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Spinal nociceptin inhibits AMPA-induced nociceptive behavior and Fos expression in rat spinal cord

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Abstract

The effects of intrathecal nociceptin (NOCI) on the nociceptive behavior (biting, scratching and licking; BSL) and the spinal Fos expression induced by intrathecal administration of *N*-methyl-D-aspartate (NMDA, 4 μg/rat) or α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, 2 μg/rat) were studied. Coadministration of NOCI (3 and 10 nmol/rat) with NMDA did not modify the NMDA-induced BSL or Fos expression. In contrast, NOCI (0.1–3 nmol/rat) dose-dependently inhibited the BSL behavior induced by AMPA. Furthermore, coadministration of NOCI (3 and 10 nmol/rat) significantly reduced the AMPA-induced expression of Fos protein in the superficial layers of the spinal dorsal horn. In order to test whether classical or opioid receptor-like type 1 (ORL1) receptors are involved in the inhibitions by NOCI of AMPA-evoked BSL, the corresponding antagonists were assayed. The administration of the nonselective opioid receptor antagonist, naloxone (10 mg/kg ip), did not modify the NOCI-induced inhibition of AMPA-evoked BSL. However, the selective ORL1 receptor antagonist, [N-Phe^1]nociceptin-(1–13)-NH_2 (90 nmol/rat it), completely prevented the NOCI-mediated inhibition of the nociceptive responses evoked by AMPA. In conclusion, NOCI, acting at ORL1 receptors can, at least in part, induce spinal analgesia by blocking the nociceptive responses produced through the stimulation of AMPA receptors.

Keywords: Nociceptin; AMPA; Spinal cord; Fos; Rat; ORL1 receptors

1. Introduction

Nociceptin (NOCI, Orphanin FQ), a recently described "new" member of the opioid peptide family, is known for its hyperalgesic properties, although it can also induce analgesia when administered spinally, especially in rats (Inoue et al., 1999; Yamamoto et al., 1997a,b; Corradini et al., 2001). Some discrepancy exists with regard to the receptors mediating the analgesic action of NOCI since either opioid receptor-like type 1 receptors (ORL1, where NOCI binds at nanomolar concentrations) or classical opioid receptors could be involved. This is a very interesting issue since, if ORL1 receptors do participate in spinal analgesia, they could be envisaged as a new target for spinal analgesics.

Recently, we have shown that spinally administered NOCI might inhibit the nociceptive behavior (biting, scratching, and licking; BSL) induced by intrathecal administration of the tachykinin-NK_1 receptor agonist, septide, through activation of ORL1 receptors (Villanueva et al., 2002). In contrast, the behavioral reaction induced by intrathecal *N*-methyl-D-aspartate (NMDA) does not seem to be affected by NOCI, thus suggesting that this peptide modulates certain spinal nociceptive signals selectively (Villanueva et al., 2002).

In this work, we examined the ability of NOCI to modulate the nociceptive reactions triggered by the two principal agonists of the ligand-gated glutamate channels: NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA). We have extended the previous behavioral observations with NMDA by a parallel immunocytochemical study on Fos expression in the spinal cord and by comparing the results obtained with NMDA with those produced by intrathecal administration of AMPA. Thus, we describe here the modulation by NOCI of the behavioral syndrome seen upon intrathecal application of NMDA or AMPA, respectively. Simultaneously, the Fos expression in

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the external laminae of the spinal cord was also monitored. The involvement of the classical opioid and/or the ORL1 receptors in the behavioral effects induced by NOCI was also examined.

2. Material and methods

2.1. Animals

Male Wistar rats, weighing 250–350 g, from the Animalario of the Universidad de Oviedo (Reg. 33044 13A) were maintained, five to a cage, in a controlled environment (60% humidity, 20–22 °C) exposed to a light–dark cycle of 12 h and supplied with water and food (A-04, Panlab) ad libitum. For intrathecal injections (Menéndez et al., 1997), rats were lightly anesthetized with ether and a small cut was then made in the skin over the lumbar area and the needle tip of a hypodermic syringe (0.33×13 mm) was introduced between the L_4 and L_5 vertebrae. The observation of a flick of the tail when the dura is perforated by the needle confirms the accuracy of the intrathecal localization. All experimental protocols were approved by the Comisión de Ensayos Clínicos y Bioética del Principado de Asturias (Spain).

