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Endogenous nociceptin/orphanin FQ signalling produces opposite spinal antinociceptive and supraspinal pronociceptive effects in the mouse formalin test: Pharmacological and genetic evidences

Anna Rizzi [a,*], Cristiano Nazzaro [a], G. Giuliano Marzola [a], Silvia Zucchini [a], Claudio Trapella [b], Remo Guerrini [b], Hanns Ulrich Zeilhofer [c], Domenico Regoli [a], Girolamo Calo' [a]

[a] *Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience Center, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy*

[b] *Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy*

[c] *Institut für Pharmakologie und Toxikologie, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland*

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Abstract

Nociceptin/orphanin FQ (N/OFQ) has been demonstrated to modulate nociceptive transmission via selective activation of N/OFQ peptide (NOP) receptors. Despite huge research efforts, the role(s) of the endogenous N/OFQ–NOP receptor system in pain processing remains incompletely understood. In the present study, we investigated the role of endogenous N/OFQ in the processing of tonic nociceptive input. To address this issue the effects of NOP-selective antagonists [Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101) and J-113397 on nociceptive behaviour, and the nociceptive phenotype of NOP receptor-deficient mice were tested in the mouse formalin test. Twenty microliters of 1.5% formalin solution was injected subcutaneously into the right hind paw causing a characteristic pattern of nociceptive behaviours (licking, biting and lifting of the injected paw). In control mice, the injection of formalin resulted in a classical biphasic nociceptive response with the first phase lasting from 0 to 10 min and the second phase from 15 to 45 min. UFP-101 at 10 nmol/mouse (but not at 1 nmol/mouse) produced antinociceptive action when injected intracerebroventricularly and a pronociceptive action when given intrathecally. Systemic administration of J-113397 (10 mg/kg, intravenously) and the genetic ablation of the NOP receptor gene both produced a significant increase of mouse nociceptive behaviour. Collectively, these results demonstrate that endogenous N/OFQ–NOP receptor signalling is activated during the mouse formalin test producing spinal antinociceptive and supraspinal pronociceptive effects. The overall effect of blocking NOP receptor signalling, by either systemic pharmacological antagonism or genetic ablation, indicates that the spinal antinociceptive action prevails over supraspinal pronociceptive effects.

Keywords: Nociceptin/orphanin FQ; NOP receptors; J-113397; UFP-101; Knockout mice; Formalin test

1. Introduction

The neuropeptide nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995) selectively binds and activates a G-protein coupled receptor named N/OFQ peptide (NOP) receptor (Cox et al., 2000). This peptide/receptor system is considered "a non-opioid branch of the opioid family" of peptides and receptors (Cox et al., 2000); this suggestion is based on the close structural and transductional similarities that contrast with the pharmacological and functional differences between the N/OFQ–NOP receptor and classical opioid

* Corresponding author. Tel.: +39 0532 291 221; fax: +39 0532 291 205.

E-mail address: a.rizzi@unife.it (A. Rizzi).

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systems (Calo et al., 2000b; Mogil and Pasternak, 2001). In particular, the role of the N/OFQ–NOP receptor system in the control of pain transmission appears to be quite different from that of opioids. In fact, most of the available data suggest that N/OFQ signalling has opposite actions at supraspinal versus spinal levels being pronociceptive and/or anti-analgesic in the brain and antinociceptive in the spinal cord (see for reviews Mogil and Pasternak, 2001; Zeilhofer and Calo', 2003). Data obtained in the formalin test by investigating the effects of exogenously applied N/OFQ are in line with this view. In fact, it has been reported that spinally administered N/OFQ produces an antinociceptive effect in the rat formalin test through the activation of naloxone-insensitive receptors (Erb et al., 1997; Yamamoto et al., 1997, 2000). On the contrary, the intracerebroventricular injection of N/OFQ increases formalin-induced pain behaviours in mice (Suaudeau et al., 1998) and rats (Zhu et al., 1997). These results, i.e., N/OFQ opposite effects at spinal (antinociception) and supraspinal (pronociception) sites, were later confirmed in the rat formalin test in parallel studies performed under the same experimental conditions (Wang et al., 1999). The spinal antinociceptive effect of exogenously applied N/OFQ in the formalin test has been also confirmed in mice by Ahmadi et al. (2001) who reported that intrathecally injected N/OFQ induced significant antinociception in wild-type ($NOP^{+/+}$) but not in NOP knockout ($NOP^{-/-}$) mice. However, these data, obtained by administering exogenous N/OFQ, do not provide information about the role of the endogenous N/OFQ signalling in modulating nociceptive transmission at spinal and supraspinal levels. To this aim, we used the NOP-selective peptide antagonist UFP-101 (Calo et al., 2002b, 2005) given intrathecally (i.t.) and intracerebroventricularly (i.c.v.) for evaluating the effects of blocking N/OFQ signalling selectively either at spinal or supraspinal sites in the mouse formalin test. In addition, the overall effect of N/OFQ signalling interruption was assessed in the same test by evaluating the action of the non-peptide NOP receptor antagonist ((±) *trans*-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzoimidazol-2-one) (J-113397) (Ozaki et al., 2000; Calo et al., 2002a) given intravenously (i.v.) and by comparing the phenotype of $NOP^{+/+}$ and $NOP^{-/-}$ mice (Nishi et al., 1997).

