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Effects of flurbiprofen and its enantiomers on the spinal c-Fos protein expression induced by noxious heat stimuli in the anaesthetized rat

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Abstract

We have evaluated the effects of either intravenous or intraplantar administration of racemic-, *S*(+)- and *R*(−)-flurbiprofen on the spinal c-Fos protein expression after a single noxious heat stimulation (52°C for 15 s) of the rat hindpaw in urethane anaesthetized rats. Two hours after noxious heat, numerous c-Fos protein immunoreactive (c-Fos-IR) nuclei (> 70 c-Fos-IR nuclei per section at the level of L4–L5 segments) were observed with essential localization in the superficial (I–II) laminae of the spinal dorsal horn, i.e. areas containing numerous neurons driven exclusively by noxious stimuli. Considering the number of c-Fos-IR nuclei in laminae I–II, the intravenous injection of racemic-flurbiprofen (0.3, 3 and 9 mg/kg) was inefficacious and *S*(+)-flurbiprofen had weak and non-dose-related effects. The same doses of *R*(−)-flurbiprofen produced dose-related effects ($r = 0.58$, $P < 0.05$) with weak, but significant, effects for doses of 3 and 9 mg/kg (18 ± 6% and 26 ± 5% reduction of the number of noxious heat-evoked c-Fos-IR nuclei in laminae I–II, $P < 0.05$ and $P < 0.01$, respectively). The weak effects of *R*(−)-flurbiprofen are probably due to the central site of action since the intraplantar injection of a relatively high dose of 30 μg is inefficacious. These results provide further evidence for weak effects of non-steroidal anti-inflammatory drugs and their enantiomers on the acute responses to nociceptive stimulus which are very efficacious upon inflammatory nociception, but not upon brief noxious heat-evoked nociception.

Keywords: c-Fos; Racemic-flurbiprofen; *S*(+)-flurbifrofen; *R*(−)-flurbifrofen; Noxious heat; Spinal dorsal horn

1. Introduction

Flurbiprofen, an anti-inflammatory/analgesic drug, is a member of the 2-arylpropionic acid class of non-steroidal anti-inflammatory drugs (NSAIDs; for review see Insel, 1991), i.e. a group of NSAIDs which possess a chiral carbon and exist as racemates. The availability of pure *S*(+) and *R*(−) enantiomers of flurbiprofen contributed to the extensive research into possible site(s) and mechanism(s) of action of NSAIDs (for review see Jamali et al., 1988; Brune et al., 1992; Wechter, 1994; Evans, 1996; Geisslinger and Shaible, 1996; Buritova and Besson, 1998). Behavioural studies have demonstrated the antinociceptive effects for both *S*(+) and *R*(−) enantiomers of flurbiprofen after oral (Brune et al., 1991), intraperitoneal (Geisslinger et al., 1994) or intrathecal (Malmberg and Yaksh, 1994) administration in the rat. In addition, an electrophysiological study (Neugebauer et al., 1995) demonstrated the antinociceptive effects after intravenous administration of either *S*(+) or *R*(−) enantiomers of flurbiprofen, and after intraplantar injection of its *S*(+), but not *R*(−), enantiomer, in a model of inflammatory nociception. Furthermore, in an immunohistochemical study using c-Fos protein technique in the carrageenan model of inflammatory nociception, we have recently provided further evidence for anti-inflammatory and antinociceptive effects of (i) the intravenous administration of racemic-, *S*(+)- and *R*(−)-flurbiprofen, and (ii) the intraplantar injection of racemic- and *S*(+)-flurbiprofen, but not its *R*(−) enantiomer (Buritova and Besson, 1998). Overall, these results observed in inflammatory pain conditions suggest that both the peripheral and central sites of action of racemic − and *S*(+)-flurbiprofen exist, and indicate that the site of action of *R*(−)-flurbiprofen could be mainly of central origin. The aim of the present study is to consider the effects of flurbiprofen and its enantiomers under non-inflammatory nociceptive conditions. In an at-

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tempt to address this issue, we have studied the effects of racemic-, *S*(+)- and *R*(−)-flurbiprofen, administered either intravenously or intraplantarly, in a model of acute, non-inflammatory, nociception due to a single noxious heat stimulation in the anaesthetised rat. For the evaluation of these effects, we have used the method of c-Fos protein immunoreactivity at the spinal cord level. The expression of nuclear protein c-Fos encoded by the immediate-early gene c-*fos* (for review see Morgan, 1991; Hughes and Dragunow, 1995; Morgan and Curran, 1995) is widely used as an indirect marker of neurons involved in spinal nociceptive transmission (for review see Munglani and Hunt, 1995; Chapman and Besson, 1997). We used a single noxious heat stimulation (52°C, 15 s), which induces the expression of c-Fos protein in the dorsal horn of the lumbar spinal cord in the anaesthetised rat (see Refs. in Abbadie et al., 1994; Buritova et al., 1996a).

