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Peripheral and/or central effects of racemic-, S(+)- and R(−)-flurbiprofen on inflammatory nociceptive processes: a c-Fos protein study in the rat spinal cord

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1 We have evaluated the effects of intravenous or intraplantar racemic-, S(+)- and R(−)-flurbiprofen on both the carrageenan-evoked peripheral oedema and spinal c-Fos immunoreactivity, an indirect index of neurons involved in spinal nociceptive processes.

2 Three hours after intraplantar injection of carrageenan (6 mg in 150 μl of saline) in awake rats, a peripheral oedema and numerous c-Fos protein-like immunoreactive (c-Fos-LI) neurons in L4–L5 segments were observed. c-Fos-LI neurons were essentially located in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn.

3 Intravenous racemic-flurbiprofen (0.3, 3 and 9 mg kg^{-1}) dose-relatedly reduced the carrageenan-evoked oedema and spinal c-Fos expression ($r=0.64$, $r=0.88$ and $r=0.84$ for paw diameter, ankle diameter and number of c-Fos-LI neurons; $P<0.05$, $P<0.001$ and $P<0.001$ respectively).

4 Similar effects to those of intravenous racemic-flurbiprofen were obtained with intravenous S(+)-flurbiprofen (0.3, 3 and 9 mg kg^{-1}) which dose-relatedly reduced the number of c-Fos-LI neurons ($r=0.69$, $P<0.01$) and diameters of paw and ankle ($r=0.56$ and $r=0.52$ respectively, $P<0.05$ for both).

5 For the dose of 0.3 mg kg^{-1} i.v., R(−)-flurbiprofen did not modify the number of c-Fos-LI neurons and produced a weak reduction of oedema at only the ankle level (23 ± 12% reduction, $P<0.05$). However, a ten times higher dose of R(−)-flurbiprofen (3 mg kg^{-1} i.v.) was necessary to obtain effects comparable to those of S(+)- or racemic-flurbiprofen (0.3 mg kg^{-1} i.v.).

6 Intraplantar racemic-flurbiprofen (1, 10 and 30 μg) dose-relatedly reduced the carrageenan-enhanced ankle diameter ($r=0.81$, $P<0.001$) and the number of c-Fos-LI neurons in L4–L5 segments ($r=0.83$, $P<0.001$), with a 60 ± 3% reduction of the number of c-Fos-LI neurons ($P<0.001$), and 30 ± 3 and 67 ± 7% reduction of paw and ankle diameter respectively ($P<0.001$ for both) for the dose of 30 μg.

7 For intraplantar S(+)-flurbiprofen (1, 10 and 30 μg) the dose-related effects ($r=0.77$, $r=0.60$ and $r=0.59$ for c-Fos-LI neurons, paw and ankle diameters respectively, $P<0.001$, $P<0.01$ and $P<0.01$) were similar to those of racemic-flurbiprofen. In contrast, intraplantar R(−)-flurbiprofen (1, 10 and 30 μg) did not have detectable effects on all studied parameters.

8 The present study provides clear evidence for potent anti-inflammatory and antinociceptive effects of both intravenous or intraplantar racemic- and S(+)-flurbiprofen. These results further demonstrate marked anti-inflammatory and antinociceptive effects of intravenous, but not intraplantar, R(−)-flurbiprofen. These results suggest that the main site of action of racemic- and S(+)-flurbiprofen is in the periphery and indicate that the site of action of R(−)-flurbiprofen is mainly of central origin.

Keywords: Carrageenan inflammation; c-Fos; dorsal horn; racemic-flurbiprofen; S(+)-flurbiprofen; R(−)-flurbiprofen; nociception; NSAIDs; rat

Introduction

Flurbiprofen is a member of the 2-arylpropionic acid (2-APA) class, also known as profens, an important group of non-steroidal anti-inflammatory drugs (NSAIDs); for review see Insel, 1991. Like other NSAIDs, the anti-inflammatory and analgesic actions of flurbiprofen are traditionally explained by the inhibition of cyclo-oxygenase (COX) mediated prostaglandin biosynthesis in the peripheral inflamed tissue (Vane, 1971; Ferreira, 1972; Flower & Vane, 1974). However, several behavioural studies (Brune *et al*., 1974; Taiwo & Levine, 1986; Malmberg & Yaksh, 1992a, b) and electrophysiological studies (Carlsson *et al*., 1988; Jurna & Brune, 1990; Jurna *et al*., 1992; Chapman & Dickenson, 1992; Neugebauer *et al*., 1994) have provided accumulating evidence that NSAIDs are also active at the level of central nervous system. Recently, the availability of pure S(+) and R(−) enantiomers of propionic acids initiated extended research into possible sites and mechanisms of action of NSAIDs (for review see Brune *et al*., 1992a, b; Wechter 1994; Evans, 1996; Geisslinger & Schaible, 1996). S(+)-flurbiprofen has been shown to be more potent than R(−)-flurbiprofen when considering the inhibition of prostaglandin E_2 release *in vitro* (Brune *et al*., 1991; Malmberg & Yaksh, 1994a). Only one *in vivo* study, to date, demonstrated anti-inflammatory effects of oral administration of relatively low doses (up to 3 mg kg^{-1} p.o.) of S(+)-flurbiprofen, but not R(−)-flurbiprofen, with the carrageenan model of inflammation (Brune *et al*., 1991). In contrast to S(+)-flurbiprofen, R(−)-flurbiprofen did not cause significant mucosal damage in the gastro-intestinal tract after oral administration in the rat (Brune *et al*., 1991, 1992a, b). Behavioural studies have demonstrated that both S(+) and R(−) enantiomers of flurbiprofen have antinociceptive effects

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after systemic (oral, Brune *et al*., 1991; intraperitoneal, Geisslinger *et al*., 1994) and intrathecal (Malmberg & Yaksh, 1994b) administration in the rat. In these studies, R(−)-flurbiprofen was almost as potent as S(+)-flurbiprofen in producing antinociception after an oral administration (Brune *et al*., 1991), while S(+)-flurbiprofen was more potent than R(−)-flurbiprofen when administered intraperitoneally (Geisslinger *et al*., 1994) and intrathecally (Malmberg & Yaksh, 1994b). Furthermore, an electrophysiological study (Neugebauer *et al*., 1995) demonstrated that the antinociceptive effects of S(+)-flurbiprofen were more potent than those of R(−)-flurbiprofen following intravenous administration. These authors also demonstrated the antinociceptive effects of intraplantar injection of S(+)-flurbiprofen, but not R(−)-flurbiprofen (see above). Unfortunately, most of the studies mentioned above did not compare the effects of racemic-flurbiprofen to those of its S(+) and R(−) enantiomers considering simultaneously the antinociceptive and anti-inflammatory effects of these compounds in the same rat.

