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Effects of nefopam on the spinal nociceptive processes: a c-Fos protein study in the rat

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Abstract

We have evaluated the effects of nefopam on the spinal c-Fos protein expression in the model of acute (noxious heat) and persistent (intraplantar injection of formalin) nociception in the rat. One and two hours after i.pl. formalin injection, c-Fos immunoreactive (c-Fos-IR) nuclei were preferentially located in the superficial (I-II) and deep (V–VI) laminae of the spinal dorsal horn of segments L4–L5, i.e. spinal areas containing numerous neurons responding exclusively, or not, to peripheral nociceptive stimuli. The doses of 15 and 30 mg/kg (s.c.) of nefopam had significant reducing effects on the formalin-evoked spinal c-Fos protein expression (36 ± 14% and 47 ± 9% reduction of the total number of c-Fos-IR nuclei per section, respectively, $P < 0.05$ for both). These reducing effects of nefopam were not detectable 2 h after formalin. These results provide evidence that the significant effects of nefopam are time-limited in the formalin model of persistent nociception. One hour after noxious heat stimulation (52 °C for 15 s), c-Fos-IR nuclei were principally located in the superficial laminae I–II of the spinal dorsal horn (about 90% of the total number of c-Fos-IR nuclei per section). Nefopam (15 mg/kg s.c.) significantly reduced the noxious heat-evoked spinal c-Fos protein expression (33 ± 3% reduction of the total number of c-Fos-IR nuclei, $P < 0.0001$). The present results provide first evidence for the reducing effects of nefopam on the noxiously evoked spinal c-Fos protein expression, principally in acute nociceptive processes. These results suggest that nefopam may produce antinociceptive effects mainly in acute pain states.

Keywords: c-Fos; Formol; Nefopam; Noxious heat; Spinal dorsal horn

1. Introduction

Nefopam hydrochloride (Acupan) is a clinically effective analgesic compound (Re, 1976; Heel et al., 1980), chemically unrelated to standard analgesic compounds, such as opioids or anti-inflammatory drugs (Heel et al., 1980). Nefopam's effect is lacking undesirable side effects that are typical for opioids or anti-inflammatory drugs (Heel et al., 1980). The antinociceptive effect of nefopam has been demonstrated in various models of nociception in animals (Mather et al., 2000; Gray et al., 1999; Kanui et al., 1993; Hunskaar and Hole, 1987; Hunskaar et al., 1987; Fasmer et al., 1987; Esposito et al., 1986; Piercey and Schroeder, 1980, 1981; Heel et al., 1980) and humans (Mimoz et al., 2001; Guirimand et al., 1999; Heel et al., 1980). Although mechanism(s) of nefopam's antinociceptive effect is not fully understood, the involvement of noradrenergic, dopaminergic and serotoninergic mechanisms has been suggested (Hunskaar et al., 1987; Esposito et al., 1986). The recent study (Guirimand et al., 1999) describing the strong depressive effect of nefopam on the nociceptive flexion (RIII) reflex in humans suggests that the spinal cord could be one site of action of this compound. This activity has been clinically confirmed by a 50% morphine-sparing effect in patients undergoing surgery (Mimoz et al., 2001).

In an attempt to further explore the effects of nefopam on spinal nociceptive processes, we have used the "c-Fos technique" which provides alternative information to that obtained by either behavioural or electrophysiological approaches. This technique is based on the immunohistochemical investigation of the c-Fos protein expression, the nuclear protein product encoded by the immediate–early gene *c-fos*, in the central nervous system (for review, see Morgan, 1991; Morgan and Curran, 1995; Hughes and Dragunow, 1995). The immunohistochemical revelation of the c-Fos protein expression provides the information on the

Abbreviations: c-Fos-IR; c-Fos protein immunoreactive.

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location of c-Fos protein immunoreactive (c-Fos-IR) nuclei. The c-Fos protein expression, especially at the spinal cord level, is widely used as an indirect marker of neurons involved in the nociceptive transmission (for review, see Munglani and Hunt, 1995; Chapman and Besson, 1997). This technique has been used by numerous groups to study the pharmacological aspects of nociceptive processes at the spinal cord level (see references in Chapman and Besson, 1997; Buritova and Besson, 1999).

In this study, we have used the c-Fos protein expression to study the effect of nefopam on the spinal nociceptive processes in the formalin model of persistent nociception and in the noxious heat model of acute nociception in the rat. Previous studies have clearly shown that the intraplantar formalin evokes the spinal c-Fos protein expression, especially at the level of the dorsal horn of the lumbar spinal cord, in the awake rat (see Presley et al., 1990; Chapman et al., 1996; Peterson et al., 1997; and references therein). Similarly, a single noxious heat stimulation (52 °C, 15 s) induces the spinal c-Fos protein expression in the anaesthetised rat (see Abbadie et al., 1994; Buritova et al., 1996; Buritova and Besson, 2000; and references therein). Importantly, various pharmacological treatments have been shown to reduce noxiously evoked spinal c-Fos protein expression in these animal models of nociception (see references above).

2. Materials and methods

2.1. Experimental animals

Experiments were performed on 78 adult male albino Sprague–Dawley rats (Charles River, France), weighing 225–250 g.

Guidelines on ethical standards of the International Association for the Study of Pain were followed for the investigations of experimental pain in animal models (Zimmermann, 1983).