2. Materials and methods

2.1. Experimental animals

Three experimental series were performed on 65 rats (i.e. 60 noxious heat-stimulated and five non-stimulated rats). All rats were adult male albino Sprague–Dawley rats (Charles River, France), weighing 225–250 g. The ethical guidelines of the International Association for the Study of Pain, for investigations of experimental pain were followed (Zimmermann, 1983). One week prior to experiments, rats were housed in a plastic breeding cage at a constant temperature of 22°C in an animal room with a 12 h alternating light–dark cycle and with free access to water and food.

2.2. Drug administration

Racemic-, *S*(+)- and *R*(−)-flurbiprofen (Knoll Pharmaceutical, Nottingham, England) were dissolved in vehicle (for 10 ml: 1 ml of ethanol, 1 ml of cremofor, 8 ml of saline) and injected either intravenously or intraplantarly in the anaesthetised rat. Both the intravenous (into the tail vein) and intraplantar (subcutaneous) injections were made with a 25-gauge needle.

2.3. Noxious heat stimulation (52°C, 15 s)

Rats were anaesthetised with urethane (1500 mg/kg i.p.; ethyl carbamate, Prolabo, France) prior to drug treatment and noxious heat stimulation, i.e. 10 min prior to intravenous or intraplantar injection of studied substances. 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 and the timing of perfusion at 2 h after heat stimulation were chosen according to previous studies of spinal c-Fos protein expression resulting from a single noxious heat stimulation (see Williams et al., 1990; Wisden et al., 1990; Abbadie et al., 1994; Buritova et al., 1996a).

2.4. Experimental protocol and immunohistochemistry

All stimulated rats were perfused 2 h after noxious heat stimulation (see above). In addition, we perfused the group of non-stimulated rats. For all rats, paw and ankle diameters were measured with calibrated callipers, under deep anaesthesia (Pentobarbital, Sanofi; 55 mg/kg i.p.), immediately before perfusion (for more details see Methods in Buritova et al., 1996b). Rats were perfused intracardially with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord was removed, postfixed for 4 h and cryoprotected in 30% sucrose overnight. Frozen serial frontal sections (40 μm) of the lumbar spinal cord were cut. Immunohistochemistry of the free floating sections was performed with polyclonal antiserum, generated in rabbits and directed against the c-Fos protein (Oncogene Science, Ab-2 solution, 0.1 mg/ml diluted at 1:4000), using the method of Hsu et al. (1981). The c-Fos protein-labeled nuclei was visualised by 1-naphtol ammonium carbonate solution (Ménetrey et al., 1992). The sections were mounted on gelatin-subbed slides and intensified by 0.025% crystal violet in bidistilled water. After bidistilled water rinses to take off the excess stain, sections were differentiated in 70% alcohol (differentiation time was evaluated using control under the microscope). Finally, the slides were air dried and coverslipped. For more details see experimental procedures previously described by Honoré et al. (1995).

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

As previously described (Honoré et al., 1995), the c-Fos-labeled nuclei, i.e. c-Fos-IR nuclei, were counted with a camera lucida attachment through four arbitrary defined regions of the spinal grey matter of the L4–L5 segments, according to the cytoarchitectonic organisation of the spinal cord (Rexed, 1952; Molander et al., 1984; Molander and Grant, 1986): superficial laminae (laminae I–II), nucleus proprius (laminae III–IV) and deep laminae (laminae V–VI) of dorsal horn and, in addition, ventral horn (laminae VII–X) of the spinal cord. For each rat, two counts were made: (1) the total number of c-Fos-labeled nuclei in the grey matter for 10 sections through L4–L5 segments, and (2) in these 10 sections, the number of c-Fos-labeled nuclei per four defined regions (see above). Plotting and counting of c-Fos-labeled nuclei was performed blind to the experimental conditions. One-way analysis of variance (ANOVA) was conducted for comparison across the experimental conditions. The Fisher's protected least-significant difference test was applied to define

which group contributed to these differences. Significance was taken as $P < 0.05$.

2.6. Experimental design

In the present study, the doses of racemic-, *S*(+)- and *R*(−)-flurbiprofen were chosen considering a previous electrophysiological study of the effects of *S*(+)- and *R*(−)-flurbiprofen in the model of knee joint inflammation induced by kaolin and carrageenan (Neugebauer et al., 1995) and our previous c-Fos protein study of effects of racemic-, *S*(+)- and *R*(−)-flurbiprofen in carrageenan-evoked inflammation (Buritova and Besson, 1998). Note that doses up to 9 mg/kg i.p. of *S*(+)- and up to 27 mg/kg i.p. of *R*(−)-flurbiprofen did not produce any sedative side effects in a behavioural test of locomotor activity of rats (Neugebauer et al., 1995).

In the first experimental series, racemic-, *S*(+)- and *R*(−)-flurbiprofen (0.3, 3 and 9 mg/kg for each substance; $n = 5$ rats for each group; volume 0.25 ml) were administered intravenously into the tail vein of the anaesthetised rat, 25 min prior to noxious heat stimulation. Control anaesthetised rats ($n = 5$) received intravenous injection of vehicle (0.25 ml), 25 min prior to noxious heat stimulation.