The aims of the present study were to evaluate the anti-inflammatory and antinociceptive effects of racemic-, S(+)- and R(−)-flurbiprofen by using the combination of the measurement of the peripheral oedema and the method of c-Fos protein immunoreactivity. These two experimental approaches were performed simultaneously in the same rat with acute inflammation evoked by an intraplantar injection of carrageenan. Intraplantar carrageenan induces an inflammatory oedema (Winter *et al*., 1962; Di Rosa *et al*., 1971 and references therein) associated with mechanical allodynia (Kayser & Guilbaud, 1987), heat and mechanical hyperalgesia (Hargreaves *et al*., 1988; Iadarola *et al*., 1988; Joris *et al*., 1990 and the expression of c-Fos protein at the spinal cord level (Draisci & Iadarola, 1989; Noguchi *et al*., 1991, 1992; Honoré *et al*., 1995). The expression of the nuclear protein c-Fos encoded by the immediate-early gene c-*fos* (for review see Morgan, 1991; Hughes & Dragunow, 1995; Morgan & Curran, 1995) is widely used as an indirect marker of neurons involved in spinal nociceptive processes (for review see Zieglgansberger & Tölle, 1993; Munglani & Hunt, 1995; Chapman & Besson, 1997). Recently, in the carrageenan model of inflammatory nociception, we have demonstrated a significant reduction of both the carrageenan-evoked oedema and spinal c-Fos protein expression by pre-treatment with various classical NSAIDs, such as indomethacin, aspirin, diclofenac, piroxicam, ketoprofen, niflumic acid and lornoxicam (see references in Buritova *et al*., 1996a; Buritova & Besson, 1998), and new NSAIDs acting against the inducible isoform of COX, such as selective COX-2 inhibitors (Buritova *et al*., 1996c). In the present study, we evaluated and compared the effects of racemic-, S(+)- and R(−)-flurbiprofen in the same experimental paradigm. Furthermore, we investigated the eventual contribution of peripheral and central sites of action of these compounds by using either systemic (intravenous) or peripheral (intraplantar) routes of administration. A preliminary account of this study has been presented on 27th Annual Meeting of Society for Neuroscience in New Orleans, October 1997 (Buritova & Besson, 1997b).

Methods

Experimental animals

Five experimental series were performed on 155 rats (150 carrageenan injected and five non-stimulated rats). In addition, the effects of all drugs, given alone, were studied in 16 non-stimulated rats (without carrageenan injection). All experiments were performed on 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 in conscious animals 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. The experimental groups were chosen by completely randomized schedule.

Drug administration

Racemic-flurbiprofen and its S(+) and R(−) enantiomers (Knoll Pharmaceutical, Nottingham, U.K.) were dissolved in vehicle {for 1 ml of vehicle: 100 μl of Ethanol, 100 μl of Cremophor EL (Sigma, France) and 800 μl of saline}. Intravenous injections were performed into the tail vein using a 25 Gauge needle. The conscious rats were restrained using a cylindrical rodent restrainer (Harvard Apparatus, Ealing, France) for about 1 min during the intravenous injection. Intraplantar injections were performed subcutaneously in the right hind paw using a 25 Gauge needle (for more details see Methods in Buritova *et al*., 1996b). The conscious rats were restrained in the hand for about 30 s during the intraplantar injection.

Carrageenan-evoked inflammation

Carrageenan (λ-carrageenan, SIGMA, France; 6 mg in 150 μl of saline) was injected, intraplantarly, in the right hind paw of non-anaesthetised rat according to the method described by Winter *et al*. (1962). Rats were perfused 3 h after intraplantar carrageenan, when the number of c-Fos-LI neurons in the dorsal horn of the lumbar spinal cord is maximal (Honoré *et al*., 1995).

Three hours after carrageenan, two indicators of the ipsilateral peripheral oedema, diameters of paw and ankle, were measured with calibrated callipers, under deep pentobarbital anaesthesia, immediately before perfusion (for more details see Methods in Buritova *et al*., 1996b). Carrageenan injection enhanced paw and ankle diameters of control group of rats (P_c, A_c, respectively) and drug treated rats (P_t, A_t, respectively) were measured. For comparison, paw and ankle diameters of non-stimulated rats (P_n, A_n respectively) were measured. The carrageenan-evoked increases of paw and ankle diameters were determined as difference of paw and ankle diameters of non-stimulated rats from those of carrageenan injected rats. The following formula for these differences when considering the carrageenan-enhanced paw and ankle diameters of drug treated rats (P_t-P_n, A_t-A_n respectively) and control rats (P_c-P_n, A_c-A_n respectively) were used. The effects of drugs were determined as percentage decreases of the carrageenan-enhanced paw and ankle diameters of drug treated rats as compared to those of control rats. For each drug treated rat, the following formula for percentage of control value of the carrageenan-enhanced paw diameter $(P_t-P_n)/(P_c-P_n)\times 100$, and the ankle diameter $(A_t-A_n)/(A_c-A_n)\times 100$, were used. Studies of peripheral oedema and spinal c-Fos protein expression were performed in the same rats, thus possible correlations between the two parameters were determined.

Immunohistochemistry

Experimental procedures have previously been described (Honoré *et al*., 1995). All stimulated rats were perfused 3 h

after carrageenan and non-stimulated rats were perfused without carrageenan injection. Rats were deeply anaesthetized (Pentobarbital, Sanofi; 55 mg kg^{-1} i.p.) and 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 Inc., Ab-2 solution 0.1 mg/ml diluted 1 : 4000), using the method of Hsu *et al.* (1981). The c-Fos protein-like immunoreactivity (c-Fos-LI) was visualized by 1-naphtol ammonium carbonate solution (Menétrey *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.

Counting of spinal c-Fos-LI neurons and statistics

As previously described (Honoré *et al.*, 1995), c-Fos-LI neurons were plotted and 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 & Grant, 1986): superficial laminae (laminae I–II), nucleus proprius (laminae III–IV) and deep laminae (laminae V–VI; neck) 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-LI neurons in the grey matter for ten sections through L4–L5 segments, and (2) in these ten sections, the number of c-Fos-LI neurons per four defined regions (see above). The investigator responsible for plotting and counting of the c-Fos-LI neurons was blind to experimental conditions of each rat. Statistical analysis was performed by using analysis of variance (ANOVA) and Fisher's protected least squares difference test for multiple comparisons. The dose-dependent effects of drugs on both the number of spinal c-Fos-LI neurons and the peripheral oedema (paw and ankle diameter) and possible correlations between these parameters were determined by using simple regression and correlation coefficient, respectively.