2.2. Formalin model of persistent nociception

Formalin (100 μl) was injected intraplantarly (i.pl.) into the plantar surface of the right paw while the awake rat was restrained manually. The concentration of 2.5% of formalin was used (i.e. 2.5 ml of saturated solution of formaldehyde (37.5%)) was added to 100 ml of sterile saline); see Methods in Abbott et al., 1995. Although the behavioural response to i.pl. formalin was not systemically observed, all rats demonstrated typical nociceptive responses, such as the intensive licking of the formalin injected paw (Hunskaar and Hole, 1987; Dubuisson and Dennis, 1977).

2.3. Model of acute thermal nociception

We have used a single noxious heat stimulation (52 °C, 15 s) in the anaesthetised rat. Rats were anaesthetised with pentobarbital (60 mg/kg, i.p.; Sanofi) prior to drug treatment and noxious heat stimulation, i.e. 10 min prior to s.c. injection of nefopam. The right hind paw of the anaesthetised rat was immersed (up to the ankle level) in a hot water bath at the regulated temperature of 52 °C for 15 s. The parameters (temperature and duration) of heat stimulation were chosen according to previous studies of spinal c-Fos protein expression resulting from a single noxious heat stimulation (see references in Abbadie et al., 1994; Buritova et al., 1996; Buritova and Besson, 2000).

2.4. Experimental protocol

Nefopam (Biocodex) was dissolved in saline (0.9%) and injected subcutaneously (s.c.) in the rat.

In the first and second experimental series, nefopam (5, 15 and 30 mg/kg, $n=6$ rats in each group) was administered 30 min prior to i.pl. injection of formalin in the awake rat. In both experimental series, control rats ($n=6$) received s.c. injection of saline, 30 min prior to i.pl. formalin. In the first and second experimental series, we studied the effects of nefopam on the spinal c-Fos protein expression, 1 and 2 h after i.pl. formalin, respectively. In both experimental series, one group of rats receiving nefopam (30 mg/kg s.c., $n=6$) alone (without noxious stimulation due to formalin injection) was included. The doses of nefopam (5, 15 and 30 mg/kg) were chosen according to previous studies that demonstrated the pharmacological activity of nefopam (10–30 mg/kg) in the formalin model (Fasmer et al., 1987; Hunskaar et al., 1987; Kanui et al., 1993).

In order to minimize the number of animals used in the noxious heat model, a single dose of nefopam (15 mg/kg) was selected according to the results previously obtained in the formalin model. In the third experimental series, nefopam (15 mg/kg s.c., $n=9$) was administered 30 min prior to noxious heat stimulation in the anaesthetised rat. Control anaesthetised rats ($n=9$) received s.c. injection of saline, 30 min prior to noxious heat stimulation. The effects of nefopam on the spinal c-Fos protein expression were studied 1 h after noxious heat stimulation.

2.5. Immunohistochemistry

Rats were perfused 1 h (formalin, noxious heat) or 2 h (formalin) after noxious stimulation. They were deeply anaesthetised with pentobarbital (60 mg/kg, i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate buffered saline 0.1 M (PBS) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord was then removed and postfixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen sections from the lumbar spinal cord, 40 μm thick, were cut and collected in PB to be processed immunohistochemically as free floating sections.

Immunohistochemistry of the free floating sections was performed with polyclonal antiserum, generated in rabbits

and directed against the c-Fos protein (Santa Cruz Biotechnology; SC-52, 0.1 mg/ml; diluted at 1:30,000), using the method of Hsu et al. (1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in phosphate buffer 0.1 M + saline 0.9% (PBS) with 0.3% Triton-X (NGST) and were then incubated 18 h at 4 °C in the primary antiserum directed against the c-Fos protein. The incubated sections were washed three times in PBS and incubated in biotinylated goat anti-rabbit antiserum (Vector Laboratories, BA-1000) for 1 h at room temperature, then washed twice in PBS and incubated for 1 h in avidin–biotin–peroxidase complex (Vector Laboratories, PK-6100). Finally, the sections were washed three times in PBS and developed in chromogen solution (Peroxidase Substrate Kit, Vector Laboratories, SK-4600) for 5 min, and were washed again three times in PB to stop the staining reaction. The sections were mounted on gelatine-subbed slides. After being air dried, sections were differentiated in 70%, 95% and 100% alcohol (differentiation time was evaluated under the microscope) and coverslipped.

2.6. Counting of c-Fos protein immunoreactive (c-Fos-IR) nuclei and statistics

Tissue sections were first examined using lightfield microscopy at 4 × to determine the segmental level according to Molander et al. (1984), as well as the grey matter landmarks. The sections were then examined under lightfield microscopy at 10 × to localize c-Fos labelled nuclei. All c-Fos-IR nuclei were analysed without considering the intensity of the staining. c-Fos-IR nuclei were plotted and counted using a camera lucida attachment. The investigator responsible for plotting and counting the c-Fos-IR nuclei was blind to the experimental situations.

To study the laminar distribution of c-Fos-IR nuclei, four regions were arbitrarily defined in the spinal grey matter of the L3–L5 segments: superficial laminae of the dorsal horn (corresponding to laminae I–II), nucleus proprius (laminae III–IV), neck of the dorsal horn (laminae V–VI) and the ventral horn (laminae VII–X), according to the cytoarchitectonic organisation of the spinal cord (Rexed, 1952; Molander et al., 1984; Molander and Grant, 1986).