In the second experimental series, racemic-, *S*(+)- and *R*(−)-flurbiprofen (30 μg in 50 μl of saline for each substance; $n = 5$ rats for each group) were injected intraplantarly in the anaesthetised rat, 5 min prior to noxious heat stimulation. Control anaesthetised rats ($n = 5$) received intraplantar injection of vehicle (50 μl), 5 min prior to noxious heat stimulation.

In the third experimental series, racemic-, *S*(+)- and *R*(−)-flurbiprofen (100 μg in 50 μl of saline for each substance; $n = 5$ rats for each group) were injected intraplantarly in the anaesthetised rat, 5 min prior to noxious heat stimulation. Control anaesthetised rats ($n = 5$) received intraplantar injection of vehicle (50 μl), 5 min prior to noxious heat stimulation.

In this study, the rats receiving intravenous vehicle alone or racemic-flurbiprofen alone (without noxious heat stimulation) were not included since we have previously shown that 3 h after intravenous injection of vehicle or racemic-flurbiprofen (9 mg/kg), the c-Fos-labeled nuclei were virtually absent in the lumbar spinal cord, and paw and ankle diameters were not modified (Buritova and Besson, 1998).

3. Results

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

Two hours after a single noxious heat stimulation (52°C, 15 s), the number of spinal c-Fos-labeled nuclei and their laminar distribution were similar in the control groups for the three experimental series (Table 1). In control groups, c-Fos-labeled nuclei were predominantly located in the superficial laminae I–II (about 70% of the number of c-Fos-labeled nuclei per section in segments L4–L5; Table 1, Figs. 1 and 2). In contrast, the number of c-Fos-labeled nuclei in the deep laminae V–VI of the dorsal horn was moderate (about 16% of the number of c-Fos-labeled nuclei). Very few c-Fos-labeled nuclei were located in nucleus proprius (laminae III–IV) and in the laminae VII–X of the ventral horn (between 5 and 8 c-Fos-labeled nuclei per section in segments L4–L5). c-Fos-labeled nuclei are virtually absent in the contralateral lumbar spinal cord (< 3 c-Fos-labeled nuclei per section in segments L4–L5).

Table 1
The spinal c-Fos protein expression and paw and ankle diameters in the control groups ($n = 5$ rats for each) of three experimental series (series I–III), 2 h after single noxious heat stimulation (52°C, 15 s) in anaesthetised rats

Stimulus	Series	Number of c-Fos-IR nuclei		Diameter	
		Total number	Laminae I–II	Paw	Ankle
Noxious heat (52°C)	I	75 ± 2	51 ± 2	0.48 ± 0.02	0.80 ± 0.02
	II	73 ± 4	52 ± 3	0.48 ± 0.01	0.79 ± 0.01
	III	85 ± 3	60 ± 3	0.49 ± 0.01	0.81 ± 0.02

Results are expressed as the 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), and as mean value (± S.E.M.) of the diameter at the paw and ankle levels (paw diameter, ankle diameter), 2 h after noxious heat. The values for paw and ankle diameters of stimulated rats did not vary from those of non-stimulated rats, which are 0.48 ± 0.02 and 0.75 ± 0.03 cm for paw and ankle diameters, respectively.

Two hours after noxious heat stimulation, there is no detectable peripheral oedema. Neither paw nor ankle diameters in control heat-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).

3.2. Effects of intravenous racemic-, S(+)- and R(−)-flurbiprofen on nociceptive processes in the anaesthetised rat

Considering the total number of noxious heat-evoked c-Fos-labeled nuclei, the doses of 3 and 9 mg/kg (i.v.) of Racemic-flurbiprofen had weak, but significant, reducing effects (Table 2; Figs. 1 and 2). Likewise, the effects of all doses of *S*(+)- and *R*(−)-flurbiprofen on the total number of c-Fos-labeled nuclei were weak, but significant (Table 2). These reducing effects were dose-related for