2. Methods

2.1. Animals

Male albino Swiss mice (25–30 g) from Morini (Reggio Emilia, Italy), male CD1-C57BL6-J129 (25–30 g) $NOP^{+/+}$ and $NOP^{-/-}$ (25–30 g) were used for the experiments. These mice were genotyped by PCR. Details of the generation and breeding of mutant mice have been published previously (Nishi et al., 1997; Gavioli et al., 2003). The animals were housed in colony cages (8–10 mice each) with free access to food and water prior to the experiments. They were maintained in climate and light controlled room (22 ± 2 °C, 12/12 h dark/light cycle with light on at 7.00 a.m.). Formalin test took place during the light phase between 14.00 and 17.00 p.m. The animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and National Italian Regulation (D.L.116/92). Each animal was used once.

2.2. Formalin test

The procedure used was essentially the same as reported by Hunskaar and Hole (1987). Approximately 30 min before testing, mice were individually placed in transparent observation chambers (32 cm high, 24 cm diameter) for adaptation. Then, the animal was taken out of the chamber, and 20 μl of 1.5% formalin solution was injected into the dorsal surface of the right hind paw with a syringe with a 30G-gauge needle (BD, Drogheda, Ireland). Immediately after formalin injection, each mouse was returned to the observation chamber, and time (s) spent by the animal displaying pain-related behaviours was measured with a hand-held stopwatch for each 5-min block for 45 min after formalin injection. The nociceptive behaviours consisted of licking, biting and lifting of the injected paw. Time (s) spent by the animal showing all these pain-related behaviours was cumulatively measured and expressed as seconds (s) of nociceptive behaviour/min.

The cumulative response times during 0–10 min and during 15–45 min were regarded as the first-phase (I° phase) and second-phase (II° phase) responses, respectively. UFP-101 (1 and 10 nmol/mouse) was applied i.c.v. (2 μl/mouse) or i.t. (5 μl/mouse) 1 min before formalin injection according to the procedure described by Hylden and Wilcox (1980) or Laursen and Belknap (1986), respectively. J-113397 (1 and 10 mg/kg) was applied i.v. (100 μl/mouse in the anterior caudal vein) 15 min before formalin injection.

2.3. Drugs

The peptides used in this study were prepared and purified as previously described (Guerrini et al., 1997). J-113397 was prepared as a racemic mixture, according to De Risi et al. (2001). The substances were solubilised in physiological medium just before performing the experiment.

2.4. Data analysis and terminology

All data are expressed as means ± standard error of the mean of *n* experiments. Data have been statistically analyzed with Student's *t*-test or one-way ANOVA followed by Dunnett's test, as specified in figure legends; *p* values less than 0.05 were considered to be significant.

3. Results

The intraplantar injection of 20 μl of 1.5% formalin solution into the dorsal surface of the right hind paw produced a biphasic nociceptive response. The first

phase (I° phase) started immediately after formalin injection and lasted for 10 min, while the second phase (II° phase) was prolonged, starting approximately 20 min after the injection and lasting for about 30 min. Mice receiving 20 μl of saline into the dorsal surface of the right hind paw did not show any pain-related behaviour (data not shown).

To test whether the processing of tonic nociceptive input was under the control of endogenous N/OFQ we injected the peptide NOP receptor antagonist UFP-101 either intracerebroventricularly or intrathecally.

In mice treated with saline i.c.v. (control), the formalin injection induced nociceptive behaviours of 31.5 ± 3.7 s/min during the I° phase and 11.5 ± 0.5 s/min during the II° phase. When UFP-101 was injected i.c.v. at a dose of 10 nmol/mouse a statistically significant antinociceptive effect occurred in the II° phase (4.2 ± 1.0 s/min) but not in the I° phase (24.5 ± 2.5 s/min) (Fig. 1). One nanomole per mouse was without effect (Fig. 1).

