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Effects of intraplantar Nocistatin and (±)-J 113397 injections on nociceptive behavior in a rat model of inflammation

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ABSTRACT

Nocistatin (NST) and Nociceptin/Orphanin FQ (N/OFQ) are derived from the same precursor protein, pre-proN/OFQ, and exert opposite effects on the modulation of pain signals. However, the role of the peripheral N/OFQ and the NOP receptor, which is located at the endings of sensory nerves, in inflammatory pain was not ascertained. NST administered intrathecally (i.t.) prevented the nociceptive effects induced by i.t. N/OFQ and PGE_2. Moreover an up regulation of N/OFQ was shown in the rat in response to peripheral inflammation. Here, we investigated the effects of intraplantar (i.pl.) administration of functional N/OFQ and NOP receptor antagonists in a rat model of inflammatory pain. Our findings showed that i.pl. injection of (±)-J 113397, a selective antagonist of the NOP receptor, and NST, the functional N/OFQ antagonist, prior to carrageenan significantly reduced the paw allodynic and thermal hyperalgesic threshold induced by the inflammatory agent. The resulting antiallodynic and antihyperalgesic effects by co-administering NST and (±)-J 113397 prior to carrageenan were markedly enhanced, and the basal latencies were restored. Thus, it is likely that the peripheral N/OFQ/NOP receptor system contributes to the abnormal pain sensitivity in an inflammatory state.

1. Introduction

Nocistatin (NST) (Okuda-Ashitaka et al., 1998) and Nociceptin/Orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995), two neuropeptides derived from the same precursor pre-proN/OFQ, are both involved in pain transmission (Civelli, 2008).

N/OFQ, the endogenous ligand of the NOP receptor, was first identified as a pronociceptive peptide in the brain (Meunier et al., 1995), but successive studies have generated conflicting results depending on the dose and the site of administration (Mogil and Pasternak, 2001; Nakano et al., 2000). Supraspinal injection of the peptide has been shown to produce hyperalgesia and allodynia; in contrast, when administered intrathecally (i.t.), N/OFQ led to analgesia (Rizzi et al., 2006; Hu et al., 2010) and, surprisingly, in some circumstances, allodynia (Mogil and Pasternak, 2001). The biologically active 17-amino acid peptide NST, prevented the effects induced by i.t. N/OFQ and PGE_2 when administered i.t. (Okuda-Ashitaka and Ito, 2000). Supraspinal NST alone did not induce analgesia or hyperalgesia and had no effect on opioid-induced analgesia, but it was shown to reverse the nociceptive effect of N/OFQ (Scoto et al., 2005) and also prevent the antagonistic effect of N/OFQ against opioid-induced analgesia (Zhao et al., 1999; Scoto et al., 2005). The effects of NST, however, are independent from the binding to the NOP receptor (Okuda-Ashitaka and Ito, 2000), so it has been considered to be a functional N/OFQ antagonist (Okuda-Ashitaka et al., 1998).

The NOP receptor, in addition to its wide distribution throughout the CNS in regions involved in pain transmission, has been clearly identified in several isolated organs and in the peripheral nervous system (Mollereau and Mouledous, 2000). In particular, the NOP receptor seems to be located at the endings of sensory nerves (Bigoni et al., 1999).

Data in the literature regarding the effects of N/OFQ at the periphery are controversial. N/OFQ can attenuate the licking/biting behavior induced by capsaicin in mice when injected into the plantar surface of the hind paw, suggesting a local peripheral antinociceptive action mediated by the NOP receptor (Ko et al., 2002; Sakurada et al., 2005). On the contrary, Inoue et al. (1998, 1999) reported that intraplantar (i.pl.) N/OFQ elicited biphasic effects depending on the doses administered: nociception at low doses and antinociception at higher doses. In acutely inflamed knee joints, N/OFQ acts on NOP receptors located on synovial mast cells and leukocytes, leading to the secondary release of proinflammatory mediators into the joint (Zhang and McDougall, 2006). A recent report (Lambert, 2008) found an up regulation of N/OFQ in the rat response to peripheral inflammation with bacterial lipopolysaccharide and a modulation of immune function in response to staphylococcal enterotoxin.