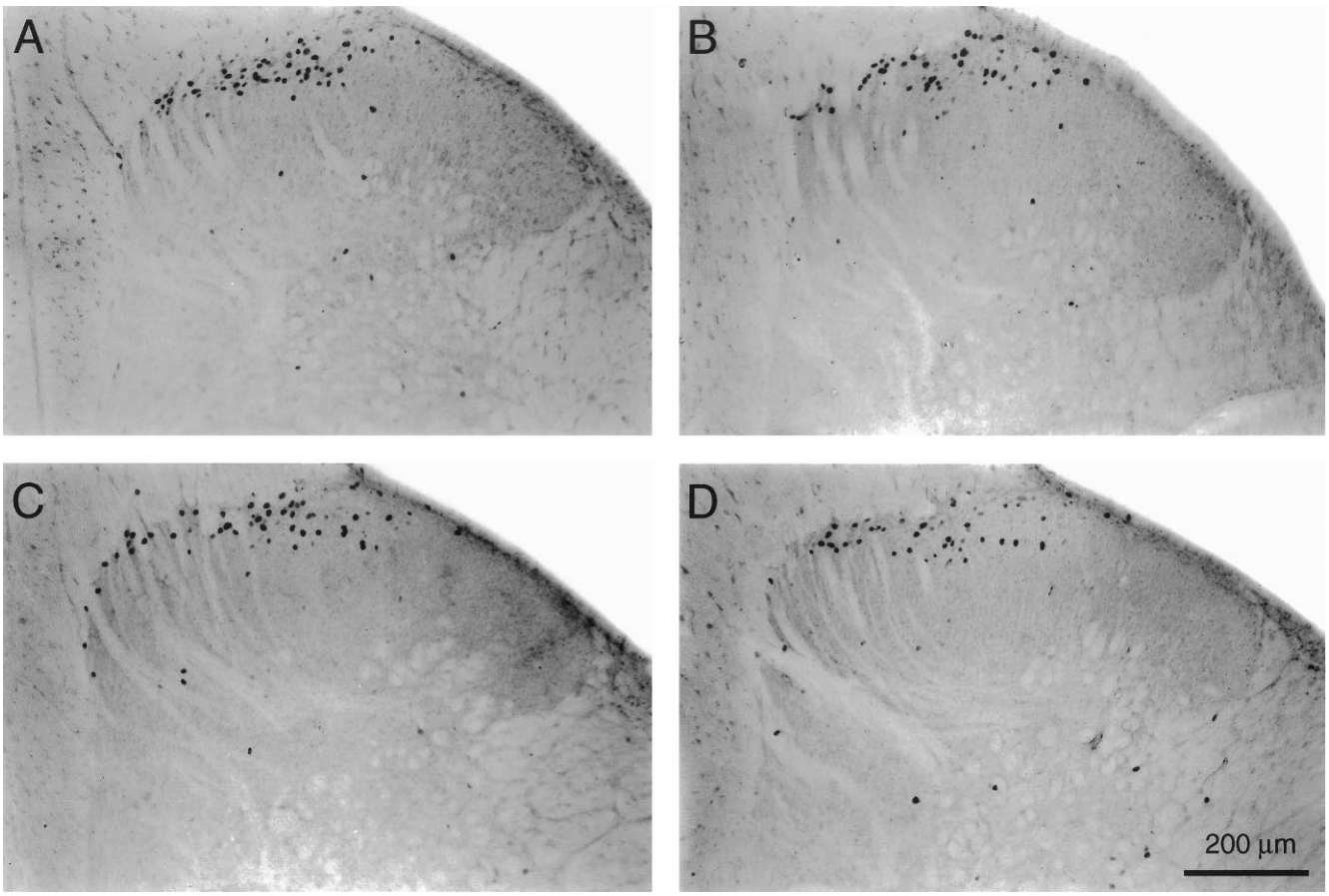

Fig. 1. Photomicrographs illustrating the effects of intravenous pre-administration of racemic-, *S*(+)- and *R*(−)-flurbiprofen on the spinal c-Fos protein expression, 2 h after noxious heat stimulation (52°C, 15 s) in anaesthetised rats. Each microphotograph is an individual representative example of one section (40 μm) at the level of L4–L5 segments including c-Fos protein-labeled nuclei (black dots) in laminae I–V of dorsal horn, in the ipsilateral side to heat stimulation. Four experimental situations are represented: noxious heat stimulation plus pre-administration of intravenous vehicle (A; control) or racemic-flurbiprofen 9 mg/kg i.v. (B), *S*(+)-flurbiprofen 9 mg/kg i.v. (C) and *R*(−)-flurbiprofen 9 mg/kg i.v. (D). Scale bar = 200 μm.

R(−)-flurbiprofen (regression coefficient $r = 0.623$; $P < 0.05$), but not for racemic- and *S*(+)-flurbiprofen (regression coefficients $r = 0.439$ and $r = 0.215$, respectively; $P > 0.05$ for both compounds). The effects of the two