Opposite results were obtained when UFP-101 was injected i.t. (i.e., into the spinal canal). In fact, as

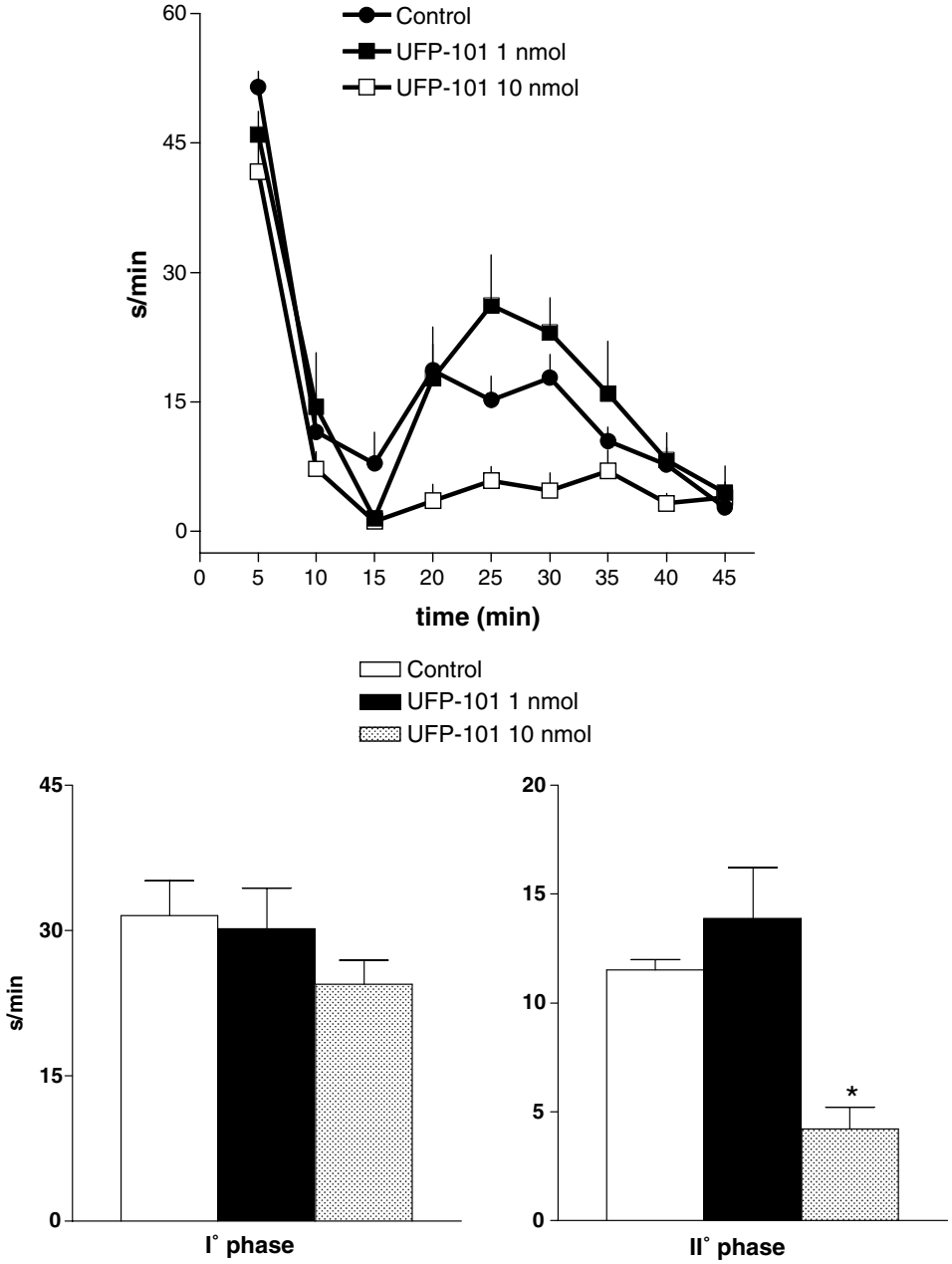

Fig. 1. (Top panel) Time course of formalin-induced pain behaviour in mice treated i.c.v. with saline (2 μl/mouse) or UFP-101 (1 and 10 nmol) 1 min before the intraplantar injection of 1.5% formalin solution into the right hind paw. The total nociceptive behaviour (s/min) is plotted versus time (min). (Bottom panel) Formalin-induced pain behaviour during the I° and II° phases. Each point represents the mean of 8–10 animals, and the vertical bars indicate the SEM. *$p < 0.05$ versus control according to ANOVA followed by Dunnett's test.

reported in Fig. 2, UFP-101 given i.t. did not modify the animal behaviour at 1 nmol while at 10 nmol produced a statistically significant pronociceptive effect during the second phase of the test (saline 9.5 ± 0.9 s/min; UFP-101 10 nmol 14.7 ± 1.2 s/min).

It is worthy of mention that 10 nmol UFP-101 either given i.t. or i.c.v. did not modify the spontaneous locomotor activity of the mice during the 45-min observation time (data not shown).