There is considerable interest in the neurochemical mechanisms that underlie the pathological conditions of hyperalgesia and allodynia in inflammatory and neuropathic pain, and the N/OFQ and NST peptides are undoubtedly involved in the changes that occur during

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inflammatory pain (Mika et al., 2011; Scoto et al., 2009). Injection of carrageenan into the hind paw (Rosén et al., 2000) or ligation of the sciatic nerve (Gabriel et al., 2004) in rat led to an augmented N/OFQ level in the spinal cord critical for the abnormal pain sensitivity in these conditions. In a previous study (Scoto et al., 2009), we showed that intra-ventrolateral periaqueductal gray (PAG) injection of UFP-101, a selective NOP antagonist, reversed the decreased allodynic threshold of rats with chronic constriction injury or inflammation induced by carrageenan, suggesting that the N/OFQ/NOP receptor system in the PAG plays a role in allodynia. Supraspinal NST also has an anti-hyperalgesic effect on the inflammatory hyperalgesia induced by carrageenan/kaolin (Nakagawa et al., 1999).

In light of these data and to investigate the possible role of the peripheral N/OFQ/NOP receptor system in a inflammatory pain condition, we administered the non-peptidergic NOP receptor antagonist (±)-J 113397 (Ozaki et al., 2000) and the N/OFQ functional antagonist NST into the hind paw of rats treated with carrageenan and assessed the mechanical allodynia and the thermal hyperalgesia produced in this model of inflammation.

2. Materials and methods

2.1. Animals

Experiments were conducted on male Sprague–Dawley rats (Harlan, San Pietro al Natisone (UD), Italy) weighing 180–200 g. The animals were kept in cages at a constant room temperature (25 ± 1 °C) under a 12:12 h light and dark cycle with free access to food and water. Each rat was used for only one experiment. On three consecutive days prior to behavioral testing, rats were regularly handled and gradually habituated to the testing equipment. All tests were carried out in a quiet, isolated room to minimize animal anxiety and were performed between 09:00 and 15:00. The behavioral tests were conducted by researchers blinded to the treatment group. Experimental procedures were approved by the local ethical committee and the Institutional Animal Care and Use Committee (IACUC), and all experiments were conducted in accordance with International Guidelines and the European Communities Council Directive and National Regulations (EEC Council 86/609 and DL 116/92).

2.2. Carrageenan model of inflammatory pain

Carrageenan was suspended in sterile isotonic 0.9% saline to a 2% solution and sonicated prior to injection (Scoto et al., 2009). A volume of 100 μl was injected i.pl. into the left hind paw, approximately halfway between the toes and heel just proximal to the interdigital pads.

2.3. Behavioral testing

2.3.1. Mechanical allodynia

The assessment of tactile allodynia consisted of measuring the withdrawal threshold of the hind paw in response to probing with a series of calibrated von Frey's filaments (Scoto et al., 2009). The rat was placed in a clear plastic testing chamber with a wire mesh bottom and allowed to acclimatize for 20 min. The ventral surface of the hind paw was mechanically stimulated from below with an ascending series of graded von Frey's filaments with bending forces ranging from 0.02 to 30 g. The withdrawal threshold was determined by the "up-down" method of sequentially increasing and decreasing the stimulus strength (Dixon, 1980) and was expressed as the mean withdrawal threshold.

Paw withdrawal thresholds were measured every hour for 6 h after i.pl. carrageenan injection. Withdrawal thresholds were also expressed as the percent change from the basal level, and the results were reported as the mean area under the curve (MAUC) (Prezzavento et al., 2008) over a 6 h testing session.