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 on neuronal activities during an acute knee joint inflammation induced by kaolin and carrageenan (Neugebauer *et al.*, 1995). Note that doses up to 9 mg kg^{-1} i.p. of S(+)-flurbiprofen and up to 27 mg kg^{-1} 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-flurbiprofen (0.3, 3 and 9 mg kg^{-1}, $n=5$ rats for each group; volume 0.25 ml) was administered intravenously, 25 min prior to intraplantar injection of carrageenan. Control carrageenan rats ($n=5$) received an equal volume (0.25 ml) of intravenous vehicle, 25 min prior to intraplantar carrageenan.

In the second experimental series, racemic-flurbiprofen (9 mg kg^{-1}, $n=5$ rats), S(+)- or R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} for both substances; $n=5$ rats for each group) were administered intravenously, 25 min prior to intraplantar carrageenan. Control carrageenan rats ($n=5$) received intravenous vehicle (0.25 ml), 25 min prior to intraplantar carrageenan.

In the third experimental series, racemic-flurbiprofen (1, 10 and 30 μg in 50 μl of vehicle; $n=5$ rats for each group) was injected intraplantarly, simultaneously with carrageenan (in the same syringe). Control carrageenan rats ($n=5$) received an equal volume (50 μl) of intraplantar vehicle simultaneously with carrageenan injection.

In the fourth experimental series, racemic-flurbiprofen (0.1 and 30 μg in 50 μl of vehicle; $n=5$ rats for each group), S(+)-flurbiprofen (0.1, 1, 10 and 30 μg in 50 μl of vehicle; $n=5$ rats for each group) or R(−)-flurbiprofen (1, 10 and 30 μg in 50 μl of vehicle; $n=5$ rats for each group) were injected intraplantarly, simultaneously with carrageenan (in the same syringe). Control carrageenan rats ($n=5$) received intraplantar vehicle (50 μl) simultaneously with carrageenan injection.

In the fifth experimental series, racemic-, S(+)- and R(−)-flurbiprofen (100 μg in 50 μl of vehicle for each substances; $n=5$ rats for each group) were injected intraplantarly, simultaneously with carrageenan (in the same syringe). Control carrageenan rats ($n=5$) received intraplantar vehicle (50 μl) simultaneously with carrageenan injection.

In addition, we assessed the effects of the administration of the highest doses of all of studied drugs, given alone, in non-stimulated rats. Racemic-, S(+)- and R(−)-flurbiprofen were administered intravenously (9 mg kg^{-1} for each substance; $n=2$ rats in each group) or intraplantarly (100 μg in 50 μl of vehicle for each substance; $n=2$ rats in each group) without carrageenan injection. Control group of rats received an injection of vehicle intravenously (0.25 ml; $n=2$ rats) or intraplantarly (50 μl; $n=2$ rats) without carrageenan injection. Rats were perfused 3 h after injection of vehicle or drug.

Results

In the preliminary study, we assessed that an intravenous administration or an intraplantar injection of vehicle or racemic-, S(+)- or R(−)-flurbiprofen did not modify paw and ankle diameters. Three hours after injection of vehicle or racemic-, S(+)- or R(−)-flurbiprofen (9 mg kg^{-1} i.v. or 100 μg in 50 μl intraplantar, $n=2$ rats for each group), the c-Fos-LI neurons were virtually absent in lumbar spinal cord (<3 c-Fos-LI neurons per section in segments L4–L5). These observations are in accordance with our previous results (Honoré *et al.*, 1995; Chapman *et al.*, 1996) showing negligible spinal c-Fos protein expression after intraplantar injection of saline (<5 c-Fos-LI neurons per section L4–L5) which was not significantly different from that observed in non-stimulated rats.

Peripheral oedema and c-Fos protein expression in the lumbar spinal cord at 3 h after intraplantar carrageenan

In good accordance with numerous previous studies (Draisci & Iadarola, 1989; Noguchi *et al.*, 1991, 1992; see Buritova & Besson, 1998 and references therein; Buritova *et al.*, 1998) an intraplantar injection of carrageenan evoked a peripheral oedema and c-Fos protein expression in the spinal cord ipsilateral to the carrageenan-inflamed hind paw in awake rats. Three hours after intraplantar carrageenan, two parameters of peripheral oedema, paw and ankle diameters, in carrageenan control rats (see results for experimental series I–V in Table 1)

Table 1 The spinal c-Fos protein expression and peripheral oedema in the control groups ($n=5$ for each group) of five experimental series (I–V), 3 h after intraplantar injection of carrageenan (6 mg in 150 μl of saline).

		Number of c-Fos-LI neurons/section L4–L5				*Diameter* (cm)	
Stimulus	*Series*	*Total*	*Laminae I–II*	*Laminae III–IV*	*Laminae V–VI*	*Ventral*	*Paw*	*Ankle*
Carrageenan	I	150±10	64±4	10±1	53±4	23±3	1.00±0.04	1.02±0.03
(6 mg/150 μl)	II	141±8	63±2	8±1	51±3	19±2	1.02±0.02	1.00±0.02
	III	133±9	61±4	9±1	46±4	17±2	1.11±0.04	1.00±0.03
	IV	127±9	53±7	7±1	47±3	20±1	0.98±0.02	1.00±0.02
	V	135+6	52±2	10±1	52±3	22±2	1.03±0.02	1.10±0.02

Results are expressed as the mean number of spinal c-Fos-LI neurons (±s.e.m), per L4–L5 segments (Total), and per laminar region (laminae I–II, III–IV and V–VI, Ventral), and as mean value (±s.e.m. in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Note that in non-stimulated rats, the value for paw and ankle diameters are 0.48±0.02 and 0.75±0.03 cm respectively.

were significantly increased as compared to non-stimulated rats (0.48±0.02 and 0.75±0.03 cm for paw and ankle diameters, respectively). Contralateral oedema was not observed.

Three hours after intraplantar carrageenan, the c-Fos-LI neurons were numerous at the level of lumbar segments L4–L5 in controls rats (for example see Figure 2A). Importantly, the number of c-Fos-LI neurons and their laminar distribution were similar in the control carrageenan groups for the five experimental series (see controls in Figures 1, 2, 6, 7 and 11, and also statistical results in Table 1). In all control carrageenan groups, c-Fos-LI neurons were essentially located in the dorsal horn of the spinal cord, with a predominant and similar distribution in both the superficial (I–II) and deep (V–VI) laminae (approximately 42 and 36% of the total number of c-Fos-LI neurons, respectively); see Laminae I–II and V–VI in Table 1. Note that the number of c-Fos-LI neurons in the ventral horn (laminae VII–X) was moderate and very few c-Fos-LI neurons were present in nucleus proprius (laminae III–IV); see Table 1. c-Fos-LI neurons are virtually absent in the contralateral lumbar spinal cord (<3 c-Fos-LI neurons per section in segments L4–L5).