To test whether the pro- or antinociceptive effect of NOP receptor antagonism prevails after systemic treatment mice were intravenously injected with the non-peptide NOP receptor antagonist J-113397, which penetrates the blood–brain barrier. As shown in Fig. 3, in control mice (animals receiving 100 μl saline i.v. 15 min before formalin), the injection of formalin produced the typical biphasic response and the nociceptive behaviour amounted to 32.2 ± 2.5 and 9.2 ± 0.9 s/min during the I° and II° phases, respectively. J-113397 (1 mg/kg) did not significantly modify formalin-induced behaviour (I° phase 36.9 ± 4.2 s/min; II° phase 9.7 ± 2.3 s/min), while at 10 mg/kg the

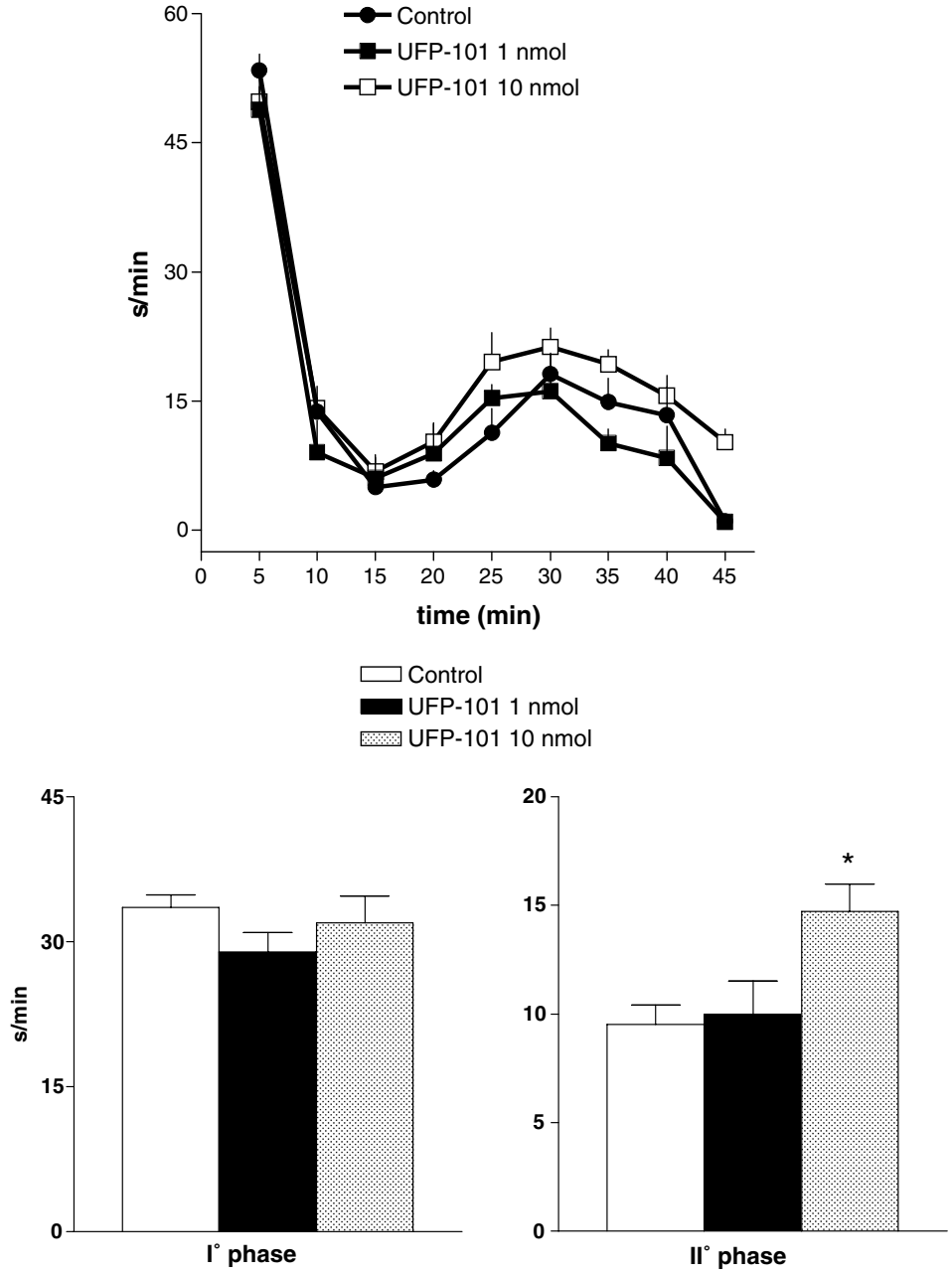

Fig. 2. (Top panel) Time course of formalin-induced pain behaviour in mice treated i.t. with saline (5 μl/mouse) or UFP-101 (1 and 10 nmol) 1 min before the intraplantar injection of 1.5% formalin solution into the right hind paw. The total nociceptive behaviour (s/min) is plotted versus time (min). (Bottom panel) Formalin-induced pain behaviour during the I° and II° phases. Each point represents the mean of 10–12 animals, and the vertical bars indicate the SEM. *$p < 0.05$ versus control according to ANOVA followed by Dunnett's test.