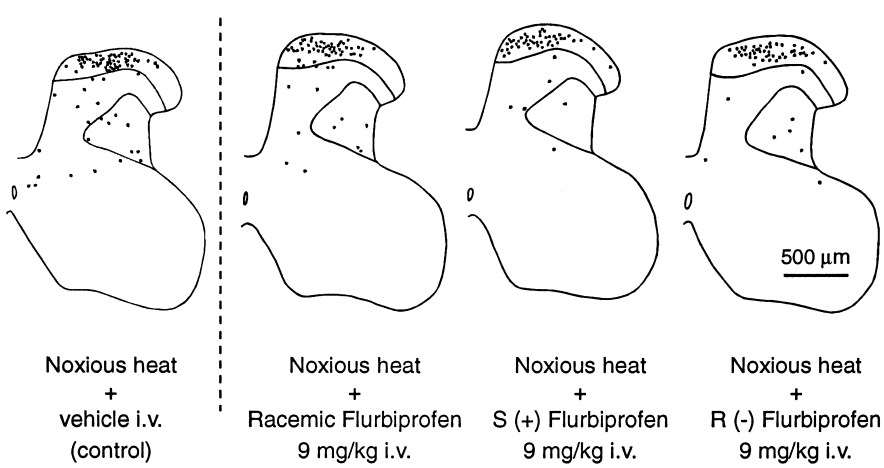

Fig. 2. Camera lucida drawings illustrating the effects of intravenous pre-administration of racemic-, *S*(+) or *R*(−)-flurbiprofen (9 mg/kg for each substance) on the spinal c-Fos protein expression, 2 h after noxious heat stimulation (52°C, 15 s) in anaesthetised rats. Each camera lucida drawing is an individual representative example of one (40 μm) section at the level of L4–L5 segments including c-Fos protein-labeled nuclei in the ipsilateral side to heat stimulation. Each dot represents one c-Fos protein-labeled nucleus. The boundaries of the superficial laminae and of the reticular part of the laminae V–VI of the dorsal horn are outlined. Scale bar = 500 μm.

Table 2
The noxious heat-evoked spinal c-Fos protein expression and paw and ankle diameters in the control group and groups receiving intravenous pre-administration of racemic-, *S*(+)- or *R*(−)-flurbiprofen (0.3, 3 and 9 mg/kg for each substance; $n = 5$ for each group) prior to noxious heat stimulation (52°C, 15 s) in anaesthetised rats

Group	Dose, mg/kg i.v.	Number of c-Fos-IR nuclei		Paw diameter	Ankle diameter
		Total number	Laminae I–II		
Controls	–	81 ± 6	56 ± 4	0.49 ± 0.01	0.75 ± 0.02
Racemic flurbiprofen	0.3	73 ± 4 (11 ± 5)	56 ± 3 (1 ± 5)	0.47 ± 0.01	0.75 ± 0.01
	3.0	69 ± 3 (16 ± 4)[a]	54 ± 4 (4 ± 6)	0.49 ± 0.02	0.75 ± 0.04
	9.0	64 ± 2 (21 ± 3)[b]	52 ± 2 (8 ± 4)	0.49 ± 0.03	0.77 ± 0.02
S(+) flurbiprofen	0.3	61 ± 2 (25 ± 3)[c]	49 ± 3 (14 ± 5)	0.50 ± 0.03	0.76 ± 0.02
	3.0	61 ± 2 (25 ± 3)[c]	47 ± 2 (17 ± 4)[a]	0.43 ± 0.01	0.76 ± 0.02
	9.0	58 ± 4 (29 ± 5)[c]	48 ± 2 (15 ± 4)	0.47 ± 0.01	0.76 ± 0.01
R(−)- flurbiprofen	0.3	67 ± 4 (18 ± 5)[b]	55 ± 4 (2 ± 6)	0.46 ± 0.02	0.75 ± 0.02
	3.0	56 ± 3 (31 ± 4)[c]	46 ± 4 (18 ± 6)[a]	0.54 ± 0.05	0.76 ± 0.03
	9.0	51 ± 3 (37 ± 4)[c]	42 ± 3 (26 ± 5)[b]	0.48 ± 0.01	0.76 ± 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), and as mean value (±S.E.M.) of the diameter at the paw and ankle levels (paw diameter, ankle diameter), 2 h after noxious heat. Results expressed as % reduction of control value of studied parameters are presented in brackets. Significance compared to control group was performed using ANOVA and Fisher's PLSD test.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

highest doses (3 and 9 mg/kg i.v.) of *R*(−)-flurbiprofen on the total number of c-Fos-labeled nuclei were significantly more pronounced than those of the same doses of racemic-flurbiprofen ($P < 0.05$ for both doses).

Since 70% of spinal c-Fos-labeled nuclei were located in superficial laminae the laminar analyses were restricted to this laminar level (laminae I–II). Racemic-flurbiprofen (0.3, 3 and 9 mg/kg i.v.) did not influence the number of c-Fos-labeled nuclei in superficial laminae I–II as compared to the control heat-stimulated group (Table 2). A weak, but significant, effect was observed with only one dose of 3 mg/kg (i.v.) of *S*(+)-flurbiprofen ($P < 0.05$) and with the two highest doses (3 and 9 mg/kg i.v.) of *R*(−)-flurbiprofen ($P < 0.05$ and $P < 0.01$, respectively), see Table 2. These reducing effects of *R*(−)-flurbiprofen on the number of c-Fos-labeled nuclei in laminae I–II were dose-related (regression coefficient $r = 0.58$, $P < 0.05$).

Note that neither racemic-, *S*(+)- nor *R*(−)-flurbiprofen (0.3, 3 and 9 mg/kg i.v. for each substance) influenced the paw and ankle diameters as compared to those of the control heat-stimulated group (Table 2).