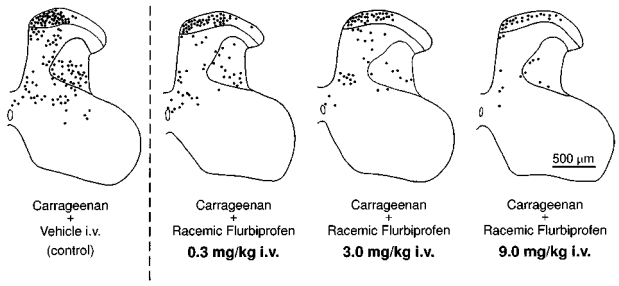

Figure 1 Camera lucida drawings, of individual representative example of segments L4–L5 of rat spinal cord, illustrating the c-Fos protein-like immunoreactivity evoked by intraplantar carrageenan (control) and the effects of intravenous pre-administration of racemic flurbiprofen (0.3, 3 and 9 mg kg^{-1}) on the number of c-Fos-LI neurons, 3 h after carrageenan. Each drawing includes all c-Fos-LI neurons in one 40-μm section of segments L4–L5; each dot represents one c-Fos-LI neuron. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. Scale bar = 500 μm.

Effects of intravenous administration of racemic-flurbiprofen

In the first experimental series, intravenous pre-administration of racemic-flurbiprofen reduced the carrageenan-evoked peripheral oedema at paw and ankle levels, and the number of c-Fos-LI neurons at spinal cord level (Figure 1, Table 2). As compared to the control carrageenan group the effects of intravenous racemic-flurbiprofen (0.3, 3 and 9 mg kg^{-1}) were significant when considering both paw and ankle diameters (ANOVA test $F_{3,16}=13.40$ and $F_{3,16}=44.28$ respectively, $P<0.001$ for both), and the total number of spinal c-Fos-LI neurons and their laminar distribution in L4–L5 segments (ANOVA test $F_{3,16}=40.37$ and $F_{3,64}=91.69$ respectively, $P<0.001$ for both). Racemic-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) dose-relatedly reduced both the paw and ankle diameters (regression coefficients $r=0.64$ and $r=0.88$, $P<0.05$ and $P<0.001$ respectively), the total number of c-Fos-LI neurons in L4–L5 segments (regression coefficient $r=0.84$, $P<0.001$) and the number of c-Fos-LI neurons in both the superficial (I–II) and deep (V–VI) laminae (regression coefficients $r=0.77$ and $r=0.87$ respectively, $P<0.001$ for both). All three doses (0.3, 3 and 9 mg kg^{-1} i.v.) of racemic-flurbiprofen had similar effects on the number of c-Fos-LI neurons in superficial (I–II) and deep (V–VI) laminae (Figure 1, Table 2) and reduced the swelling of both the paw and ankle, with a greater effect on the ankle (Table 2). Interestingly, the relatively low dose of 0.3 mg kg^{-1} of racemic-flurbiprofen was sufficient to produce a marked reduction of carrageenan-evoked peripheral oedema and spinal c-Fos protein expression; see Figure 1, Table 2. The relatively high dose of 9 mg kg^{-1} of racemic-flurbiprofen greatly reduced the carrageenan evoked spinal c-Fos expression and blocked the extension of peripheral oedema to the ankle (Figure 1, Table 2). Furthermore, the effects of intravenous racemic-flurbiprofen on the carrageenan-evoked peripheral oedema and the total number of spinal c-Fos-LI neurons were positively correlated (correlation coefficient $r=0.76$ and $r=0.79$, $P<0.01$ and $P<0.001$ for the paw and ankle diameters respectively); see Figure 5A.

Effects of intravenous administration of racemic-, S(+)- and R(−)-flurbiprofen

In the second part of the study, we investigated the effects of intravenously pre-administered S(+)- and R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} for both substance) on carrageenan-evoked peripheral oedema and the number of spinal c-Fos-LI neurons (Figures 2–4 , Table 3). For comparison, one dose of racemic-flurbiprofen (9 mg kg^{-1} i.v.) was used as the dose of reference.

As compared to the control carrageenan group, the effects of both S(+)- and R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v. for both substance) on carrageenan-evoked spinal c-Fos expression were significant when considering the total number of c-Fos-LI neurons (ANOVA test $F_{3,20}=33.59$ and $F_{3,20}=13.23$ respectively, $P<0.001$ for both substance) and their laminar distribution in L4–L5 segments (ANOVA test

$F_{3,80}=68.83$ and $F_{3,80}=13.49$ respectively, $P<0.001$ for both substances). S(+)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) dose-relatedly reduced the total number of c-Fos-LI neurons in L4–L5 segments (regression coefficient $r=0.69$, $P<0.01$) and the number of c-Fos-LI neurons in both the superficial (I–II) and deep (V–VI) laminae (regression coefficients $r=0.59$ and $r=0.69$, $P<0.05$ and $P<0.01$ respectively). R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) dose-relatedly reduced the total number of spinal c-Fos-LI neurons (regression coefficient $r=0.68$, $P<0.01$) and the number of c-Fos-LI neurons in the deep laminae (regression coefficient $r=0.63$, $P<0.01$). In the superficial laminae, the effects of R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) were not dose-related (regression coefficient $r=0.163$, $P>0.05$).

The relatively low dose of 0.3 mg kg^{-1} of S(+)-flurbiprofen was sufficient to markedly reduce the total number of carrageenan-evoked spinal c-Fos-LI neurons (more than 40% reduction; see Figure 3, Table 3). The highest studied dose of S(+)-flurbiprofen (9 mg kg^{-1} i.v.) has similar effects as compared to that of the same dose of racemic-flurbiprofen (more than 65% reduction of the total number of carrageenan-evoked c-Fos-LI neurons; see Figure