2.2. Drugs

NMDA and (*RS*)-AMPA (both from Tocris) dissolved in 25 μl of distilled water were administered intrathecally either alone or coinjected with NOCI (Tocris). Naloxone hydrochloride (Sigma) dissolved in saline was injected intraperitoneally in a volume of 10 ml/kg 15 min prior to the intrathecal treatment. The ORL1 receptor antagonist, [N-Phe^1]nociceptin-(1–13)-NH_2 (Neosystem), dissolved in 25 μl of distilled water was injected intrathecally, either alone or coinjected with AMPA and/or NOCI. In all cases, the control groups received the appropriate solvents intraperitoneally or intrathecally.

2.3. BSL behavior assays

As described in our previous report (Menéndez et al., 1997), immediately after the intrathecal administration of drugs, rats were placed into a transparent plastic cage and the time spent in BSL the hindquarters was measured. NMDA and AMPA were administered intrathecally either alone or coadministered with NOCI and BSL behavior was measured during three consecutive periods of 5 min. When the effect induced by NOCI was assayed in the presence of naloxone or [N-Phe^1]nociceptin-(1–13)-NH_2, the total time spent in BSL during the 15 min period was considered. The intrathecal administration of solvent, NOCI (10 nmol/rat) or [N-Phe^1]nociceptin-(1–13)-NH_2 (90 nmol/rat) did not produce any behavioral effect by themselves.

2.4. Tissue preparation and Fos immunocytochemistry

The basic procedure to perform these experiments was adapted from the protocol described in a previous report (Bester et al., 1997). Two hours after intrathecal administration of solvent, NMDA (4 μg/rat) or AMPA (2 μg/rat), alone or coadministered with NOCI (3 or 10 nmol/rat), rats were deeply anesthetized with pentobarbital (55 mg/kg ip) and, after an intracardiac injection of heparin (25 UI/rat) they were perfused transcardiacally with 100 ml of 0.04 M phosphate-buffered saline (PBS) followed by 200–250 ml of cold 4% paraformaldehyde in 0.15 M (pH 7.4) phosphate buffer. Lumbar spinal cords were removed, postfixed for 6–8 h in 4% paraformaldehyde, and then cryoprotected overnight (12 h at least) in phosphate buffer containing 30% sucrose and 0.02% sodium azide.

The following day, transverse serial frozen sections (50 μm) of the lumbar L_4–L_5 segments were cut and collected as free floating slices in a 50% mixture glycerol–PBS. The sections were kept at 4 °C for the following 2 h. Then, sections were washed in 0.15 M PBS (pH 7.4) for three consecutive periods of 10 min, and next incubated in phosphate-buffered 0.1% H_2O_2 for 30 min. After three 10-min consecutive periods in PBS, sections were maintained for 30 min in 0.1% bovine serum albumin/0.3% Triton X-100/0.15 M phosphate buffer (BSA–T–PB). All these procedures were performed under agitation at room temperature. Using BSA–T–PB as solvent, the free floating sections were incubated overnight at 4 °C with polyclonal primary antiserum generated in rabbits against the Fos protein (Ab-5, Oncogene) and diluted 1:8000. On the third day, sections were washed under agitation at room temperature for three consecutive 10-min periods in 0.15 M PBS and incubated for 30 min in a goat biotinylated anti-rabbit IgG (H+L) antibody (Vector Laboratories) at a 1:500 dilution. After washing thrice 10 min in 0.15 M PBS, slices were incubated at room temperature for 30 min under darkness with the commercially available kit of avidin–biotin–peroxidase complex (Elite, Vectastain ABC Kit, Vector Laboratories). After three new washes of 10 min in Tris buffer (0.15 M, pH 7.6), sections were kept for 5 min in a solution containing 0.05% of 3,3′-diaminobenzidine (DAB, Sigma) in Tris phosphate buffer (0.15 M, pH 7.6). Next, 0.006% of H_2O_2 was added and when the staining was adequate, the reaction was stopped by a further 10-min wash in Tris buffer (0.05 M, pH 7.6). As before, all procedures were performed at room temperature under agitation. Then the slices were mounted on gelatine-coated slides, dried, and after soaking into VectaMount medium (Vector Laboratories), they were coverslipped for optical microscopical observation.