For each animal, two counts were made: (1) the total number of c-Fos-IR nuclei in the grey matter for 10 sections through L4–L5 segments (mean ± S.E.M.), and (2) in these 10 sections, the number of c-Fos-IR nuclei per four defined regions (mean ± S.E.M.). One-way analysis of variance (ANOVA) was conducted for comparison across the experimental conditions. Fisher's protected least-significant difference test was applied to define which group contributed to these differences. Significance was taken as $P < 0.05$.

3. Results

In the absence of noxious stimulation, nefopam (30 mg/kg s.c.) did not induce the c-Fos protein expression in the lumbar spinal cord of the rat.

3.1. Spinal c-Fos protein expression induced by noxious formalin stimulation in the awake rat

One and two hours after i.pl. injection of formalin, the c-Fos-IR nuclei were numerous (73 ± 9 and 117 ± 5 c-Fos-IR nuclei per section of lumbar segments L4–L5, respectively) in the spinal cord ipsilaterally to the formalin-injected hind paw in control awake rats (Table 1). One hour after i.pl. formalin, c-Fos-IR nuclei were predominantly located in the superficial laminae I–II of the spinal dorsal horn (about 54% of the total number of c-Fos-IR nuclei per section in the segments L4–L5; Table 1, Fig. 1). Two hours after i.pl. formalin, the number of c-Fos-IR nuclei increased with their essential localisation in the superficial (I–II) and deep (V–VI) laminae (44% and 35% of the total number of c-Fos-IR nuclei per section in L4–L5 segments, respectively). At both time points after i.pl. formalin, c-Fos-IR nuclei were virtually absent in the contralateral lumbar spinal cord (< 3 c-Fos-IR nuclei per section).

Table 1
Effects of nefopam (5, 15 and 30 mg/kg s.c., $n = 6$ for each group) on the spinal c-Fos protein expression, 1 h after i.pl. injection of formalin in the awake rat

Group	Dose (mg/kg s.c.)	Number of c-Fos-IR nuclei		Paw diameter (cm)	Ankle diameter (cm)
		Total number	Laminae I–II		
Controls	–	73 ± 9	39 ± 4	0.71 ± 0.02	0.82 ± 0.02
Nefopam	5	56 ± 8 (23 ± 11)	30 ± 4 (24 ± 11)	0.69 ± 0.01	0.79 ± 0.02
	15	47 ± 10 (36 ± 14)[a]	28 ± 5 (29 ± 12)	0.69 ± 0.03	0.79 ± 0.02
	30	38 ± 7 (47 ± 9)[a]	21 ± 3 (45 ± 8)[b]	0.64 ± 0.03[a]	0.78 ± 0.01

Results are expressed as mean value (± S.E.M.) of the number of c-Fos protein immunoreactive (c-Fos-IR) nuclei per section in L4–L5 segments (total number) and in superficial laminae (laminae I–II) of the spinal cord, and as mean value (± S.E.M.) of the diameter at the paw and ankle levels (paw diameter, ankle diameter), 1 h after i.pl. injection of formalin. Results expressed as % reduction of control value of studied parameters are presented in brackets. Significance compared to control group ($n = 6$) was performed using ANOVA and Fisher's PLSD test.

[a] $P < 0.05$.

[b] $P < 0.01$.

