and 30 min after injection in the tail-flick test. The effects of the coadministration of these same doses of CPP with 4 μg of NMDA were studied in a single group of animals both in the tail-flick (B) and the BSL (C) tests ($n = 6$–8). In B, the mean and standard errors of the tail-flick latencies obtained 15 and 30 min after the administration of solvent, NMDA alone (4 μg) or NMDA (4 μg) coinjected with 10, 30, or 100 ng of CPP are represented. In C, the mean and standard error of the BSL observed in the same group of animals is shown. In the tail-flick test, the CPP-treated groups were compared with the solvent- or NMDA-treated group at each time. In the BSL test, the comparisons were made with the NMDA-treated group. In all cases, an ANOVA followed by a post hoc Newman–Keuls test were used. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with solvent-treated group; $^{+}p < 0.05$, compared with the NMDA-treated group.

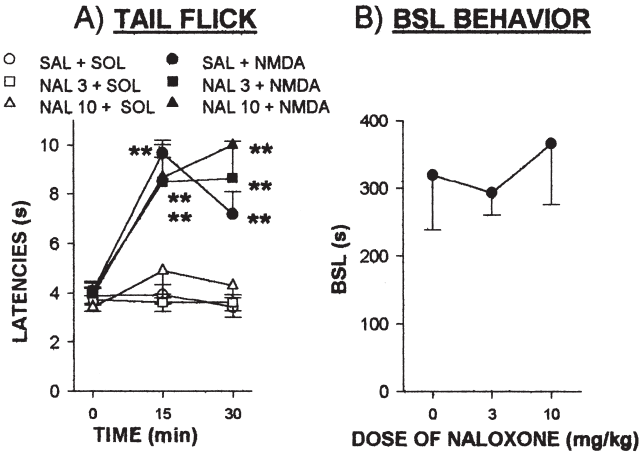

FIG. 4. Effect of the intraperitoneal administration of naloxone (3 and 10 mg/kg) or saline (SAL) 15 min before the administration of IT NMDA (4 μg) or solvent (SOL) studied in a single group of animals both in the tail-flick (A) and the BSL (B) tests (n = 6–8). In A, the mean and standard errors of the tail-flick latencies obtained 15 and 30 min after the intrathecal administration of solvent or NMDA (4 μg) are represented. In B, the mean and standard errors of the BSL behavior obtained in the same group of NMDA-treated animals is shown. In the tail-flick test, the naloxone-treated groups were compared with the solvent-treated group at each time. In the BSL test, the comparisons were made with the NMDA-treated group. In all cases, an ANOVA followed by a post hoc Newman–Keuls test were used. $^{**}p < 0.01$.

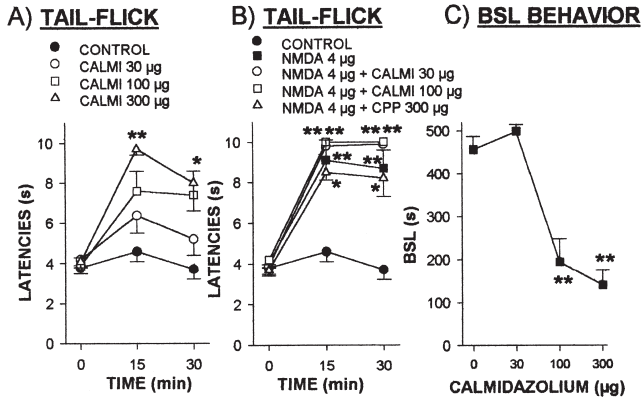

FIG. 5. (A) Effect of the intrathecal administration of calmidazolium (30, 100, and 300 μg) or solvent measured 15 and 30 min after injection in the tail-flick test. The effects of the coadministration of these same doses of calmidazolium with 4 μg of NMDA were studied in a single group of animals both in the tail-flick (B) and the BSL (C) tests (n = 6–8). In B, the mean and standard errors of the tail-flick latencies obtained 15 and 30 min after the administration of solvent, NMDA alone (4 μg) or NMDA (4 μg) coinjected with 30, 100, or 300 μg of calmidazolium are represented. In C, the mean and standard errors of the BSL observed in the same group of animals is shown. In the tail-flick test, the calmidazolium-treated groups were compared with the solvent-treated group at each time. In the BSL test, the comparisons were made with the NMDA-treated group. In all cases, an ANOVA followed by a post hoc Newman–Keuls test were used. $^{*}p < 0.05$; $^{**}p < 0.01$.

tained were in all cases significantly higher than those of the control group (Fig. 5B).