2.3.2. Thermal hyperalgesia

Thermal hyperalgesia was quantified using the method described by Hargreaves et al. (1988). Briefly, rats were placed in a plexiglass box (17×23×14 cm) on a glass surface of the apparatus (Plantar test, Ugo Basile, Italy), and a beam of radiant heat was applied through the glass to the plantar surface of the left hind paw. Rats were allowed to habituate to the apparatus until exploratory behavior was no longer observed. The basal pre-drug latency was established between 8 and 10 s and was calculated as the average of two measurements performed at 5 min intervals with a cut-off latency of 20 s to avoid tissue damage. After baseline testing, the rat received an i.pl. injection of carrageenan, and withdrawal latencies were determined every hour for 6 h. The results were also expressed as the percent change from basal level and were reported as MAUC (Prezzavento et al., 2008) over a 6 h testing session.

2.4. Experimental procedure

Animals were randomly assigned to one of 10 groups with 8–10 animals per group. Mechanical allodynia and thermal hyperalgesia thresholds were assessed in different animal groups, as follows:

Groups 1 and 2: i.pl. injection of 0.9% sterile saline (100 μl/rat) as controls.
Group 3 and 4: i.pl. injection of 2% carrageenan (100 μl/rat) to assess the time course.
Group 5 and 6: i.pl. injection of (±)-J 113397 (1 nmol/100 μl/rat) before i.pl. carrageenan.
Group 7 and 8: i.pl. injection of NST (1 nmol/100 μl/rat) before i.pl. carrageenan.
Group 9 and 10: i.pl. injection of (±)-J 113397 (1 nmol/100 μl/rat) plus NST (1 nmol/100 μl/rat) before i.pl. carrageenan.

After the completion of the experiment, the animals were sacrificed under deep anesthesia.

2.5. Drugs

(±)-J 113397 and Nocistatin (NST) were purchased from Tocris (Bristol, UK); carrageenan was supplied by Sigma Aldrich as a mixture of κ and λ carrageenan. All drugs were dissolved in 0.9% sterile saline.

2.6. Statistical analysis

The data are expressed as the mean ± S.E. Intergroup comparisons were assessed using an initial two-way analysis of variance (ANOVA) followed by Duncan's multiple range post-hoc test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Mechanical allodynia

On the test day, all animals had a baseline paw mechanical response of approximately 13.0 ± 0.7 as determined using von Frey's filaments. Carrageenan injected i.pl. (2% in saline, 100 μl/rat) into the left hind paw induced a significant decrease in mechanical thresholds (Fig. 1, panels A, B and C). This allodynic response became significant 2 h after injection (2.5 ± 1.8 g vs. 13.5 ± 0.8 g for saline), reached its maximum at 3 h (0.8 ± 0.2 g vs. 13.3 ± 0.6 g) and lasted several hours. The injection of (±)-J 113397, a selective antagonist of the NOP receptor (1 nmol/100 μl/rat), given i.pl. immediately prior to carrageenan, significantly increased ($P<0.05$) the paw allodynic threshold values induced by the inflammatory agent (6.44 ± 1.4 g, 5.66 ± 1.5 g, 7.7 ± 2.1 g and 6.7 ± 1.5 g vs. 2.5 ± 1.8 g, 0.8 ± 0.2 g and 1.5 ± 0.7 g and 3.0 ± 1.4 g at 2, 3, 4 and 5 h after carrageenan i.pl. injection, respectively) (Fig. 1,