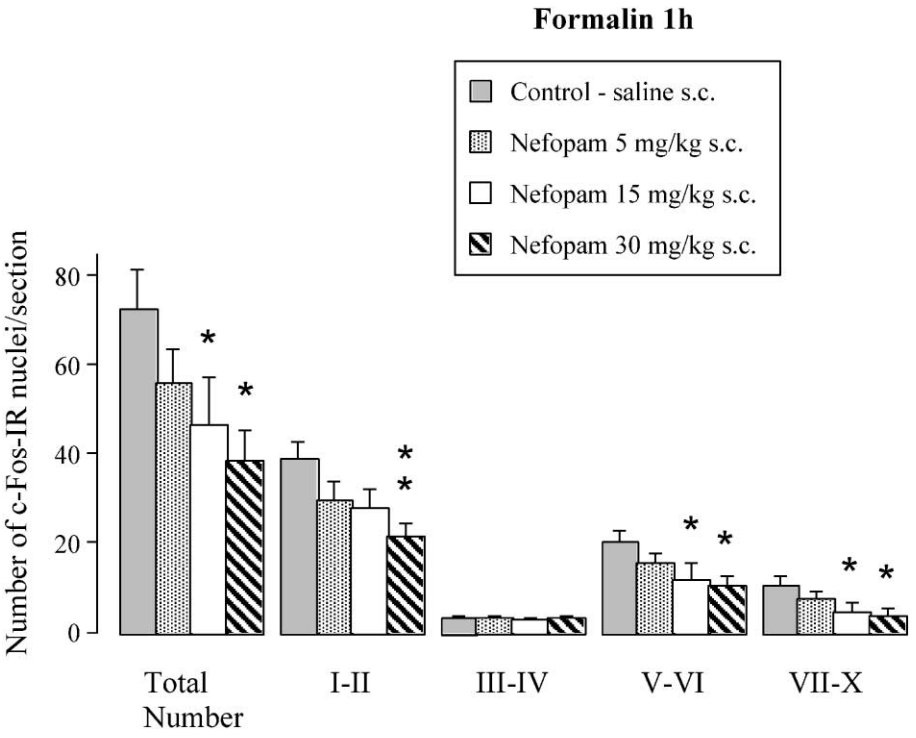

Fig. 1. Effects of nefopam (5, 15 and 30 mg/kg s.c., $n=6$ for each group) on the spinal c-Fos protein expression, 1 h after i.pl. injection of formalin in the awake rat. Results are expressed as mean value (± S.E.M.) of the number of c-Fos protein immunoreactive (c-Fos-IR) nuclei per section in L4–L5 segments (total number), in superficial laminae (I–II), nucleus proprius (III–IV), deep laminae (V–VI) of dorsal horn and in ventral horn (VII–X) of the spinal cord, 1 h after i.pl. injection of formalin. Significance compared to control group ($n=6$) was performed using ANOVA and Fisher's PLSD test (* $P<0.05$, * * $P<0.01$).

3.2. Effects of nefopam on the formalin-induced spinal c-Fos protein expression and peripheral edema

One hour after i.pl. formalin, a low dose of nefopam (5 mg/kg s.c.) had no significant effect on the formalin-evoked spinal c-Fos protein expression. Two higher doses of 15 and 30 mg/kg had significant reducing effects (36 ± 14% and 47 ± 9% reduction of the total number of formalin-evoked c-Fos-IR nuclei per section in L4–L5 segments, respectively, $P<0.05$ for both; Table 1; Fig. 1). These reducing effects of nefopam were not dose-related (regression coefficient $r=0.348$; $P>0.05$). Laminar analysis revealed that the reducing effects of 30 mg/kg of nefopam were due to a decrease of the number of c-Fos-IR nuclei in both superficial and deep laminae (45 ± 8%, 49 ± 11% and 64 ± 14% reduction of the number of c-Fos-IR nuclei in laminae I–II, V–VI and VII–X, $P<0.01$, $P<0.05$ and $P<0.05$, respectively; Fig. 1). The dose of 15 mg/kg of nefopam significantly reduced the number of c-Fos-IR nuclei in deep laminae (41 ± 18% and 57 ± 19% reduction of the number of c-Fos-IR nuclei in laminae V–VI and VII–X, respectively, $P<0.05$ for both; Fig. 1).

Considering the total number of c-Fos-IR nuclei, the effects of nefopam (5, 15 and 30 mg/kg s.c.) were not detectable 2 h after i.pl. formalin. However, the highest dose of nefopam (30 mg/kg s.c.) produced a weak, but significant, reduction of the number of c-Fos-IR nuclei in superficial laminae (19 ± 8% reduction of the number of c-Fos-IR nuclei in laminae I–II, $P<0.05$).

Ipsilateral paw and ankle diameters in control formalin-stimulated rats (Table 1) were significantly enhanced as compared to those in non-stimulated rats (0.48 ± 0.02 and 0.75 ± 0.03 cm for paw and ankle diameters, respectively). As compared to the control group, the highest dose of nefopam (30 mg/kg s.c.) produced a weak, but significant, decrease of the formalin-enhanced paw diameter, 1 h after i.pl. formalin (28 ± 8% reduction of the paw diameter, $P<0.05$; Table 1). This effect was not detectable 2 h after i.pl. formalin.

3.3. Spinal c-Fos protein expression induced by noxious heat stimulation in the anaesthetised rat

One hour after a single noxious heat stimulation (52 °C, 15 s), c-Fos-IR nuclei were numerous in the spinal cord ipsilaterally to the heat-stimulated hind paw in control rats (55 ± 2 c-Fos-IR nuclei per section in L4–L5 segments; Figs. 2A and 3). c-Fos-IR nuclei were principally located in the superficial laminae I–II of the dorsal horn of the spinal cord (about 90% of the total number of c-Fos-IR nuclei per section; Figs. 2B and 3). In contrast, very few c-Fos-IR nuclei were located in the nucleus proprius (laminae III–IV), deep laminae V–VI of the dorsal horn, and in the laminae VII–X of the ventral horn (< 4 c-Fos-IR nuclei per