3.3. Effects of intraplantar racemic-, S(+)- and R(−)-flurbiprofen on nociceptive processes in the anaesthetised rat

Neither intraplantar racemic-, nor *S*(+)- nor *R*(−)-flurbiprofen (30 μg for each substance) modified the number of spinal c-Fos-labeled nuclei induced 2 h after single noxious heat stimulation (Table 3). The very high dose of 100 μg, for each substance studied, was necessary to obtain a weak reduction of the total number of noxious heat-evoked c-Fos-labeled nuclei (between 16% and 20% of reduction; Table 4). Laminar analyses revealed that the weak effects of 100 μg of racemic-, *S*(+)- or *R*(−)-flur-

Table 3
The noxious heat-evoked spinal c-Fos protein expression and paw and ankle diameters in the control group and groups receiving intraplantar (i.pl.) racemic-, *S*(+)- or *R*(−)-flurbiprofen (30 μg for each substance; $n = 5$ for each group) prior to noxious heat stimulation (52°C, 15 s) in anaesthetised rats

Group	Dose, μg i.pl.	Number of c-Fos-IR nuclei		Paw diameter (cm)	Ankle diameter (cm)
		Total number	Laminae I–II		
Controls	–	73 ± 4	52 ± 3	0.48 ± 0.01	0.79 ± 0.01
Racemic flurbiprofen	30	70 ± 3	53 ± 3	0.51 ± 0.02	0.80 ± 0.02
S(+)- flurbiprofen	30	68 ± 3	52 ± 3	0.49 ± 0.01	0.79 ± 0.02
R(−)- flurbiprofen	30	73 ± 3	53 ± 2	0.51 ± 0.02	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), and as mean value (±S.E.M.) of the diameter at the paw and ankle levels (paw diameter, ankle diameter), 2 h after noxious heat. No significance compared to control group was revealed using ANOVA and Fisher's PLSD test.

Table 4
The noxious heat-evoked spinal c-Fos protein expression and paw and ankle diameters in the control group and groups receiving intraplantar (i.pl.) racemic-, *S*(+)- or *R*(−)-flurbiprofen (100 μg for each substance; $n = 5$ for each group) prior to noxious heat stimulation (52°C, 15 s) in anaesthetised rats

Group	Dose, μg i.pl.	Number of c-fos-IR Nuclei		Paw diameter (cm)	Ankle diameter (cm)
		Total number	Laminae I–II		
Controls	–	85 ± 3	60 ± 3	0.49 ± 0.01	0.81 ± 0.03
Racemic flurbiprofen	100	69 ± 3 (18 ± 4)[a]	50 ± 3 (17 ± 5)[a]	0.48 ± 0.02	0.78 ± 0.02
S(+)-flurbiprofen	100	71 ± 3 (16 ± 4)[a]	52 ± 2 (14 ± 3)[b]	0.50 ± 0.02	0.81 ± 0.01
R(−)- flurbiprofen	100	68 ± 2 (20 ± 3)[a]	49 ± 2 (19 ± 4)[a]	0.47 ± 0.01	0.79 ± 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), and as mean value (±S.E.M.) of the diameter at the paw and ankle levels (paw diameter, ankle diameter), 2 h after noxious heat. Results expressed as % reduction of control value of studied parameters are presented in brackets. Significance compared to control group was performed using ANOVA and Fisher's PLSD test.
[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.001$.

biprofen were essentially due to a decrease of the number c-Fos-labeled nuclei in superficial laminae (see Laminae I–II in Table 4). Neither racemic-, nor *S*(+)- nor *R*(−)-flurbiprofen (30 μg or 100 μg i.p. for each substance) influenced the paw and ankle diameters as compared to those of the control heat-stimulated group (Tables 3 and 4).

4. Discussion

In the present study, 2 h after a single noxious heat stimulation (water bath at 52°C for 15 s) of the hindpaw in urethane anaesthetised rats, c-Fos protein-labeled nuclei were numerous and predominantly localized in the superficial laminae (I–II) of the dorsal horn of L4–L5 segments. This predominant localization of noxious heat-evoked c-Fos-labeled nuclei in superficial laminae (I–II) is in good agreement with previous studies using the same stimulation in anaesthetised rats (Williams et al., 1990; Wisden et al., 1990; Abbadie et al., 1994; Buritova et al., 1996a) or radiant noxious heat in awake rats (Hunt et al., 1987). Quantitatively, our results were highly reproducible in the control groups of the three experimental series when considering the number of c-Fos-labeled nuclei and their laminar distribution (Table 1). The laminar pattern of c-Fos-labeled nuclei after noxious heat stimulation is in good keeping with the electrophysiological studies demonstrating a high proportion of superficial neurons driven exclusively by 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). Taken together, these data are in favour of the use of the c-Fos protein as an indirect preferential marker of neurons involved in nociceptive transmission at the spinal cord level after noxious heat stimulation (see Williams et al., 1990).

Numerous previous studies have demonstrated strong reduction of noxious heat-evoked spinal c-Fos protein expression by intravenous administration of morphine (see Refs. in Chapman and Besson, 1997), but not by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin (Abbadie et al., 1994) and ketoprofen (Buritova et al., 1996a) in the anaesthetised rat. In the present study, we extended these investigations by considering the effects of flurbiprofen and its enantiomers *S*(+) and *R*(−) on the spinal c-Fos protein expression induced by a single noxious heat stimulation. For intravenous administration of racemic-, *S*(+)- or *R*(−)-flurbiprofen, we used the doses (0.3, 3 and 9 mg/kg for each compounds) which have been previously shown to reduce spinal c-Fos protein expression evoked by inflammatory noxious stimulation in awake rats (Buritova and Besson, 1998).