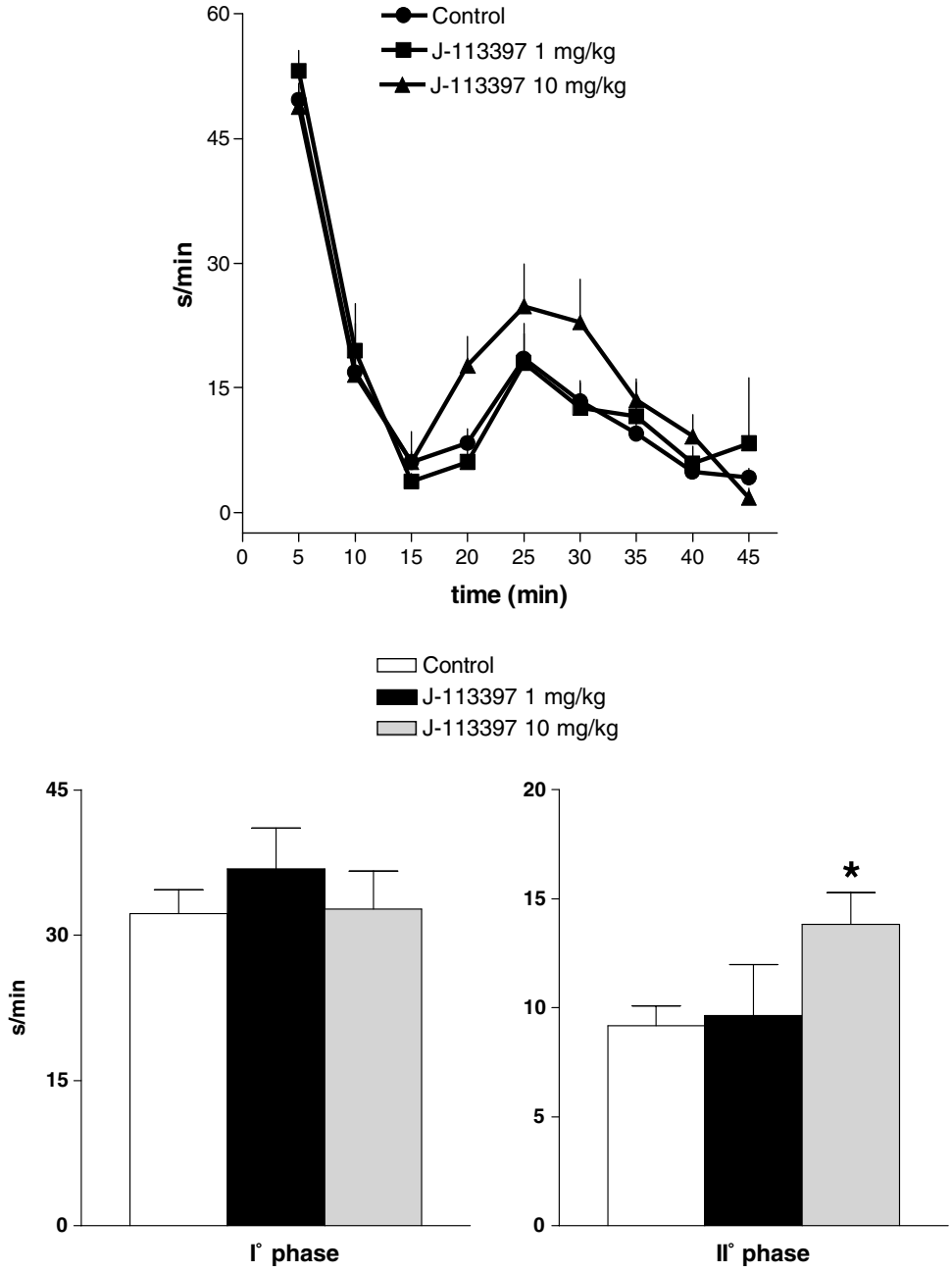

Fig. 3. (Top panel) Time course of formalin-induced pain behaviour in mice treated i.v. with 100 μl saline or J-113397 1 and 10 mg/kg 15 min before formalin. (Bottom panel) Formalin-induced pain behaviour during the I° and II° phases. Each point represents the mean of 10–12 animals, and the vertical bars indicate the SEM. *$p < 0.05$ versus control according to ANOVA followed by Dunnett's test.

antagonist enhanced in a statistically significant manner the nociceptive behaviour during the II° (13.8 ± 1.5 s/min) but not the I° phase (32.7 ± 3.9 s/min). J-113397 (10 mg/kg i.v.) did not modify spontaneous locomotor activity of mice (data not shown).