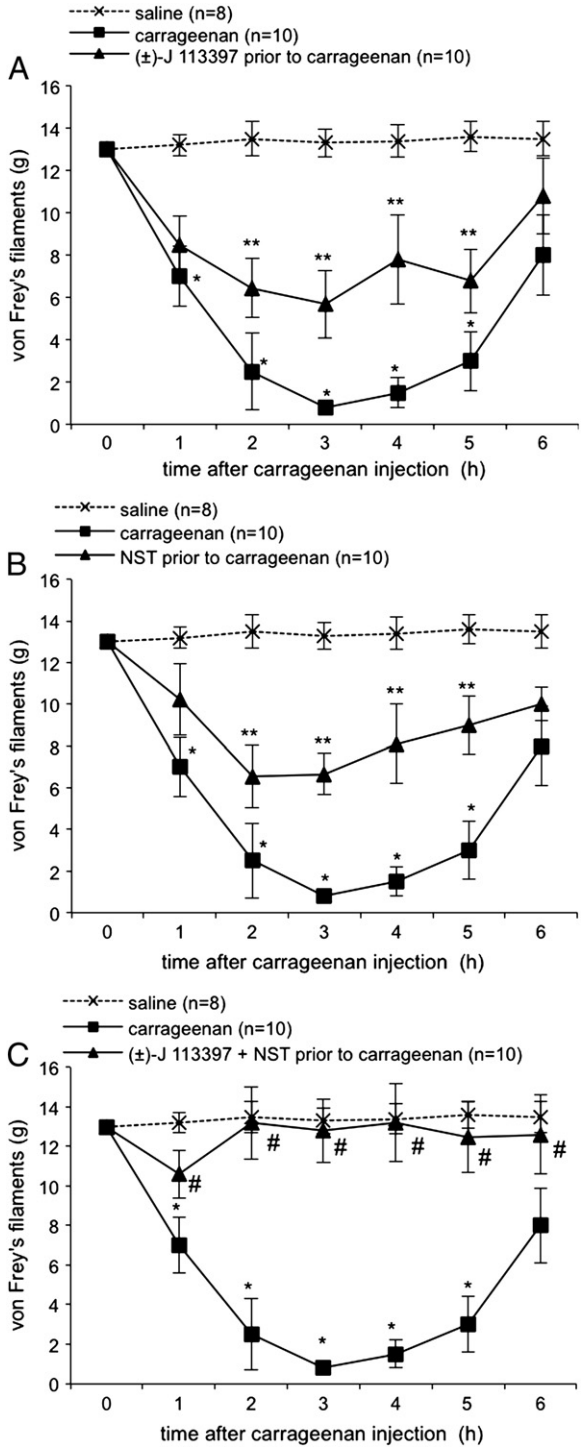

Fig. 1. Effect of (±)-J 113397 and NST on carrageenan-induced allodynia.Time course of the effects of i.pl. (±)-J 113397 (1 nmol/100 μl/rat) (panel A), NST (1 nmol/100 μl/rat) (panel B) and (±)-J 113397 (1 nmol/100 μl/rat) plus NST (1 nmol/100 μl/rat) (panel C) when injected prior to i.pl. carrageenan (2%/100 μl/rat) on mechanical allodynia measured with von Frey's filaments. The results are expressed in grams (g). The data are means ± S.E. from 8 to 10 rats. $^{*}P<0.05$ vs. i.pl. saline-treated rats; $^{**}P<0.05$ vs. carrageenan-treated rats; $^{\#}P<0.05$ vs. carrageenan-treated rats.

panel A). The dose of (±)-J 113397 (1 nmol/100 μl/rat) was chosen after the evaluation of the dose response curve. Its antiallodynic effect was lost at 0.25 nmol.

In addition, i.pl. injection of NST, the functional N/OFQ antagonist, given under the same experimental protocol, caused a significant increase of the carrageenan-induced mechanical allodynic threshold, up to 6.55 ± 1.5 g, 6.6 ± 1.0 g, 8.11 ± 1.9 g and 9.0 ± 1.4 at 2, 3, 4 and 5 h after carrageenan, respectively (Fig. 1, panel B); these results were significant ($P<0.05$) when compared to values obtained after carrageenan injection at the same time points (2.5 ± 1.8 g, 0.8 ± 0.2 g, 1.5 ± 0.7 g and 3.0 ± 1.4 g). The dose of NST (1 nmol/100 μl/rat) was chosen after the evaluation of the dose response curve. Its antiallodynic effect was lost at 0.5 nmol.