Neurons containing Fos immunoreactivity (Fos-IR) were identified throughout the superficial laminae I and

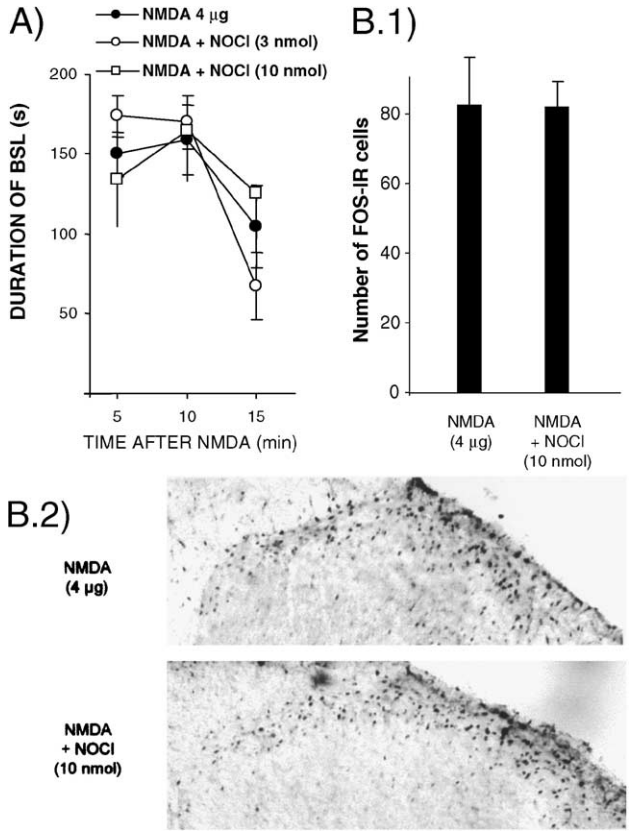

Fig. 1. (A) Lack of effect of the coadministration of NOCI (3–10 nmol/rat) on the nociceptive behavior (BSL) induced by NMDA (4 μg/rat i.t.). Means and S.E. ($n \geq 6$) are seen. (B) Fos-IR neurons seen in the superficial layers of the dorsal horn of rats treated with NMDA (4 μg/rat i.t.) alone or coinjected with NOCI (10 nmol/rat). The means and S.E. ($n \geq 6$) (B.1) and a representative example (× 10) of Fos-IR cells seen in each group (B.2) are shown.

II of the dorsal horn spinal segments corresponding to L_4–L_5 by a light microscope (objective × 10). From the sections of each animal, five were selected, those showing the most intense Fos-IR. Then, on each slide the total of Fos-IR nuclei was counted by two independent observers. The mean of these two measures (always showing an error less than 15%) was considered. In each experimental group, the spinal cords of at least six animals were included.

The intrathecal administration of solvent or NOCI (10 nmol) alone did not produce any significant Fos expression in the spinal cord.

2.5. Statistical analysis

For each group, the mean duration of nociceptive behavior and the average of Fos containing neurons were calculated. Intergroup comparisons were made by one-way analysis of variance (ANOVA) followed by the Dunnett's *t* test or the Newman–Keuls tests, when appropriate. The level of significance was set at $P < .05$.

3. Results

3.1. Lack of effect of NOCI on nociceptive behavior and spinal Fos expression induced by NMDA (both administered intrathecally)

As can be seen in Fig. 1A, the present results confirm that the coadministration of NOCI (3 and 10 nmol/rat) with NMDA (4 μg/rat) did not modify the nociceptive behavior (BSL) induced by the excitatory amino acid at any of the three 5-min time intervals studied (0–15 min after injection). In addition, the NMDA-induced intense expression of Fos protein in the superficial layers of the spinal cord (82.5 ± 13) was unaffected by coadministration of 10 nmol of NOCI (82.0 ± 7) (Figs. 1B.1 and B.2).