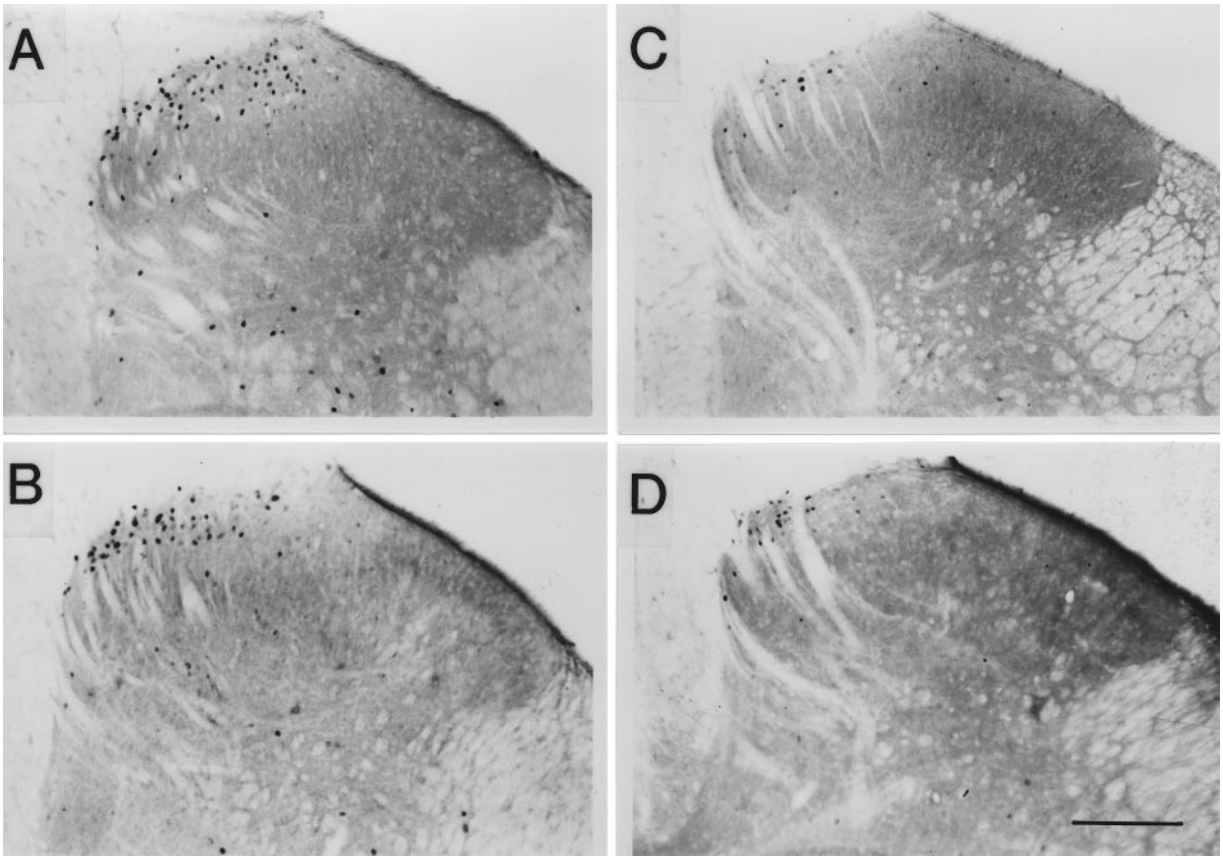

Figure 2 Microphotographs, of individual representative example of 40-μm sections of dorsal horn of segments L4–L5 of rat spinal cord, illustrating the effects of intravenous pre-administration of racemic-, S(+)- and R(−)-flurbiprofen on the c-Fos protein-like immunoreactivity evoked by intraplantar injection of carrageenan. Each microphotograph includes nuclei of c-Fos-LI neurons (black dots) in laminae I–V of dorsal horn. Four experimental situations are represented: intraplantar injection of carrageenan plus pre-administration of intravenous vehicle (A; control) or R(−)-flurbiprofen 9 mg kg^{-1} i.v. (B), racemic-flurbiprofen 9 mg kg^{-1} i.v. (C) and S(+)-flurbiprofen 9 mg kg^{-1} i.v. (D). Scale bar = 200 μm.

Table 2 The carrageenan-evoked spinal c-Fos protein expression and peripheral oedema in the control carrageenan group and groups receiving racemic flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group) prior to intraplantar carrageenan.

Group	*Dose* (mg/kg i.v.)	*c-Fos-LI neurons in L4–L5 segments*			*Peripheral oedema*	
		Total	*Laminae I–II*	*Laminae V–VI*	*Paw diameter*	*Ankle diameter*
Controls	–	150±10	64±4	53±4	1.00±0.4	1.02±0.03
Racemic flurbiprofen	0.3	86±3 (43±2)***	42±2 (35±2)***	29±2 (46±4)***	0.86±0.02 (27±4)**	0.90±0.02 (44±6)***
	3.0	67±8 (55±5)***	34±4 (48±6)***	24±2 (55±5)***	0.80±0.02 (38±3)***	0.81±0.02 (78±7)***
	9.0	38±6 (75±4)***	23±4 (64±6)***	10±3 (82±5)***	0.74±0.04 (49±8)***	0.73±0.01 (106±5)***

Results for spinal c-Fos expression are expressed as mean number (±s.e.m.) of c-Fos-LI neurons per L4–L5 segments (Total), and per laminar region (laminae I–II, and V–VI). Results for peripheral oedema are expressed as mean value (±s.e.m. in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Note that in non-stimulated rats, the value for paw and ankle diameter are 0.48±0.02 and 0.75±0.03 cm respectively. Results expressed as % reduction of control value of studied parameters are presented in brackets. For the calculation of the % reduction for paw and ankle diameters see Methods. Significance compared to control carrageenan group was performed using ANOVA and Fisher's PLSD test (**$P<0.01$, ***$P<0.001$).

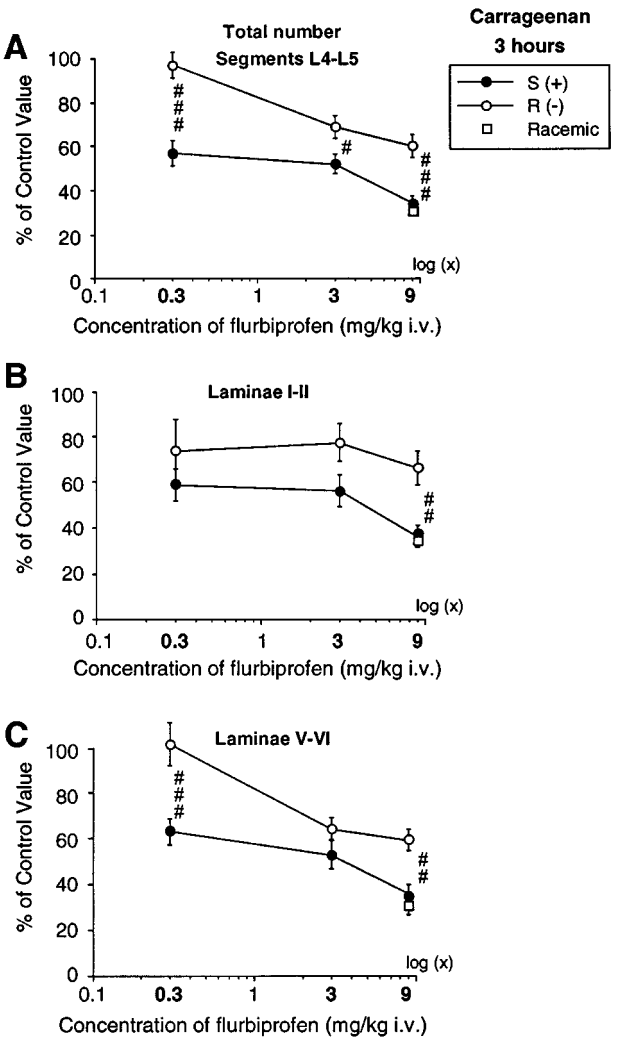

Figure 3 Effects of pre-administered racemic-flurbiprofen (9 mg kg^{-1} i.v.; $n=5$; □), S(+)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group; ●) and R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group; ○) on the total number of c-Fos-LI neurons (Total number; A), the number of superficial (laminae I–II; B) and deep (laminae V–VI; C) c-Fos-LI neurons in the L4–L5 segments, 3 h after intraplantar carrageenan. Results are expressed as % of the control value for the total number of c-Fos-LI neurons and their number in each region (± s.e.m.). Statistical comparisons between the effects of S(+)-flurbiprofen versus R(−)-flurbiprofen were performed using ANOVA and Fisher's PLSD test (#$P<0.05$, ##$P<0.01$, ###$P<0.001$).