Genetic ablation of the NOP receptor gene produced a statistically significant increase of nociceptive behaviour of the mutant mice both in the I° and II° phases compared to wild-type animals ($NOP^{+/+}$: I° phase 21.4 ± 2.8 s/min; II° phase 15.2 ± 2.9 s/min; $NOP^{-/-}$: I° phase 35.7 ± 2.4 s/min; II° phase 32.6 ± 3.4 s/min) (Fig. 4).

4. Discussion

Collectively, the present findings demonstrate that endogenous N/OFQ–NOP receptor signalling is activated during the formalin test in mice producing spinal antinociceptive and supraspinal pronociceptive effects. Moreover, data obtained using J-113397 and $NOP^{-/-}$

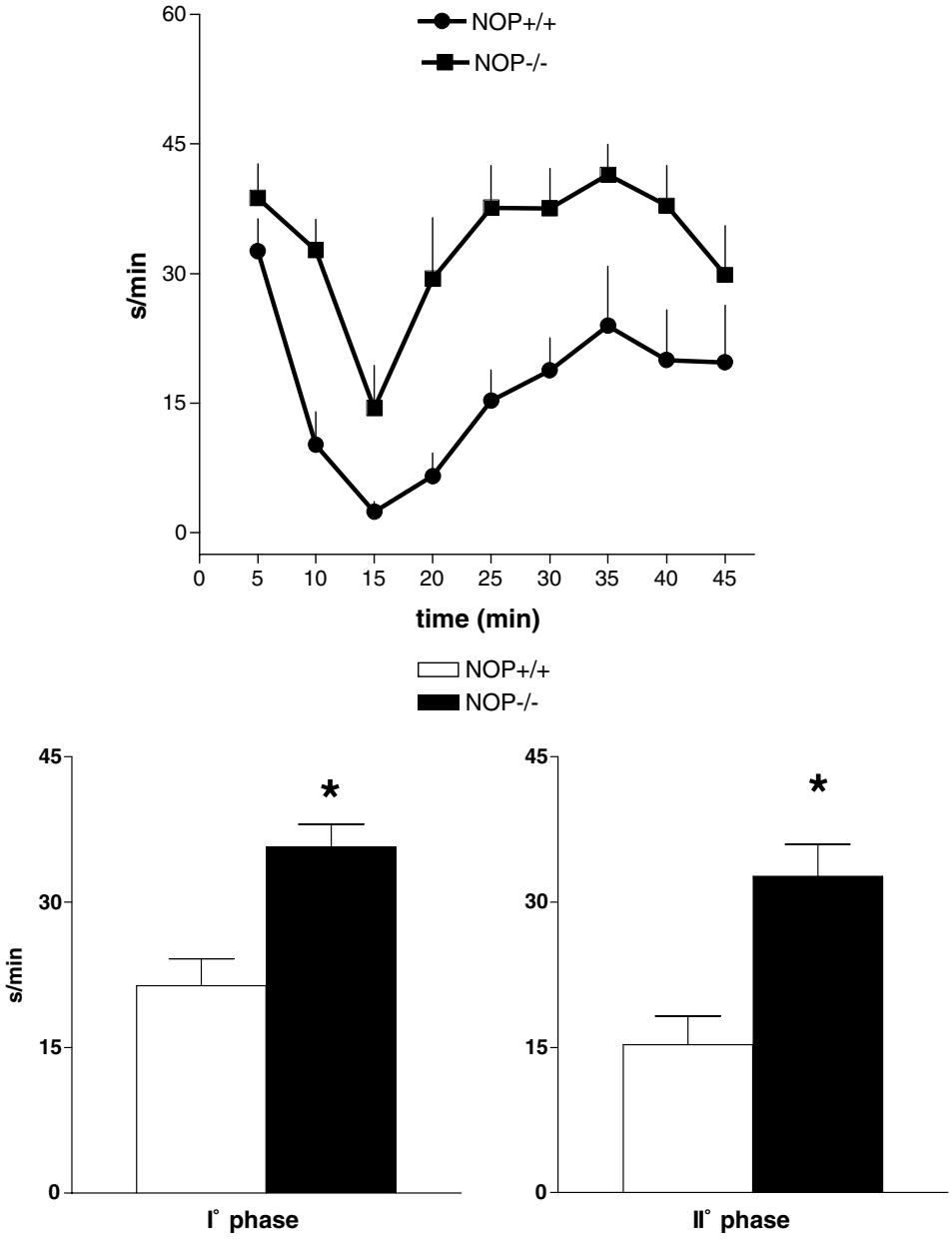

Fig. 4. (Top panel) Time course of formalin-induced pain behaviour in $NOP^{+/+}$ and $NOP^{-/-}$ mice. The total nociceptive behaviour (s/min) is plotted versus time (min). (Bottom panel) Formalin-induced pain behaviour during the I° and II° phases. Each point represents the mean of eight animals, and the vertical bars indicate the SEM. $*p < 0.05$ versus $NOP^{+/+}$ according to Student's *t*-test for unpaired data.

mice suggest that the spinal action prevails over supraspinal effects.