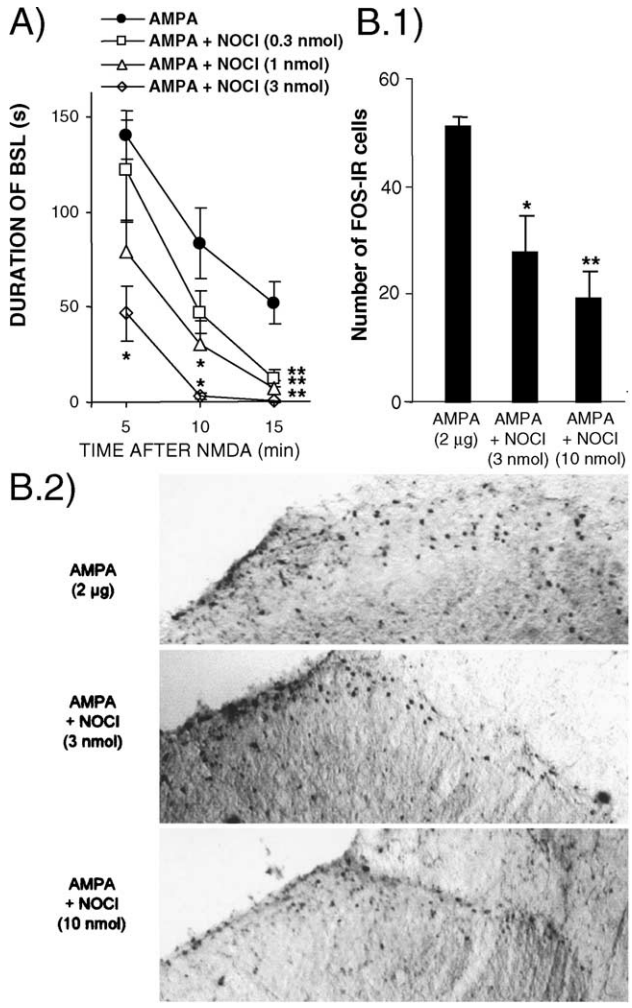

Fig. 2. (A) Inhibition of NOCI (0.3–3 nmol/rat) of the nociceptive reaction (BSL) produced by AMPA (2 μg/rat i.t.). Means and S.E. ($n \geq 6$) are shown. (B) Fos-IR neurons detected in the superficial layers of the dorsal horn of rats treated with AMPA (2 μg/rat i.t.) alone or coinjected with NOCI (3 and 10 nmol/rat). The means and S.E. ($n \geq 6$) (B.1) and a representative example (× 10) of Fos-IR cells obtained in each group (B.2) are shown. * $P < .05$, ** $P < .01$, Dunnett's *t* test.

3.2. Antagonism by NOCI of nociceptive behavior and spinal Fos expression induced by AMPA (both administered intrathecally)

Contrary to NMDA, the AMPA-induced pain behaviors were dose-dependently antagonized by NOCI (Fig. 2A). This inhibitory effect was already significant with the dose of 0.3 nmol during the last 5-min period studied (10–15 min). In the presence of higher doses, the inhibition appeared earlier. In fact, the coadministration of 3 nmol of NOCI produced a significant inhibition of BSL for the three periods studied.

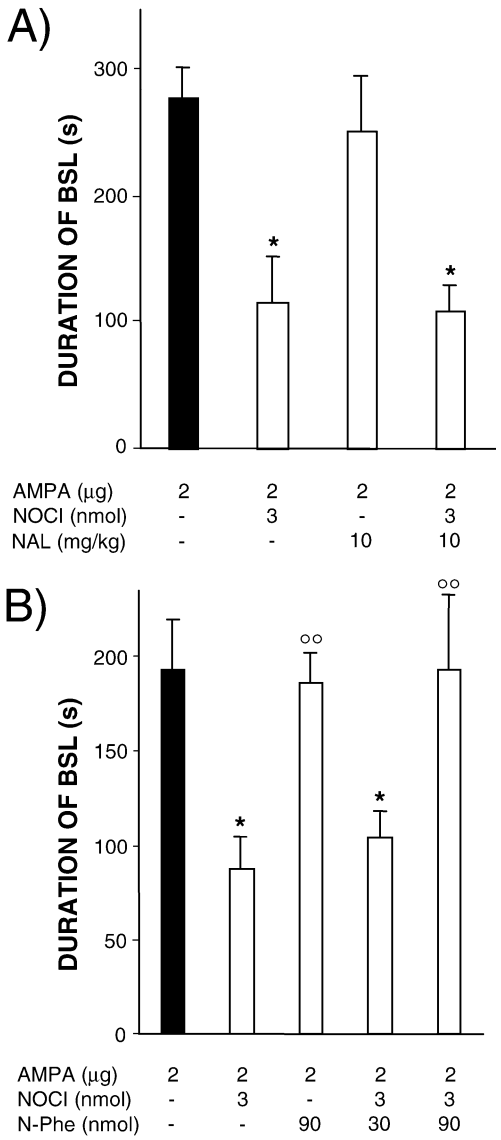

Fig. 3. (A) Lack of effect of naloxone (NAL, 10 mg/kg i.p.) on the inhibition by NOCI (3 nmol/rat) of the AMPA (2 μg/rat)-evoked nociceptive behavior (BSL). (B) Antagonism by [N-Phe^1]nociceptin-(1–13)-NH_2 (N-Phe, 30–90 nmol/rat) of the inhibition by NOCI (3 nmol/rat) of AMPA-evoked BSL. The means and S.E. ($n \geq 6$) are shown. * $P < .05$ as compared to AMPA-treated group, °° $P < .01$ as compared to AMPA- and NOCI-treated group, Newman–Keuls test.