When considering the total number of c-Fos-labeled nuclei, the present results showed a weak, but significant, reduction of the noxious heat-evoked spinal c-Fos protein expression by intravenous administration of flurbiprofen and its *S*(+) and *R*(−) enantiomers in the urethane anaesthetised rat. These reducing effects were dose-related for *R*(−)-flurbiprofen, but not for racemic- and *S*(+)-flurbiprofen. Laminar analyses revealed that the effects of racemic-, *R*(−)- and *S*(+)-flurbiprofen on the total number of noxious heat-evoked c-Fos-labeled nuclei were due principally to the significant effects of these compounds on the number of c-Fos-labeled nuclei in deep laminae (V–VI) of the spinal dorsal horn (Table 2). However, these results must be taken with caution since there is a possible bias due to the fact that the number of c-Fos-labeled nuclei was low in these later laminae (i.e. 16 ± 2 c-Fos-labeled nuclei in laminae V–VI, 2 h after noxious heat). For such reason, our quantitative analyses essentially concern superficial laminae (I–II; Table 5) where numerous neurons express c-Fos protein following noxious stimulation. Moreover, in these spinal regions corresponding to the main termination sites of projection of peripheral nociceptors, a high propor-

Table 5
Comparison of the effects of intravenous pre-administration of racemic-, *S*(+)- or *R*(−)-flurbiprofen (0.3, 3 and 9 mg/kg for each substance; $n = 5$ for each group) on the spinal c-Fos protein expression following noxious stimulation due to noxious heat (52°C, 15 s; present study) and intraplantar injection of carrageenan (modified from Buritova and Besson, 1998)

Group	Dose, mg/kg i.v.	% reduction of the number of c-Fos-IR nuclei in laminae I–II	
		Heat	Carrageenan
Racemic flurbiprofen	0.3	−1 ± 5	−35 ± 2[a]
	3.0	−4 ± 6	−48 ± 6[a]
	9.0	−8 ± 4	−64 ± 6[a]
S(+)-flurbiprofen	0.3	−14 ± 5	−41 ± 7[a]
	3.0	−17 ± 4[b]	−44 ± 7[a]
	9.0	−15 ± 4	−64 ± 4[a]
R(−)-flurbiprofen	0.3	−2 ± 6	−26 ± 14[b]
	3.0	−18 ± 6[b]	−23 ± 8[a]
	9.0	−26 ± 5[a]	−34 ± 7[a]

Results are expressed as % reduction of control value (± S.E.M.) of the number of c-Fos protein immunoreactive (c-Fos-IR) nuclei in superficial laminae (Laminae I–II) per section at the level of L4–L5 segments. Significance compared to corresponding control group was performed using ANOVA and Fisher's PLSD test.
[a] $P < 0.001$.
[b] $P < 0.05$.

tion of neurons are driven exclusively by noxious stimuli (see above). Racemic- and *S*(+)-flurbiprofen were inefficacious as considering the number of c-Fos-labeled nuclei in laminae I–II. For *R*(−)-flurbiprofen, the effects in the superficial laminae I–II were significant and dose-related.

The comparison of the present data with those previously obtained with the same doses of racemic-, *S*(+)- or *R*(−)-flurbiprofen in the carrageenan model of inflammatory pain (Buritova and Besson, 1998) clearly demonstrate that the effects of flurbiprofen and its enantiomers *S*(+) and *R*(−) are partly dependent on the stimulus modality (i.e. acute vs. persistent, non-inflammatory vs. inflammatory). Interestingly, comparison of the two c-Fos protein studies indicates that in the superficial dorsal horn, the number of noxiously evoked c-Fos-labeled nuclei in control groups is in the similar order in the two models studied: $n = 56 \pm 4$ or $n = 64 \pm 4$ c-Fos-labeled nuclei in superficial laminae after noxious heat (present study) or intraplantar injection of carrageenan (Buritova and Besson, 1998), respectively. Thus, the comparison between two studies is valuable. As shown in Table 5, racemic-flurbiprofen is not efficacious on noxious heat-evoked c-Fos protein expression, even for the highest dose (9 mg/kg i.v.), while the three doses studied induced highly significant effects on carrageenan-evoked c-Fos protein expression ($P < 0.001$ for the three doses studied), these effects being clearly dose-related (Buritova and Besson, 1998). Similarly, the effects of *S*(+)-flurbiprofen are dose-related and highly significant ($P < 0.001$ for the three doses studied) in the carrageenan model (Buritova and Besson, 1998), while they are extremely weak and not dose-related in the noxious heat model (see Table 5).