The effects of NST and (±)-J 113397 co-administration prior to the inflammatory agent were also evaluated (Fig. 1, panel C). The resulting antiallodynic effect was robustly enhanced ($P<0.02$), and this double treatment allowed a restoration of the control thresholds (13.2 ± 1.8 g, 12.8 ± 1.6 g, 13.2 ± 1.9 g, 12.5 ± 1.8 g and 12.6 ± 2, recorded starting at 2 h and over the entire observation period). The result was well observed also in Fig. 3, panel A.

3.2. Thermal hyperalgesia

On the test day, all the animals had baseline paw withdrawal latencies (PWL) of approximately 9.0 ± 0.5 s as determined by the Plantar test. Injection of i.pl. carrageenan induced the development of heat hyperalgesia, which was significant at 2 h after injection (3.3 ± 1.0 s vs. 8.0 ± 0.1 s after saline injection), reached its peak at 3 h (3.1 ± 1.1 s vs. 8.1 ± 0.14 s) and remained stable until 6 h (4.5 ± 1.12 s vs. 8.8 ± 0.18 s) (Fig. 2, panel A, B and C).

The i.pl. injection of (±) J 113397 prior to carrageenan induced a significant increase of PWL. This antihyperalgesic effect was significant ($P<0.05$) from 2 h until 6 h of treatment when compared to the values registered in the carrageenan injected paw (9.7 ± 2.0 s, 7.6 ± 1.9 s, 8.0 ± 2.2 s, 7.4 ± 2.0 and 8.0 ± 1.8 s vs. 3.3 ± 1.0 s, 3.1 ± 1.1 s, 2.5 ± 1.2 s, 3.0 ± 1.0 s and 4.5 ± 1.1 s) (Fig. 2, panel A). The dose of (±)-J 113397 (1 nmol/100 μl/rat) was chosen after the evaluation of the dose response curve. Its antihyperalgesic effect was lost at 0.25 nmol.

NST, a N/OFQ functional antagonist, showed a significant antihyperalgesic effect ($P<0.05$) 2 h after carrageenan when compared to that observed in the rat paw injected with carrageenan (9.04 ± 1.9 s vs. 3.3 ± 1 s) (Fig. 2, panel B). The dose of NST (1 nmol/100 μl/rat) was chosen after the evaluation of the dose response curve. Its antihyperalgesic effect was lost at 0.5 nmol.

Co-administration of NST and (±)-J 113397 prior to carrageenan restored the PWL values close to control over the entire period of observation (8.8 ± 1.8 s, 8.6 ± 2 s, 8 ± 2.3 s, 8.5 ± 2.3 s, 8.9 ± 2 s and 9.1 ± 1.9 s) (Fig. 2, panel C). This antihyperalgesic effect induced by the co-administration of the two antagonists is evident from the MAUC values (Fig. 3 panel B).

4. Discussion

The results presented here show that either i.pl. administration of NST, a N/OFQ functional antagonist, or i.pl. injection of (±)-J 113397, a competitive and selective antagonist of the NOP receptor, significantly reduced the induction of mechanical allodynia and thermal hyperalgesia following subcutaneous injection of the inflammatory agent carrageenan. Furthermore, this study shows how the co-administration of functional and receptor antagonists cooperates to abolish nociceptive symptoms.

A role for the NOP receptor in sensory transmission, especially nociceptive, is supported by its expression in spinal dorsal and ventral horns and dorsal root ganglion (DRG) (Mollereau and Mouledous, 2000; Mogil and Pasternak, 2001). The NOP receptor is synthesized in the DRG; thus, the bipolar fibers may express functional receptors not only at the central but also at the peripheral terminals (Wick et al., 1994). Moreover, mRNA expression of pre-proN/OFQ and the NOP receptor has been found in peripheral tissues, and this system has been implicated in the modulation of inflammatory pain and immunity (Grandi et al., 2011) as a component of the neuroimmune axis (Lambert, 2008).