The administration of 2 μg of AMPA also induced, as NMDA did, Fos protein expression in the superficial laminae of the spinal dorsal horn (51.1 ± 1.8). When 3 or 10 nmol of NOCI were coadministered with AMPA, the Fos expression was reduced significantly (Fig. 2B.1 and B.2).

3.3. Effects of the opioid antagonist, naloxone, and of the ORL1 receptor antagonist, [N-Phe^1]nociceptin-(1–13)-NH_2, on the inhibition induced by NOCI on the AMPA-evoked nociceptive behavior

A dose of 3 nmol of NOCI was selected for testing the type of receptors, classical opioid or ORL1, involved in the inhibitory effect that NOCI exerted on AMPA-induced BSL. As shown in Fig. 3A, prior administration of a high dose of naloxone (10 mg/kg i.p.) 15 min before the AMPA administration did not prevent the inhibition by NOCI of the AMPA-induced BSL. In contrast, when the ORL1 antagonist, [N-Phe^1]nociceptin-(1–13)-NH_2 (90 nmol/rat), was coadministered together with AMPA and NOCI, the inhibitory effect of NOCI on the AMPA-induced BSL completely disappeared (Fig. 3B).

4. Discussion

The aim of this set of experiments was to further characterize the analgesia induced by spinal application of NOCI. We examined its ability to modulate the pain responses induced by the agonists of excitatory amino acid ligand-gated channel receptors, AMPA and NMDA, respectively. NOCI can selectively inhibit the nociceptive behavior and the spinal Fos expression induced by the intrathecal injection of AMPA without affecting any of the effects induced by NMDA.

Our data indicate that NOCI modulates differentially the excitatory nociceptive signals triggered through the activation of AMPA or NMDA receptors, respectively. Firstly, the results obtained with NMDA in the BSL test confirm our previous results (Villanueva et al., 2002) indicating that spinal NOCI does not affect the NMDA-mediated BSL. This is also in accordance with the inability of NOCI to reduce spinal Fos expression induced by intrathecal injection of the same dose of NMDA, as shown here. However, both the behavioral and immunohystochemical events triggered by AMPA are significantly reduced by coadministration of NOCI.

To our knowledge, no previous report has described the induction of Fos expression by intrathecal administration of NMDA or AMPA, although the relationship between the activation of their receptors and spinal Fos expression is well established. Among others, the presence of NMDA receptors in spinal neurons showing Fos-IR has been demonstrated (Zhang et al., 1998). Pharmacologically, antagonists of both NMDA (Huang and Simpson, 1999) and AMPA (Kakizaki et al., 1996) receptors are able to

reduce spinal Fos expression after painful stimuli in rats. It is remarkable that the results obtained in behavioral studies fit with those found by measuring Fos expression. Namely, NOCI does not affect either the BSL or Fos expression induced by NMDA but does inhibit the similar actions of AMPA.

The inhibition by NOCI of AMPA-mediated responses is in accordance with previous reports describing that NOCI can inhibit the excitatory responses of dorsal horn medullary (Wang et al., 1999) or lumbar spinal (Ahmadi et al., 2001) neurons induced by AMPA. Related to the involvement of ORL1 or classical opioid receptors in the effect of NOCI on AMPA-evoked responses, the results obtained with selective antagonists seem to indicate that ORL1 receptors are involved in this effect. In fact, even though a high dose of naloxone did not modify the analgesic effect of NOCI, the intrathecal coadministration of the ORL1 peptidic antagonist, [N-Phe^1]nociceptin-(1–13)-NH_2 (90 nmol/rat), completely blocked the action of NOCI. This is in accordance with the lack of any inhibitory action by NOCI on AMPA-induced excitatory responses in the spinal cord of ORL1 receptor-deficient mice (Ahmadi et al., 2001). Thus, although the ORL1 receptor presents about 60% homology with the other opioid receptors, the classical opioid receptor antagonist, naloxone, binds it with very low affinity, so explaining why some effects of NOCI such as the spinal analgesia it induces in rats are naloxone-insensitive (Yamamoto et al., 1997a).

Based on our present data and those obtained earlier, we may conclude that in the spinal cord, postsynaptic AMPA and NK_1 receptors are involved in the analgesic action of NOCI, but the NMDA receptors are not. As for the NK_1 receptors, we reported earlier (Villanueva et al., 2002) that the excitatory responses elicited by their agonist, septide, were suppressed by NOCI via ORL1 receptors. Nevertheless, either modulation of the effects of other spinal neurotransmitters or presynaptic inhibition of neurotransmitter release (Meis and Pape, 2001; Liebel et al., 1997), could also contribute to NOCI-induced spinal analgesia.

Acknowledgements

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