To investigate the role of endogenously released N/OFQ at spinal and supraspinal sites in the mouse formalin test we examined the effects of the local (i.t. and i.c.v.) injection of the potent and selective NOP receptor peptide antagonist, UFP-101 (Calo et al., 2002b). The pure antagonistic and NOP receptor-selective properties of this compound have been confirmed in the last few years in a variety of pharmacological assays and with different techniques (for a review, see Calo et al., 2005). UFP-101 was tested at 1 and 10 nmol (both i.c.v. and i.t.) because in this range of doses the compound antagonized in the mouse tail withdrawal assay both the pronociceptive (Calo et al., 2002b) and the antinociceptive (Rizzi et al., 2004) effects of 1 nmol N/OFQ given i.c.v. and i.t., respectively. At 10 nmol UFP-101 given i.c.v. elicited a robust antinociceptive effect in the second phase of the mouse formalin test. These results are in line with data obtained in the past using different NOP peptide antagonists ($[Nphe^1]N/OFQ(1–13)NH_2$, retronociceptin methylester, UFP-101) that elicited naloxone-resistant antinociceptive actions in the tail flick, tail withdrawal and hot plate test (Calo et al., 2000a, 2002b; Jinsmaa et al., 2000; Di Giannuario et al., 2001). Moreover, the

non-selective antagonist naloxone benzoylhydrazone produces antinociceptive effects in $NOP^{+/+}$ but not in $NOP^{-/-}$ mice (Noda et al., 1998). However, non-peptide antagonists selective for the NOP receptor such as J-113397 and SB-612111 were found to be inactive in similar assays (Ozaki et al., 2000; Zaratin et al., 2004) and no difference in terms of sensitivity to acute thermal and mechanical nociceptive stimuli has been displayed by $NOP^{+/+}$ and $NOP^{-/-}$ mice (Nishi et al., 1997; Ahmadi et al., 2001; Di Giannuario et al., 2001). Thus, these discrepant findings preclude firm conclusions on the activation of the endogenous N/OFQ–NOP receptor system in analgesiometric assays based on a phasic stimulus of high intensity and characterized by a short lasting nociceptive exposure, such as the tail flick, tail withdrawal and hot plate test. On the contrary, in the formalin test the nociceptive stimulus evoked by subcutaneous injection of formalin is tonic and induces behavioural responses lasting for about 1 h, thus allowing us to assess endogenous modulatory mechanisms triggered by the stimulus itself. Thus, the antinociceptive action of supraspinal UFP-101 might be due to the blockade of pronociceptive effects of N/OFQ released during prolonged nociceptive stimulation. In addition, a facilitation of stress-induced analgesia, by supraspinal UFP-101, might contribute. In this regard it is worthy of mention that the selective NOP antagonist $[Nphe^1]N/OFQ(1–13)NH_2$ (given i.c.v.) blocks the inhibitory effect of N/OFQ and facilitates the analgesic response to forced swimming in mice (Rizzi et al., 2001).

Opposite results have been obtained by investigating the effect of intrathecally injected UFP-101. Under these experimental conditions, UFP-101 enhanced in a statistically significant manner the nociceptive behaviour in the II° phase of the test. Thus, formalin injection seems to be able to promote endogenous N/OFQ release also in the spinal cord where the peptide evokes an antinociceptive effect (Mogil and Pasternak, 2001; Zeilhofer and Calo', 2003). Interestingly enough, in the mouse tail withdrawal assay the intrathecal administration of 10 nmol UFP-101 prevented the antinociceptive action of exogenously administered N/OFQ being per se inactive (Rizzi et al., 2004; Calo et al., 2005). Similar results, i.e., block of N/OFQ antinociceptive action without any effect per se, have been reported with the NOP-selective $[Nphe^1]N/OFQ(1–13)NH_2$ given i.t. in analgesiometric assays characterized by phasic stimulus of high intensity (Lu et al., 2001; Yu et al., 2002). Therefore, the spinal N/OFQ–NOP receptor signalling seems to require tonic nociceptive stimulation while phasic stimuli even of high intensity are not sufficient to promote spinal N/OFQ release.

Collectively, the results obtained in this study with UFP-101 and those reported in the literature suggest that the net effect on nociceptive behaviour of blocking N/OFQergic signalling strongly depends on both the level of interference (spinal versus supraspinal) and on the type of nociceptive stimulus adopted (phasic versus tonic).