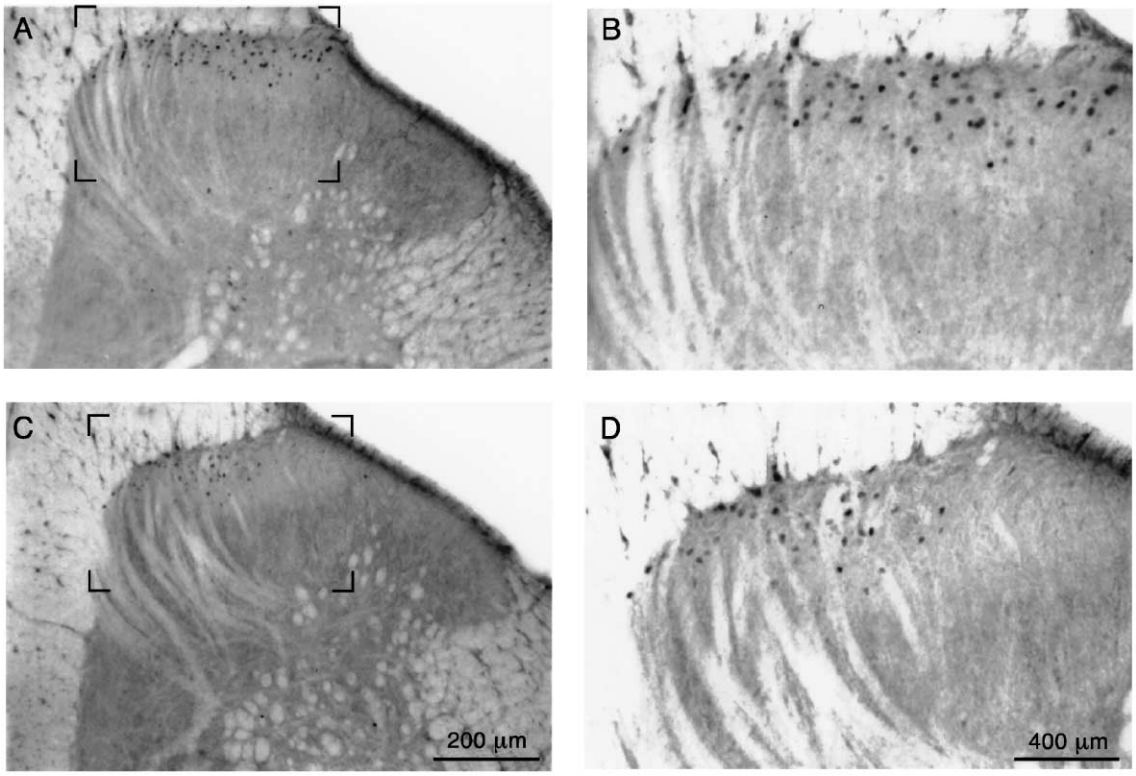

Fig. 2. Photomicrographs illustrating the effects of nefopam (15 mg/kg s.c.) on the spinal c-Fos protein expression, 1 h after noxious heat stimulation (52 °C, 15 s) in the anaesthetised rat. Each photomicrograph is an individual representative example of one section (40 μm) at the level of L4–L5 segments including c-Fos protein immunoreactive nuclei (black dots) in laminae I–V of dorsal horn, in the ipsilateral side to heat stimulation. Two experimental situations are represented: noxious heat stimulation plus pre-administration of saline (A,B; control) or nefopam (15 mg/kg s.c.) (C,D). Framed regions (corresponding to laminae I–II) in (A,C) are shown with a higher magnification in (B,D), respectively. Scale bar: 200 and 400 μm for (A,C) and (B,D), respectively.

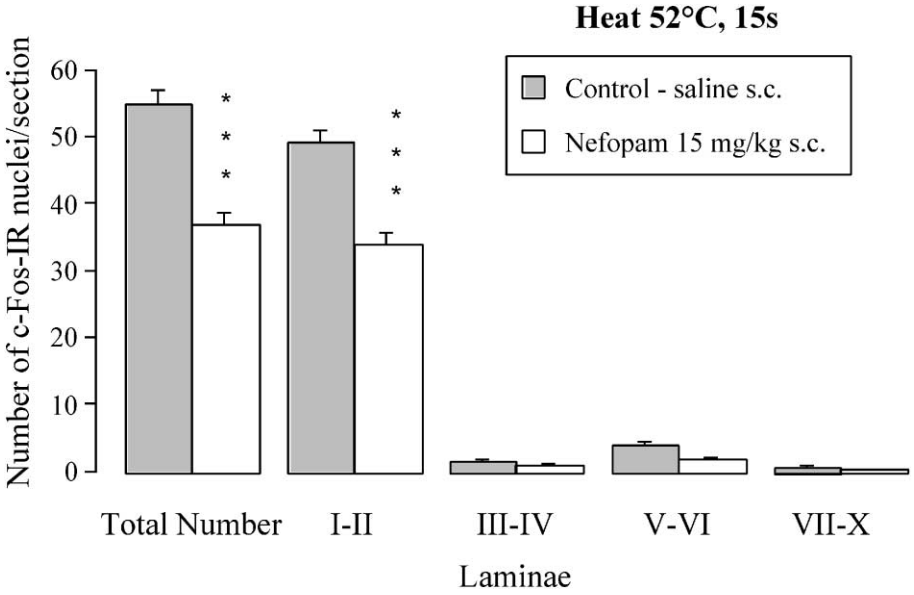

Fig. 3. Effects of nefopam (15 mg/kg s.c., $n=9$) on the spinal c-Fos protein expression, 1 h after noxious heat stimulation (52 °C, 15 s) in the anaesthetised rat. Results are expressed as mean value (± S.E.M.) of the number of c-Fos protein immunoreactive (c-Fos-IR) nuclei per section in L4–L5 segments (total number), in superficial laminae (I–II), nucleus proprius (III–IV), deep laminae (V–VI) of dorsal horn and in ventral horn (VII–X) of the spinal cord, 1 h after noxious heat. Significance compared to control group ($n=9$) was performed using ANOVA and Fisher's PLSD test (* * * $P<0.001$).

section in L4–L5 segments; Figs. 2A and 3). c-Fos-IR nuclei were virtually absent in the contralateral lumbar spinal cord.