For *R*(−)-flurbiprofen, the dose of 0.3 mg/kg (i.v.) is efficacious in inflammatory pain conditions (Buritova and Besson, 1998), but not for brief noxious heat (Table 5). For the highest dose (9 mg/kg i.v.) of *R*(−)-flurbiprofen, the significant depressive effect ($P < 0.001$) was obtained whatever the modality of noxious stimulus (Table 5). However, even for the highest dose, the percentage of depressive effects of *R*(−)-flurbiprofen was moderate (26 ± 5% and 34 ± 7% reduction of the noxious heat- and carrageenan-evoked number of c-Fos-labeled nuclei, respectively; Table 5). Statistical analyses (ANOVA test) revealed that in superficial laminae I–II, there is no significant difference of effects of *R*(−)-flurbiprofen on the number of c-Fos-labeled nuclei induced by two modalities of noxious stimulation (noxious heat vs. carrageenan). In contrast, the same ANOVA analyses indicated that the effects of *S*(+) enantiomer were significantly more pronounced in inflammatory pain conditions than in non-inflammatory conditions due to the brief noxious heat ($P < 0.01$, $P < 0.05$ and $P < 0.001$ for 0.3, 3 and 9 mg/kg of *S*(+)-flurbiprofen, respectively).

The lack of effect of intravenous racemic- and *S*(+)-flurbiprofen on the noxious heat-evoked c-Fos protein expression in the superficial dorsal horn could be due to the fact that we used a single and brief noxious heat stimulation which did not induce any clinical signs of inflammation. This notion is strongly supported by data which demonstrated that in the present study using noxious heat, the intraplantar injection of 30 μg of racemic- or *S*(+)-flurbiprofen was inefficacious. In contrast, the intraplantar injection of these two compounds strongly decreased both the carrageenan-evoked inflammatory signs and spinal c-Fos protein expression (Buritova and Besson, 1998). For example, extremely low doses of intraplantar racemic-flurbiprofen (1 μg) and *S*(+)-flurbiprofen (0.1 μg) significantly reduced the number of c-Fos-labeled nuclei in the carrageenan model of inflammatory nociception ($P < 0.01$ and $P < 0.05$, respectively). The depressive

effects of these two compounds on the carrageenan-evoked spinal c-Fos protein expression were dose-related and, in addition, correlated with those on peripheral oedema (Buritova and Besson, 1998). As an example, the intraplantar injection of 30 μg of racemic- and *S*(+)-flurbiprofen markedly decreased the carrageenan-evoked spinal c-Fos expression (52 ± 5% and 54 ± 4% reduction of the number of c-Fos-labeled nuclei in laminae I–II, respectively, $P < 0.001$ for both) and inflammatory signs (67 ± 7% and 64 ± 7% reduction of the carrageenan-enhanced ankle diameter, respectively, $P < 0.001$ for both). Nevertheless, it must be noted that in the non-inflammatory conditions due to noxious heat, a very high dose of 100 μg of intraplantar racemic-, *S*(+)- or *R*(−)-flurbiprofen had weak effects. These results are questionable since the observed weak modifications could be due, in part, to vascular reabsorption.

In good agreement with our previous study (Buritova and Besson, 1998), the intraplantar injection of 30 μg of *R*(−)-flurbiprofen is totally inefficacious in the model of noxious heat while its intravenous administration induces a weak, but significant, decrease of spinal c-Fos protein expression. These data suggest that the site of action of *R*(−)-flurbiprofen is principally at the central level; i.e. at least a part, at the spinal cord level (see also Neugebauer et al., 1995; Buritova and Besson, 1998). In this respect the direct spinal site of antinociceptive action of various NSAIDs is well documented (for review see Jurna, 1997; Yaksh et al., 1998; Yaksh, 1999).

In conclusion, contrary to the effects of flurbiprofen and its enantiomers on inflammatory conditions, the present results provide evidence for weak effects of intravenous administration of racemic-, *S*(+)- and *R*(−)-flurbiprofen on the noxious heat-evoked c-Fos protein expression at spinal cord level of the anaesthetised rat. The dose-related effects has been obtained only for *R*(−)-flurbiprofen, but not for racemic- and *S*(+)-flurbiprofen. The weak effects of these compounds are probably due to the central site of action since the intraplantar injection of a relatively high dose of 30 μg (for each compound) is inefficacious. It must be underlined that in inflammatory states, the intraplantar injection of 1 μg of racemic- and *S*(+)-flurbiprofen, but not its *R*(−) enantiomer, is sufficient to significantly decrease both the carrageenan-evoked peripheral inflammatory oedema and spinal c-Fos protein expression. Overall, these results suggest that the efficacy of flurbiprofen and its enantiomers depend on the modality of the noxious stimulus; i.e. it is more efficacious upon inflammatory nociception than upon brief noxious heat-evoked nociception. These results are in good keeping with the current knowledge about the effects of NSAIDs in different models of nociception. It is believed that NSAIDs may affect the development or maintenance of hyperalgesic states associated with tissue injury, but not the acute responses to nociceptive stimulus (for review see Yaksh et al., 1998; Yaksh, 1999).

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