To investigate the overall effect of blocking the NOP signalling at the spinal and supraspinal levels in the mouse formalin test we adopted a combined pharmacological and genetic approach investigating the effects of the systemic administration of the non-peptide NOP antagonist J-113397 and comparing the phenotype of $NOP^{+/+}$ and $NOP^{-/-}$ mice. J-1133397 is a potent and quite selective non-peptide receptor antagonist widely used to characterize the biological action of N/OFQ. In vitro, J-113397 behaves as a potent and competitive antagonist (pA_2 values in the range 7.5–8.4) at both recombinant NOP receptors and at native receptor in peripheral tissues, brain membranes, synaptosomes and slices. Moreover, the NOP antagonist properties of J-113397 were also confirmed in vivo where the compound antagonized several N/OFQ biological actions (see for review Calo et al., 2002a). On the other hand, $NOP^{-/-}$ mice (Nishi et al., 1997) were successfully used for investigating the involvement of the N/OFQ–NOP system in various biological functions such as hearing (Nishi et al., 1997), morphine tolerance (Ueda et al., 1997, 2000), memory processes (Manabe et al., 1998), locomotor activity (Marti et al., 2004), and mood regulation (Gavioli et al., 2003, 2004).

In the present study, the systemic injection of J-113397 induced clear and dose-dependent pronociceptive effects in the second phase of the formalin test. These data confirm previous findings obtained with the same agent in the rat formalin test (Yamamoto et al., 2001). Different results have been obtained with another NOP receptor non-peptide antagonist, namely JTC-801 (Shinkai et al., 2000). In fact, JTC-801 at doses effective in blocking the actions of exogenously administered N/OFQ produced per se antinociceptive effects in the mouse hot plate test and in the rat formalin test (Shinkai et al., 2000; Yamada et al., 2002). However, in our opinion, the low selectivity of action of JTC-801 over classical opioids receptors (Yamada et al., 2002) does not allow to interpret these in vivo actions as NOP dependent. It is worthy of mention that J-113397 (as well as another non-peptide NOP-selective antagonist SB-612111) does not modify pain threshold to phasic stimuli of high intensity (Ozaki et al., 2000; Zaratin et al., 2004; Rizzi et al., unpublished results).

Knockout studies corroborate the results obtained with NOP receptor antagonists in the formalin assay. In fact, in the present study $NOP^{-/-}$ mice displayed a clear pronociceptive phenotype in the formalin test. These results confirm previous findings comparing the phenotype of $NOP^{-/-}$, $ppN/OFQ^{-/-}$ and double knockout mice with those of their wild-type littermates (Depner et al., 2003). All these mutant mice displayed stronger nociceptive responses in the formalin test and

increased inflammatory hyperalgesia, while their response to acute noxious heat and strong mechanical stimulation were indistinguishable from those of wild-type mice. Thus, evidence from knockout and receptor antagonist studies converges in indicating that endogenous N/OFQ–NOP receptor signalling is activated and negatively controls pain transmission only during prolonged nociceptive stimulation. Our results obtained with UFP-101 clearly suggest that this activation of N/OFQ signalling likely occurs at the spinal level (see below), however, a peripheral site of action cannot be excluded especially considering the robust inhibitory effect elicited by N/OFQ on C-fibres (Zeilhofer and Calo', 2003).

Since the cellular actions of NOP receptors (inhibition of neurotransmitter release and postsynaptic inhibition of cell activity by potassium channel activation) are similar at both spinal and supraspinal sites, the opposing behavioural effects of spinal versus supraspinal N/OFQergic systems are probably due to a different cellular localization of NOP receptors. In the spinal cord dorsal horn, N/OFQ selectively inhibits excitatory neurons and reduces the synaptic release of L-glutamate (Liebel et al., 1997; Luo et al., 2002), an action which is absent in $NOP^{-/-}$ mice (Ahmadi et al., 2001) and blocked by UFP-101 (Nazzaro et al., 2005). At this site, N/OFQ is mainly expressed by local inhibitory interneurons located in the superficial layers where primary nociceptive afferents terminate (Neal et al., 1999). At supraspinal sites, N/OFQ inhibits various types of neurons through activation of an inwardly rectifying K^+ conductance in brain nuclei relevant for nociceptive transmission such as the nucleus raphe magnus (Pan et al., 2000), the dorsal raphe nucleus (Vaughan and Christie, 1996) and the periaqueductal grey (Vaughan et al., 1997), an action which is again sensitive to NOP-selective antagonists including J-113397 (Chiou and Fan, 2002) and UFP-101 (Chiou et al., 2005). Both the N/OFQ peptide and NOP receptor are heavily expressed in these areas (Neal et al., 1999).

In conclusion, the present study confirms and extends previous findings, demonstrating that endogenous N/OFQ–NOP receptor signalling is activated by the prolonged nociceptive input elicited by formalin injection and produces supraspinal pronociceptive and spinal antinociceptive effects. In addition, receptor antagonist and knockout studies clearly indicate that the spinal action prevails over supraspinal effects.

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