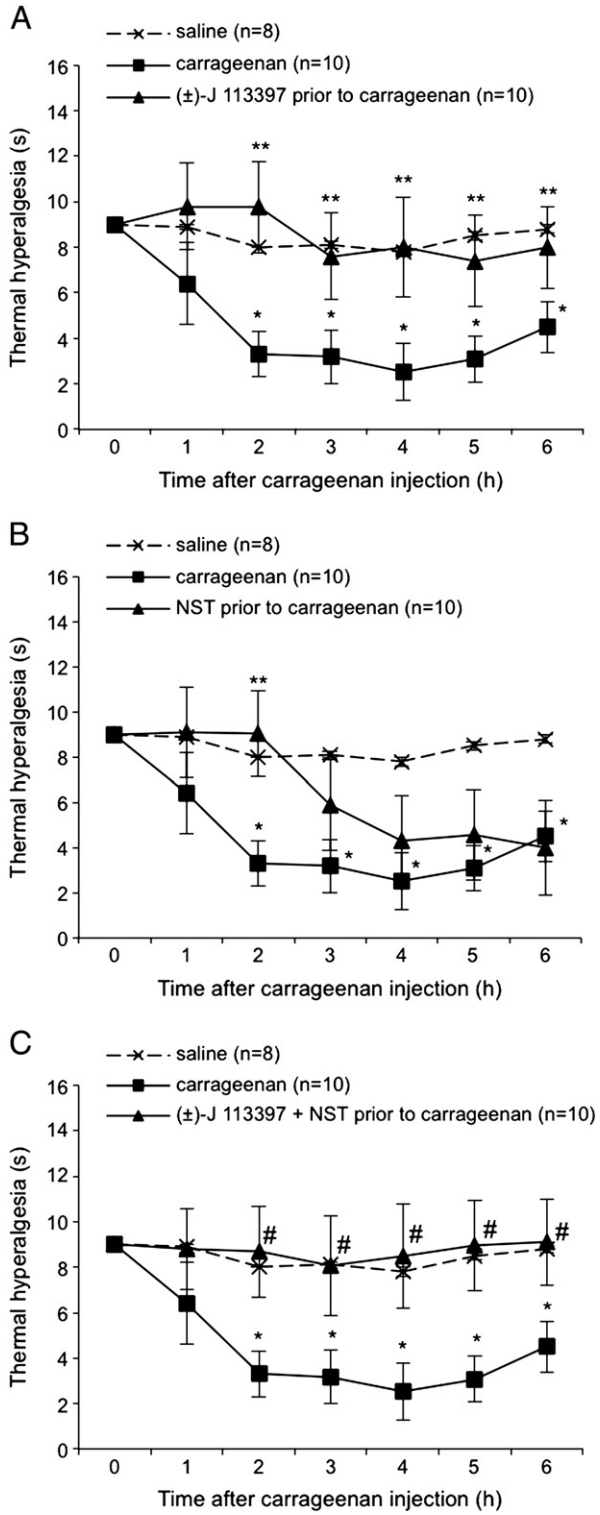

Fig. 2. Effect of (±)-J 113397 and NST on carrageenan-induced hyperalgesia.Time course of the effect of i.pl. (±)-J 113397 (1 nmol/100 μl/rat) (panel A), NST (1 nmol/100 μl/rat) (panel B) and (±)-J 113397 (1 nmol/100 μl/rat) plus NST (1 nmol/100 μl/rat) (panel C) when injected prior to i.pl. carrageenan (2%/100 μl/rat) on thermal hyperalgesia measured with the Plantar test. The results are expressed in seconds (s). The data are means ± S.E. from 8 to 10 rats. $^{*}P<0.05$ vs. i.pl. saline-treated rats; $^{**}P<0.05$ vs. carrageenan-treated rats; $^{\#}P<0.05$ vs. carrageenan-treated rats.