One hour after noxious heat stimulation, there was no detectable peripheral edema. Neither paw nor ankle diameters in control heat-stimulated rats were significantly enhanced as compared to those in non-stimulated rats.

3.4. Effects of nefopam on the noxious heat-induced spinal c-Fos protein expression

As compared to the control group, the effects of nefopam (15 mg/kg s.c.) on the noxious heat-evoked spinal c-Fos protein expression were significant when considering the total number of spinal c-Fos-IR nuclei per section in L4–L5 segments of the anaesthetised rat (ANOVA test $F_{1,16}=40.42$, $P<0.0001$). One hour after noxious heat stimulation, nefopam (15 mg/kg s.c.) significantly reduced the total number of spinal c-Fos-IR nuclei ($33 \pm 3\%$ reduction, $P<0.0001$; Figs. 2C and 3). Since the majority of noxious heat-evoked spinal c-Fos-IR nuclei were located in superficial laminae I–II (49 ± 2 c-Fos-IR nuclei per section; Figs. 2D and 3), the laminar analyses were restricted to this laminar level. At this laminar level, the reducing effects of nefopam (15 mg/kg s.c.) were significant ($31 \pm 4\%$ reduction of the number of c-Fos-IR nuclei in laminae I–II, $P<0.0001$; Figs. 2D and 3).

4. Discussion

In the present c-Fos protein study, we have evaluated the effects of nefopam in the formalin model of persistent nociception and in the noxious heat model of acute nociception in the rat. We have used the c-Fos protein expression, at the spinal cord level, as an indirect marker of neurons involved in spinal nociceptive transmission (for review, see Munglani and Hunt, 1995; Chapman and Besson, 1997). In agreement with previous studies, i.pl. injection of formalin (see Presley et al., 1990; Chapman et al., 1996; and references therein) or noxious heat (see Abbadie et al., 1994; Buritova and Besson, 2000; and references therein) evoked an abundant c-Fos protein expression in the ipsilateral spinal cord of the rat. In the present study, noxious heat-evoked c-Fos immunoreactive (c-Fos-IR) nuclei were principally localized in the superficial laminae (I–II) of the spinal dorsal horn (about 90% of the total number of c-Fos-IR nuclei per section). This predominant localization in laminae I–II is in good agreement with previous studies using the same noxious heat stimulation in anaesthetised rats (Williams et al., 1990; Wisden et al., 1990; Abbadie et al., 1994; Buritova et al., 1996; Buritova and Besson, 2000) or radiant noxious heat in awake rats (Hunt et al., 1995). Moreover, this laminar pattern of c-Fos-IR nuclei is in accordance with the electrophysiological data demonstrating a high proportion of superficial neurons driven exclusively, or not, by peripheral noxious stimuli, and with the fact that the majority of nociceptive primary afferents terminate in the superficial dorsal horn of the spinal cord (for review, see Besson and Chaouch, 1987; Willis and Coggeshall, 1991).

The acute noxious stimulation (brief noxious heat) mainly induces the c-Fos protein expression in superficial laminae (I–II) of the spinal cord, while for persistent stimuli (i.pl. injection of formalin) c-Fos-IR nuclei are also numerous in deeper laminae (V–VI) of the dorsal horn and in the ventral horn of the spinal cord (see Presley et al., 1990; Chapman et al., 1996; and references therein). In the present study, the formalin-evoked c-Fos-IR nuclei were predominantly located in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn (44% and 35% of the total number of c-Fos-IR nuclei per section, respectively). This laminar localisation of c-Fos-IR nuclei is in concordance with the spinal areas containing neurons activated by noxious stimuli driven by C- and Aδ-fibres (see references in Besson and Chaouch, 1987; Willis and Coggeshall, 1991). Moreover, morphine, an analgesic drug of reference, has been shown to depress the spinal c-Fos protein expression induced by various types of noxious stimuli, including those due to i.pl. formalin or noxious heat (see references in Chapman and Besson, 1997).

In the present study, the subcutaneous pre-administration of nefopam (5, 15 and 30 mg/kg) reduced the number of noxiously evoked c-Fos-IR nuclei in the formalin model of persistent nociception. These effects were not dose-related and were only significant for the doses of 15 and 30 mg/kg ($P<0.05$ for both). These results correspond with, and complete, previous studies demonstrating the antinociceptive effects of nefopam on behavioural responses to i.pl. injection of formalin in mice (Mather et al., 2000; Hunskaar and Hole, 1987; Hunskaar et al., 1987; Fasmer et al., 1987) and naked mole-rats (Kanui et al., 1993). In the present study, the reducing effects of nefopam on the spinal c-Fos protein expression were detectable 1 h after i.pl. injection of formalin, but not 2 h after. These results suggest the time-limitation of the effects of nefopam on persistent nociceptive processes in the formalin model.

For comparison of the effects of nefopam on persistent versus acute nociceptive processes, we evaluated the effects of this compound in the model of acute nociception due to a single noxious heat stimulation. To perform this study, we have selected the dose of 15 mg/kg (s.c.) of nefopam, sufficient to produce the significant reduction of the noxiously evoked spinal c-Fos protein expression in the formalin model. As discussed above, the noxious heat-evoked spinal c-Fos-IR nuclei were essentially located in superficial laminae I–II containing numerous neurons implicated in the spinal nociceptive transmission. The reducing effects of nefopam (15 mg/kg s.c.) at this laminar level were highly significant ($P<0.001$). These results are reminiscent of previous behavioural observations of antinociceptive effects of nefopam on acute thermal nociceptive processes (hot

plate test) in mice (Mather et al., 2000; Pallapies et al., 1994, Hunskaar et al., 1987; Fasmer et al., 1987; Esposito et al., 1986; Piercey and Schroeder, 1981).