3, Table 3). In contrast, the effects of R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) were less pronounced as compared to that of the same doses of S(+)-flurbiprofen (Figure 3, Table 3). For the highest studied dose of R(−)-flurbiprofen (9 mg kg^{-1} i.v.), the effects were significantly smaller than those of the same doses of S(+)-flurbiprofen when considering the total number of spinal c-Fos-LI neurons (Figure 3A) and the number of c-Fos-LI neurons in superficial and deep laminae (Figure 3B and C respectively). Despite the significantly more important effects of S(+)-flurbiprofen, 9 mg kg^{-1} of R(−)-flurbiprofen produced more than a 40% reduction of the number of carrageenan-evoked c-Fos-LI neurons (Figures 2 and 3, Table 3). The smallest dose of R(−)-flurbiprofen (0.3 mg kg^{-1} i.v.) studied did not have significant effects on the total number of spinal c-Fos-LI neurons (Table 3). However, for this dose of R(−)-flurbiprofen, the laminar analysis revealed a weak, but significant, reduction of the number of c-Fos-LI neurons in superficial laminae (Table 3).

As compared to the control carrageenan group, the effects of intravenous S(+)- and also R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} for both substance) on the carrageenan evoked oedema were significant when considering both the paw diameter (ANOVA test $F_{3,20}=35.88$, $P<0.001$ and $F_{3,20}=7.48$, $P<0.01$ respectively) and the ankle diameter (ANOVA test $F_{3,20}=40.58$ and $F_{3,20}=11.33$ respectively, $P<0.001$ for both substances). As shown in Figure 4 and Table 3, both S(+) and R(−) enantiomers of flurbiprofen reduced the induction of the swelling of both paw and ankle diameters, with a stronger effect at the ankle level. The effects of S(+)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.) on the paw and ankle diameters were dose-related (regression coefficients $r=0.56$ and $r=0.52$ respectively, $P<0.05$ for both). The effects of the same doses of R(−)-flurbiprofen on the peripheral oedema were not dose-related (regression coefficient $r=0.49$, $P<0.05$ and $r=0.42$, $P>0.05$ for the paw and ankle diameters respectively). Interestingly, although the effects S(+)-flurbiprofen on carrageenan-evoked oedema were significantly more pronounced in comparison to that of R(−)-flurbiprofen (Figure 4), the effects of the highest dose of R(−)-flurbiprofen (9 mg kg^{-1} i.v.) studied were marked with a 32±6 and 57±8% reduction of the paw and ankle diameters respectively ($P<0.001$ for both); see Figure 4 and Table 3. This same dose of S(+)- and racemic-flurbiprofen (9 m kg^{-1} i.v. for both) had very similar effects on both the carrageenan-evoked peripheral oedema and spinal c-Fos protein expression (Figures 3 and 4, Table 3). As shown in Figure 4 and Table 3, S(+)- and racemic-flurbiprofen (9 mg kg^{-1} i.v. for both) strongly reduced, in the same manner, the carrageenan-enhanced paw diameter and blocked the swelling of ankle diameter. Furthermore the effects of S(+)-flurbiprofen on the carrageenan-enhanced paw diameter and the total number of spinal c-Fos-LI neurons were positively correlated (correlation coefficient $r=0.64$, $P<0.01$); see Figure 5B. As shown in Figure 5, after intravenous pre-administration either racemic-flurbiprofen (first experimental series; Figure 5A) or S(+)-flurbiprofen (second experimental series; Figure 5B), the number of spinal c-Fos-LI neurons decreased in parallel with a decrease in carrageenan-evoked oedema at the paw level. In contrast, the effects of R(−)-flurbiprofen on the peripheral oedema and spinal c-Fos expression were not well correlated (correlation coefficient $r=0.49$, $P<0.05$); Figure 5C.

Effects of intraplantar injection of racemic-flurbiprofen

In the third part of the study, intraplantar racemic-flurbiprofen (1, 10 and 30 μg) reduced the carrageenan-evoked peripheral oedema at paw and ankle levels, and associated spinal c-Fos protein expression (Figure 6, Table 4). As compared to the control carrageenan group, the effects of intraplantar racemic-flurbiprofen were significant when considering both the paw and ankle diameters (ANOVA test $F_{3,16}=7.57$, $P<0.01$ and $F_{3,16}=10.93$, $P<0.001$ respectively), and the total number of c-Fos-LI neurons and their laminar distribution in L4–L5 segments (ANOVA test $F_{3,16}=24.27$ and $F_{3,64}=51.40$ respectively, $P<0.001$ for both). Racemic-flurbiprofen (1, 10 and 30 μg intraplantar) significantly reduced the paw diameter (Table 4) and dose-relatedly reduced the ankle diameter (regression coefficient $r=0.81$, $P<0.001$), the total number c-Fos-LI neurons in L4–L5 segments (regression coefficient $r=0.83$, $P<0.001$) and the number c-Fos-LI neurons in the superficial (I–II) and deep (V–VI) laminae (regression

Table 3 The carrageenan-evoked spinal c-Fos protein expression and peripheral oedema in the control carrageenan group and groups receiving racemic flurbiprofen (9 mg kg^{-1} i.v.; $n=5$), S(+)- and R(−)-flurbiprofen (0.3, 3 and 9 mg kg i.v. for both substances; $n=5$ for each group) prior to intraplantar carrageenan.