Inoue et al. (1998, 1999) demonstrated that N/OFQ elicited a nociceptive flexor reflex at remarkably low doses after i.pl. injection into the hind paw of mice. The authors correlated this effect of N/OFQ to the local release of Substance P because it can be blocked by capsaicin and by NK1 antagonists and in tachykinin-knockout mice. On the contrary, an higher dose of N/OFQ, i.pl. injected (Inoue et al., 1999) or subcutaneously applied to the tail (Kolesnikov and Pasternak, 1999) was analgesic. In acutely inflamed knee joints, N/OFQ acts on NOP receptors located on synovial mast cells and leukocytes leading to the secondary release of proinflammatory mediators into the joint (Zhang and McDougall, 2006). Peripheral administration of N/OFQ produced a dose-dependent excitation of dorsal horn neurons and a degree of sensitization to mechanical stimuli, which was unchanged after inflammation (Carpenter et al., 2000). These conflicting data reiterate the complexity of N/OFQ involvement in controlling inflammation and nociception (Zhang and McDougall, 2006).

During an inflammatory event, pain onset is the consequence of a complex interaction between a number of inflammatory mediators, including prostaglandins, some of which are known to play a critical role in the generation and maintenance of the nociceptive response (Samad et al., 2002; Kassuya et al., 2007). In particular, peripherally injected PGE_2 produces hyperalgesia and allodynia, effects related to the ability of prostaglandin to sensitize peripheral, small-diameter terminals to thermal, chemical and mechanical stimuli (Kassuya et al., 2007). The effect of PGE_2 did not occur in mice lacking the N/OFQ propeptide (Okuda-Ashitaka et al., 2006). Peripheral blood neutrophils also express a functional NOP receptor, and it was been demonstrated that these inflammatory cells are a novel source of the peptide (Fiset et al., 2003). Moreover, N/OFQ induces histamine release in mast cell preparations (Kimura et al., 2000) that in turn potentiates the inflammatory response in the skin and the pain stimuli. Indeed, intradermal injection of N/OFQ dose-dependently increased vascular permeability in the skin with a potency almost similar to other inflammatory mediators, such as Substance P and bradykinin (Kimura et al., 2000).

Our results are in accordance with previous reports in which i.t. administration of NST attenuated pain evoked by PGE_2 injection (Okuda-Ashitaka et al., 1998) and prevented allodynia induced by i.t. N/OFQ injection. Moreover, NST reversed the effect of i.t. N/OFQ on thermal hyperalgesia induced by inflammatory pain conditions in rats (Ma et al., 2003). The spinal injection of NST diminished the flinching behavior in phase 1, but not phase 2, of the formalin test in rats (Yamamoto and Sakashita, 1999), while supraspinal injection of the peptide has an antihyperalgesic effect on the inflammatory hyperalgesia induced by carrageenan/kaolin (Nakagawa et al., 1999) and reversed the nociceptive effect of N/OFQ and its antagonistic effect against analgesia caused by the selective opioid agonists (Scoto et al., 2005). NST, however, does not displace [3H]N/OFQ-binding (Nicol et al., 1998) or attenuate N/OFQ inhibition of forskolin-induced cAMP accumulation in cells transfected with the NOP receptor (Okuda-Ashitaka et al., 1998) and neither mimics nor blocks NOP receptor-mediated Ca^{2+} current inhibition (Connor et al, 1999). However NST either failed to affect the N/OFQ-induced actions or even behaves as an agonist (Ishihara et al., 2002; Xu et al., 1999; Zádori et al., 2008). Therefore, NST behaves as a "functional antagonist" of N/OFQ, interacting with neurons and/or cells that also respond to N/OFQ via different receptors. The relationship between NST and N/OFQ, different products of the same gene that modulate nociceptive behavior in opposite ways (Zeilhofer et al., 2000; Gavioli et al., 2002), is particularly attractive and seems to be similar to that of the two peptides obestatin and ghrelin, which are also processed from the same precursor. In fact, ghrelin, a circulating appetite-inducing hormone, stimulates food intake and gastric emptying activity, whereas obestatin antagonizes the effects of ghrelin by acting via a different receptor (Liu et al., 2006).