The present study provides the first evidence for the reducing effects of nefopam on the spinal c-Fos protein expression evoked 1 h after noxious stimulation in both acute and persistent models of nociception in the rat. These results are reminiscent of those previously obtained with morphine in the same models of nociceptive processes (Abbadie et al., 1994; Presley et al., 1990). Previous c-Fos studies demonstrated that systemic pre-administration of morphine (2.5 mg/kg i.v. or 2.5 mg/kg s.c.) results in 42% and 53% reduction of the total number of c-Fos-IR nuclei, 2 h after noxious heat (Abbadie et al., 1994) and i.pl. formalin (Presley et al., 1990), respectively. Interestingly, systemic nefopam (15 mg/kg s.c.) produces 33% and 36% reduction of the total number of c-Fos-IR nuclei, 1 h after noxious heat and i.pl. formalin, respectively. However, this comparison must be taken with caution since numerous parameters of experiments are different (route of administration, doses, survival delays, etc.).

Considering the antinociceptive effects of nefopam, the negative data have been previously reported by electrophysiological studies in the cat (Besson et al., 1979). This is not in contrast with the present results since these studies (Besson et al., 1979) used small doses of nefopam (<10 mg/kg) in the model of acute nociception due to mechanical stimulation in healthy cats. As previously discussed, the effects obtained with higher doses of nefopam in the present study are in good agreement with previous behavioural studies in the formalin model of persistent nociception (Fasmer et al., 1987; Hunskaar et al., 1987; Kanui et al., 1993).

The present c-Fos data demonstrate the effects of nefopam on the neuronal populations (laminae I–II and V–VI) involved in the nociceptive transmission at the spinal cord level in rats. The reducing effects of nefopam on the number of noxiously evoked spinal c-Fos-IR nuclei reflect a reduction in the spinal nociceptive transmission. However, we cannot distinguish between the direct spinal depressive effect of nefopam and its effects on the activity of various descending pain modulating systems. Our data suggest that the site of action of nefopam is principally at the central level, i.e. at least a part, at the spinal cord level. In addition, considering the weak, but significant, effect of nefopam on the formalin-evoked peripheral edema, a peripheral site of its action cannot be excluded. However, according to the study of Guirimand et al. (1999) recording the nociceptive flexion (RIII) reflex in humans, the central mechanisms (spinal and/or supraspinal) of nefopam's effects are probably predominant (see also Fasmer et al., 1987; Piercey and Schroeder, 1981).

In conclusion, the present results provide first evidence for the reducing effects of nefopam on the noxiously evoked spinal c-Fos protein expression principally in acute nociceptive processes in the rat. In the formalin model of persistent nociception, the significant effects of nefopam are time-limited. These results suggest that nefopam may produce short lasting antinociceptive effects mainly in acute pain states.

References

Abbadie, C., Honoré, P., Fournié-Zaluski, M.C., Roques, B.P., Besson, J.M., 1994. Effects of opioids and non-opioids on c-Fos immunoreactivity induced in rat lumbar spinal cord neurons by noxious heat stimulation. Eur. J. Pharmacol. 258, 215–227.

Abbott, F.V., Franklin, K.B., Westbrook, R.F., 1995. The formalin test scoring properties of the first and second phases of the pain response in rats. Pain 60, 91–102.

Besson, J.M., Chaouch, A., 1987. Peripheral and spinal mechanisms of nociception. Physiol. Rev. 67, 67–186.

Besson, J.M., Le Bars, D., Menétrey, D., 1979. Nefopam and convergent dorsal horn units: a summary. Clin. Ther. 2 (Suppl. B).

Buritova, J., Besson, J.M., 1999. Spinal c-Fos protein expression and inflammatory nociceptive processes: pharmacological studies with nonsteroidal anti-inflammatory drugs and their associations in the awake rat. J. Musculoskeletal Pain 7, 71–92.

Buritova, J., Besson, J.M., 2000. Effects of flurbiprofen and its enantiomers on the spinal c-Fos protein expression induced by noxious heat stimuli in the anaesthetised rat. Eur. J. Pharmacol. 406, 59–67.

Buritova, J., Honoré, P., Besson, J.M., 1996. Ketoprofen produces profound inhibition of spinal c-Fos protein expression resulting from an inflammatory stimulus but not from noxious heat. Pain 67, 379–389.

Chapman, V., Besson, J.M., 1997. Pharmacological studies of nociceptive systems using the c-Fos immunohistochemical technique: an indicator of noxiously activated spinal neurones. In: Dickenson, A., Besson, J.M. (Eds.), The Pharmacology of Pain. Springer, Berlin, pp. 235–279.

Chapman, V., Buritova, J., Honoré, P., Besson, J.M., 1996. Physiological contributions of Neurokinin 1 receptor activation, and interaction with NMDA receptors, to inflammatory-evoked spinal c-Fos expression. J. Neurophysiol. 76, 1817–1827.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161–174.