	Dose (mg/	*c-Fos-LI neurons in L4–L5 segments*			*Peripheral oedema*	
Group	kg i.v.)	*Total*	*Laminae I–II*	*Laminae V–VI*	*Paw diameter*	*Ankle diameter*
Controls	–	141±8	63±2	51±3	1.02±0.2	1.00±0.02
Racemic flurbiprofen	9.0	43±3 (69±2)***	22±2 (65±3)***	16±2 (69±3)***	0.74±0.02 (51±3)***	0.77±0.01 (93±7)***
S(+)	0.3	81±8 (43±6)***	37+4 (41±7)***	32±3 (37±6)***	0.81±0.03 (39±6)***	0.83±0.02 (70±9)***
flurbiprofen	3.0	74±6 (48±4)***	35±4 (44±7)***	27±3 (47±6)***	0.78±0.01 (43±2)***	0.80±0.01 (80±1)***
	9.0	48±4 (66±3)***	23±3 (64±4)***	18±2 (64±4)***	0.73±0.02 (52±3)***	0.77±0.02 (93±8)***
R(—)	0.3	138±9 (2±6)	46±9 (26±14)*	52±5 (0±9)	0.96±0.03 (11±6)	0.94±0.03 (23±12)*
flurbiprofen	3.0	97±7 (31±5)***	48±5 (23±8)***	33±2 (36±5)***	0.87±0.03 (28±6)***	0.87±0.02 (53±7)***
	9.0	85±8 (40±5)***	41±5 (34±7)***	30±2 (41±5)***	0.84±0.03 (32±6)***	0.86±0.02 (57±8)***

Results for spinal c-Fos expression are expressed as mean number (±s.e.m.) of c-Fos-LI neurons per L4–L5 segments (Total), and per laminar region (laminae I–II and V–VI). Results for peripheral oedema are expressed as mean value (±s.e.m.; in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Results expressed as % reduction of control value of studied parameters are presented in brackets. Note that in non-stimulated rats, the value for paw and ankle diameter are 0.48±0.02 and 0.75±0.03 cm respectively. For the calculation of the % reduction for paw and ankle diametes see Methods. Significance compared to control carrageenan group was performed using ANOVA and Fisher's PLSD test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

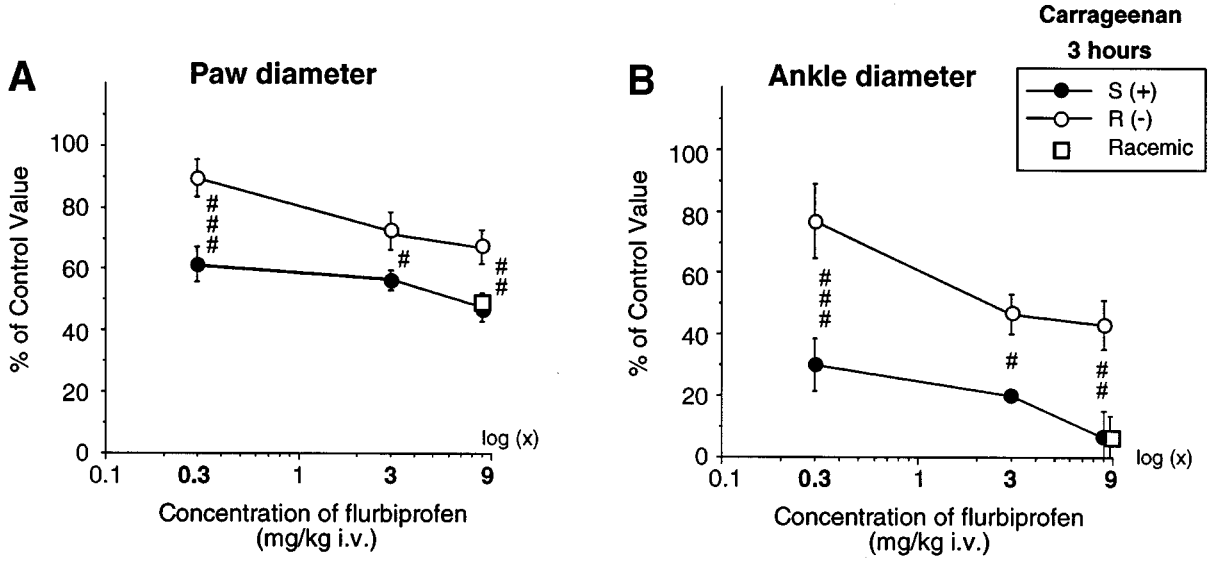

Figure 4 Effects of pre-administered racemic-flurbiprofen (9 mg kg^{-1} i.v.; $n=5$;□), S(+)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group; ●) and R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group; ○) on the carrageenan-enhanced paw (A) and ankle (B) diameters, 3 h after intraplantar carrageenan. Results are expressed as % of the control value of the carrageenan-enhanced paw and ankle diameter (± s.e.m.). Statistical comparisons between the effects of S(+)-flurbiprofen versus R(−)-flurbiprofen were performed using ANOVA and Fisher's PLSD test (#$P<0.05$, ##$P<0.01$, ###$P<0.001$).

coefficients $r=0.76$ and $r=0.73$, $P<0.001$ and $P<0.01$ respectively). All three doses (1, 10 and 30 μg) of racemic-flurbiprofen had similar effects in the superficial (I–II) and deep (V–VI) laminae (Figure 6, Table 4). Furthermore, the effects of intraplantar racemic-flurbiprofen on the carrageenan-evoked peripheral oedema and the total number of spinal c-Fos-LI neurons were positively correlated (correlation coefficient $r=0.62$ and $r=0.55$ for the paw and ankle diameters respectively, $P<0.05$ for both); see Figure 10A.

Effects of intraplantar injection of racemic-, S(+)- and R(−)-flurbiprofen

In the fourth experimental series, the effects of intraplantarly injected S(+)-flurbiprofen (0.1, 1, 10 and 30 μg) and R(−)-flurbiprofen (1, 10 and 30 μg) were studied (Figures 7–9, Table 5). Intraplantar injection of racemic-flurbiprofen (0.1 and 30 μg) was used as the drug of reference.

Intraplantarly injected S(+)-flurbiprofen reduced the carrageenan-evoked peripheral oedema at both the paw and ankle level, and the associated spinal c-Fos protein expression (Figures 7–9, Table 5). As compared to the control carrageenan group, the effects of intraplantar injection of S(+)-flurbiprofen (0.1, 1, 10 and 30 μg) were significant when considering the carrageenan-enhanced paw and ankle diameters (ANOVA test $F_{4,20}=8.79$, $P<0.01$ and $F_{4,20}=8.75$, $P<0.001$ respectively), and the total number of c-Fos-LI neurons and their laminar distribution in L4–L5 segments (ANOVA test $F_{4,20}=26.68$ and $F_{4,80}=32.65$ respectively, $P<0.001$ for both). Intraplantar S(+)-flurbiprofen (0.1, 1, 10 and 30 μg) dose-relatedly reduced the total number of c-Fos-LI neurons (regression coefficient $r=0.77$, $P<0.001$), the

number of c-Fos-LI neurons in both the superficial (I–II) and deep (V–VI) laminae (regression coefficients $r=0.68$ and $r=0.67$ respectively, $P<0.01$ for both) and the diameters of the paw and ankle (regression coefficients $r=0.60$ and $r=0.59$ respectively, $P<0.01$ for both). Two doses of 0.1 and 30 μg of racemic-flurbiprofen had similar effects as the same doses of S(+)-flurbiprofen; see Figures 7 and 8 for spinal c-Fos expression, Figure 9 for peripheral oedema, and Table 5 for both. In contrast, intraplantar injection of R(−)-flurbiprofen (1, 10 and 30 μg) did not produce detectable effects on all parameters studied; carrageenan-enhanced paw and ankle diameters (ANOVA test $F_{3,16}=1.54$ and $F_{3,16}=0.95$ respectively, $P>0.05$ for both), and the total number of c-Fos-LI neurons and their laminar distribution in L4–L5 segments (ANOVA test $F_{3,16}=0.32$ and $F_{3,64}=0.54$ respectively, $P>0.05$ for both); see Figures 7–9, Table 5.