The role of the NOP receptor at peripheral sites in the carrageenan model of inflammation was confirmed by the antinociceptive effects of i.pl. injection of the potent and selective non-peptidic NOP receptor antagonist, (±)-J 113397. Its antagonistic and selective properties on the NOP receptor have been ascertained in recent years in a variety of pharmacological assays and with different techniques (Ozaki et al., 2000; Kawamoto et al., 1999; Chiou et al., 2007; Parenti and Scoto,

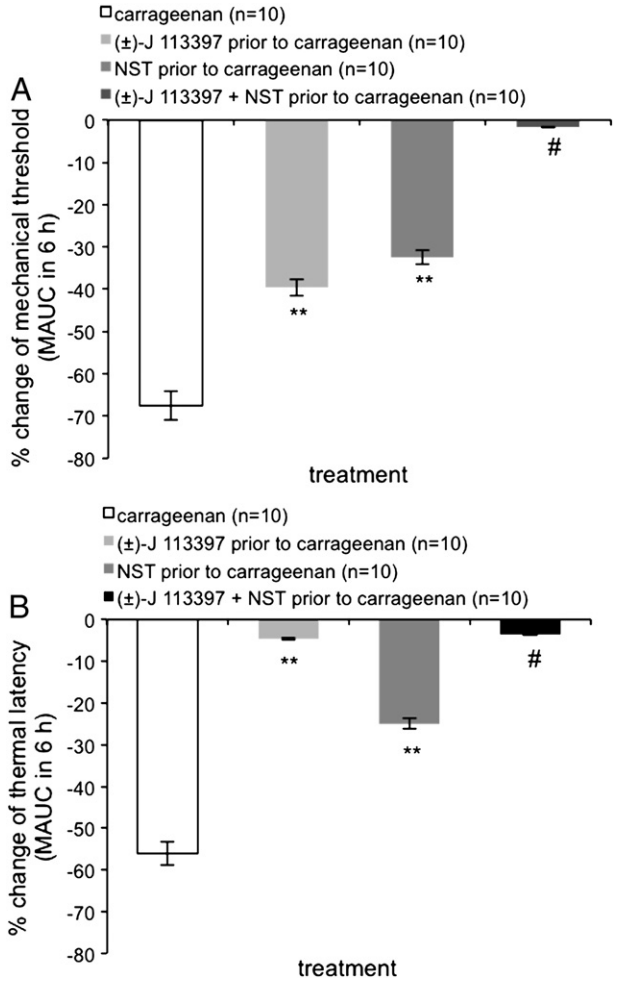

Fig. 3. Effect of (±)-J 113397 and NST on carrageenan-induced allodynia and hyperalgesia expressed as MAUC.Percent change of mechanical allodynia (panel A) and thermal hyperalgesia (panel B) after i.pl. injection of (±)-J 113397 (1 nmol/100 μl/rat), NST (1 nmol/100 μl/rat) and (±)-J 113397 (1 nmol/100 μl/rat) plus NST (1 nmol/100 μl/rat) prior to i.pl. carrageenan (2%/100 μl/rat), measured, respectively, with von Frey's filaments and the Plantar test. The results are expressed as the mean area under the curve (MAUC) after the last injection over the 6 h testing period. Columns represent the means ± S.E. from 8 to 10 rats. $^{**}P<0.05$ vs. carrageenan-treated rats; $^{\#}P<0.05$ vs. carrageenan-treated rats.

2010). Previously, it was observed that intra-ventrolateral PAG UFP-101, a competitive and selective peptidic antagonist of the NOP receptor, prevented tactile allodynia in two animal models of chronic pain, neuropathic and inflammatory, but did not change the basal nociceptive threshold (Scoto et al., 2009).

The present findings support the hypothesis that NOP receptor signaling could be involved at a peripheral level in pain sensitivity during inflammation induced by injection of carrageenan. Thus, it is possible to speculate that the effect of functional and receptor NOP antagonism may contribute to suppress the peripheral role of the endogenous N/OFQ-NOP receptor system in inflammatory pain, suggesting that N/OFQ could be one of the transmitters involved in nociceptive behavior induced by an inflammatory injury.

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