Esposito, E., Romandini, S., Merlo-Pich, E., Mennini, T., Samanin, R., 1986. Evidence of the involvement of dopamine in the analgesic effect of nefopam. Eur. J. Pharmacol. 128, 157–164.

Fasmer, O.B., Berge, O.G., Jorgensen, H.A., Hole, K., 1987. Antinociceptive effects of (±)-, (+)- and (−)-nefopam in mice. J. Pharm. Pharmacol. 39, 508–511.

Gray, A.M., Nevinson, M.J., Sewell, R.D.E., 1999. The involvement of opioidergic and noradrenergic mechanisms in nefopam antinociception. Eur. J. Pharmacol. 365, 149–157.

Guirimand, F., Dupont, X., Bouhassira, D., Brasseur, L., Chauvin, M., 1999. Nefopam strongly depresses the nociceptive flexion (RIII) reflex in humans. Pain 80, 399–404.

Heel, R.C., Brogden, R.N., Pakes, G.E., Speight, T.M., Avery, G.S., 1980. Nefopam: a review of its pharmacological properties and therapeutic efficacy. Drug 19, 249–267.

Hsu, S., Raine, L., Fanger, H., 1981. Use of avidin–biotin–peroxydase complex (ABC) in immunoperoxydase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Hughes, P., Dragunow, M., 1995. Induction of immediate–early genes and the control of neurotransmitter-regulated expression within the nervous system. Pharmacol. Rev. 47, 133–178.

Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.

Hunskaar, S., Fasmer, O.B., Broch, O.J., Hole, K., 1987. Involvement of central serotonergic pathways in nefopam-induced antinociception. Eur. J. Pharmacol. 138, 77–82.

Hunt, S.P., Pini, A., Evan, G., 1995. Induction of c-Fos-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632–634.

Kanui, T.I., Karim, F., Towett, P.K., 1993. The formalin test in the naked mole-rat (*Heterocephalus glaber*): analgesic effects of morphine, nefopam and paracetamol. Brain Res. 600, 123–126.

Mather, G.G., Labroo, R., Le Guern, M.E., Lepage, F., Gillardin, J.M., Levy, R.H., 2000. Nefopam enantiomers: preclinical pharmacology/toxicology and pharmacokinetic characteristics in healthy subjects after intravenous administration. Chirality 12, 153–159.

Mimoz, O., Incagnoli, P., Josse, C., Gillon, M.-C., Kuhlman, L., Mirand, A., Soilleux, H., Fletcher, D., 2001. Analgesic efficacy and safety of nefopam vs. propacetamol following hepatic resection. Anaesthesia 56, 520–525.

Molander, C., Grant, G., 1986. Laminar distribution and somatotopic organization of primary afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. Neuroscience 19, 297–312.

Molander, C., Xu, Q., Grant, G., 1984. The cytoarchitectonic organization of the spinal cord in the rat: I. The lower thoracic and lumbosacral cord. J. Comp. Neurol. 230, 133–141.

Morgan, J.I., 1991. Proto-oncongene expression in the nervous system. Discussions in Neuroscience. Elsevier, Amsterdam.

Morgan, J.I., Curran, T., 1995. Immediate–early genes: ten years on. Trends Neurosci. 18, 66–67.

Munglani, R., Hunt, S.P., 1995. Molecular biology of pain. Br. J. Anaesth. 75, 186–192.

Pallapies, D., Peskar, B.A., Brune, K., Zeilhofer, H.U., 1994. Modulation of nitric oxide effects by flurbiprofen enantiomers and nefopam and its relation to antinociception. Eur. J. Pharmacol. 271, 335–340.

Peterson, M.A., Basbaum, A.I., Abbadie, C., Rohde, D.S., McKay, W.R., Taylor, B.K., 1997. The differential contribution of capsaicin-sensitive afferents to behavioral and cardiovascular measures of brief and persistent nociception and to Fos expression in the formalin test. Brain Res. 755, 9–16.

Piercey, M.F., Schroeder, L.A., 1980. A quantitative analgesic assay in the rabbit based on the response to tooth pulp stimulation. Arch. Int. Pharmacodyn. 248, 294–304.

Piercey, M.F., Schroeder, L.A., 1981. Spinal and supraspinal sites for morphine and nefopam analgesia in the mouse. Eur. J. Pharmacol. 74, 135–140.

Presley, R.W., Menétrey, D., Levine, J.D., Basbaum, A.I., 1990. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. 10, 323–335.

Re, O.N., 1976. Nefopam hydrochloride (Acupan), a new analgesic. Adv. Pain. Res. Ther. 1, 537–541.

Rexed, B., 1952. The cytoarchitectonic organization of the spinal cord in the cat. J. Comp. Neurol. 96, 415–495.

Williams, S., Evan, G.I., Hunt, S.P., 1990. Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. Neuroscience 36, 73–81.

Willis, W.D., Coggeshall, R.E., 1991. Sensory Mechanisms of the Spinal Cord. Plenum, New York, pp. 79–151.

Wisden, W., Errington, M.L., Williams, S., Dunnett, S.B., Waters, C., Hitchcock, S., Evan, G., Bliss, T.V.P., Hunt, S.P., 1990. Differential expression of immediate early genes in the hippocampus and spinal cord. Neuron 4, 603–614.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.