DISCUSSION

Our results show that the intrathecal injection of NMDA leads to an increase in the nociceptive threshold measured by the tail-flick test. This is a dose-dependent and long-lasting antinociceptive effect that appears after the initial aversive reaction of biting, scratching, and licking (BSL) produced by the drug. The NMDA-induced antinociceptive effect is produced by doses that also produce BSL, although the excitatory behavior is not necessarily followed by analgesia. In fact, as can be seen, the lowest dose of NMDA (1 μg/rat) induces BSL but does not increase the tail-flick latencies. The only previous report that simultaneously describes both a BSL behavior induced by IT NMDA and an analgesic effect measured in the tail-flick test (14) shows a short-term analgesia (no longer detected 8 min after injection) that is produced by a dose lower than that necessary to induce BSL. Our study shows that, in addition to the reported short-term analgesia, IT NMDA can induce a long-lasting analgesia (of at least 90 min) that can be seen once the excitatory BSL reaction disappears.

Because the neurotoxic potential of NMDA in neural cells is well established (5), we assessed the possibility that the inhibitory effect of the nociceptive reflex induced by NMDA could be due to a toxic irreversible action, as previously suggested (2). However, the increase in the nociceptive threshold described by us was no longer measured 24 h after NMDA administration, and thus, does not seem to be a toxic-like effect due to the loss of spinal nociceptive neurons.

To discard that this inhibitory effect on the nociception produced by one excitatory molecule (NMDA) could derivate from a nonspecific action, we have evaluated the involvement of NMDA receptors in the NMDA-induced analgesia by assaying the effect of the competitive antagonist, CPP (10–100 ng/rat). The coadministration of CPP with NMDA (4 μg), produced a decrease in the BSL behavior, supporting the involvement of NMDA receptor activation in this excitatory effect produced by NMDA. The study of the effect of CPP on the NMDA-induced analgesia in the tail-flick test was much more complex, because, in accordance with previous reports (9), intrathecal administration of CPP induces analgesia by itself in this test. On these circumstances in which the administration of either the agonist (NMDA) and the antagonist (CPP) of one particular receptor leads to the same effect (tail-flick analgesia), the most likely result of their combination could be a bell-shaped curve, in which the reversion of the effect might only be seen in a narrow dose range. In our experiment, the coadministration of either 10 or 100 ng/rat of CPP with NMDA (4 μg) produced similar effects to those obtained with NMDA alone, probably by reflecting two different situations. On one hand, 10 ng of CPP was a dose unable to modify BSL behavior, and thus, it could be considered too low to compete with NMDA binding. On the other, 100 ng of CPP was a dose that almost totally supressed BSL, suggesting that CPP binding prevails at NMDA receptors, being the probable responsible of the analgesia obtained in the tail-flick test. Only by the coadministration of NMDA with an intermediate dose of CPP (30 ng) that induces by itself a clear-cut, but not maximal analgesic effect, the increase in the tail-flick latencies was avoided. Thus, the fact that the result of combining two analgesic treatments (4 μg of NMDA and 30 ng CPP) is the disappearance of the analgesia suggests the competition of both drugs for the same target, namely the NMDA receptor.

Because a previous report described the involvement of the opiod system in one type of analgesic response induced by NMDA (14), we next tested the effect of the opioid antago-

nist naloxone. The IP injection of naloxone (3 and 10 mg/kg) did not modify either the BSL score or the analgesia induced by NMDA in the tail-flick test. This result discards that opioid mechanisms could be underlying these effects.

Finally, we tested the effect of the inhibitor of calmodulin, calmidazolium, to study whether calmodulin activation could be involved in the analgesia induced by NMDA. We have previously reported that calmodulin inhibitors can decrease the NMDA-induced BSL behavior (13), suggesting that calmodulin activation participates in this nociceptive reaction induced by NMDA. In addition we have also described that the calmodulin inhibitors, calmidazolium and W-7, produce analgesia by themselves in the tail-flick test (12). The results now obtained confirm that calmidazolium inhibited the BSL induced by NMDA, but, in contrast, the increase in the tail-flick latencies obtained by the administration of NMDA persisted, suggesting that this analgesic effect is not mediated through the activation of calmodulin. These data seem to indicate that the intracellular processes responsible for the excitatory (BSL) and inhibitory (tail-flick analgesia) effects produced by NMDA can be separately modulated and, thus, could be independent.

In conclusion, our results show that intense analgesia can be evoked by spinal administration of NMDA, being a reversible and receptor-mediated phenomenon that, does not involve opioid mechanism and, in contrast to the excitatory BSL behavior, is not modified by calmodulin inhibition. These data are in line with other previous reports suggesting that the consequences of spinal NMDA receptor activation might not be limited to the amplification of the nociceptive transmission, but that inhibitory mechanisms might also come into play. Taking into account the large body of evidence indicating that painful events can activate endogenous systems of analgesia (10), it could be thought that the activation of NMDA receptors could be one of the pharmacological systems involved in this nociception-induced analgesia. The functionality of such an endogenous mechanism mediated by NMDA in physiological conditions remains to be demonstrated. However, a recent report describes that the application of foot shocks produces an analgesic effect in rats that can be antagonized by the NMDA-receptor channel blocker MK-801 (15), thus suggesting that the NMDA receptor activation could be able to trigger the activation of endogenous systems of analgesia.

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