Since the highest dose of intraplantar R(−)-flurbiprofen (30 μg) studied did not have any detectable effect on the carrageenan-evoked peripheral oedema or the spinal c-Fos protein expression (Figure 7–9, Table 5) we studied the effects of a higher dose of R(−)-flurbiprofen (100 μg) in comparison to those of the same dose for racemic- and S(+)-flurbiprofen (Figure 11, Table 6). As compared to the control carrageenan group, intraplantar injection of racemic- or S(+)-flurbiprofen (100 μg for both substances) significantly reduced the carrageenan-evoked peripheral oedema at paw and ankle levels, and associated spinal c-Fos protein expression (Figure 11, Table 6). Importantly, the effects of 100 μg of racemic- and S(+)-flurbiprofen were similar to those of 30 μg of these drugs; for comparison see Tables 5 and 6. The effects of 100 μg of R(−)-flurbiprofen were extremely weak with this relatively high dose producing only a 20% reduction of the number of c-Fos-LI neurons (Figure 11, Table 6). This dose of R(−)-flurbiprofen did not influence the carrageenan-enhanced paw diameter and had weak effects on the ankle diameter (Table 6).

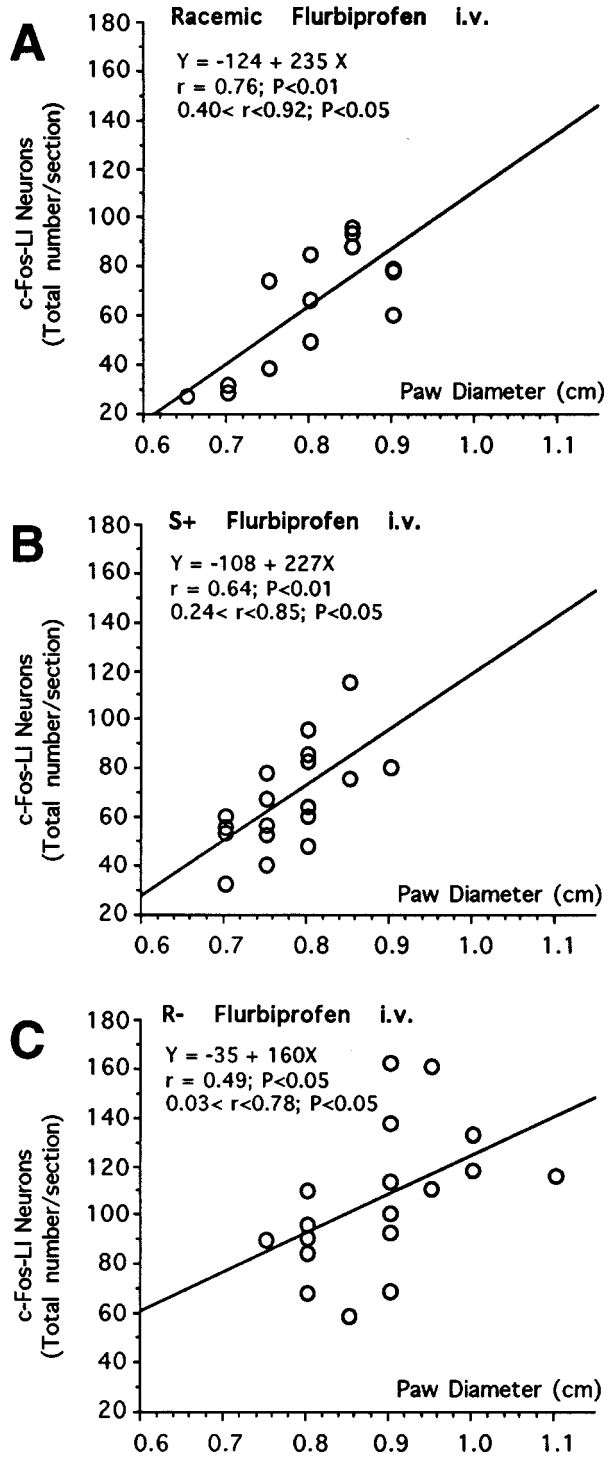

Figure 5 Correlation between the effects of racemic-, S(+)- and R(−)-flurbiprofen (0.3, 3 and 9 mg kg^{-1} i.v.; $n=5$ for each group) on the carrageenan-evoked paw oedema (Paw diameter) and total number of c-Fos-LI neurons per section of L4–L5 segments (c-Fos-LI Neurons).

Discussion

In agreement with previous studies (Draisci & Iadarola, 1989; Noguchi *et al*., 1991, 1992; Honoré *et al*., 1995; Buritova *et al*., 1996a and references therein), 3 h after intraplantar carrageenan, c-Fos-LI neurons were numerous in the superficial (I–II) and deeper (V–VI) laminae of the L4–L5 segments of lumbar spinal cord in awake rats. This localization is in concordance with the spinal areas containing neurons activated by noxious stimuli driven by C- and Aδ-fibres (see Besson & Chaouch, 1987; Willis & Coggeshall, 1991). Importantly, the number of carrageenan-evoked spinal c-Fos-LI neurons was well reproducible and homogenous, with a similar laminar distribution, for the control carrageenan groups of the five experimental series performed

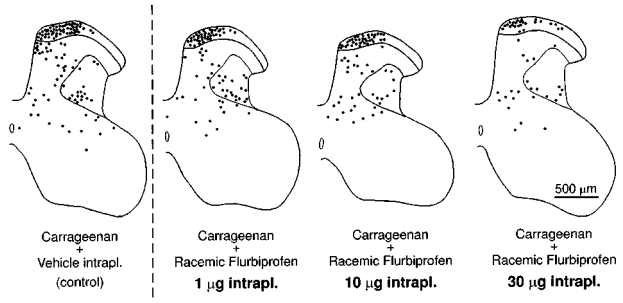

Figure 6 Camera lucida drawings, of individual representative example of segments L4–L5 of rat spinal cord, illustrating the c-Fos protein-like immunoreactivity evoked by intraplantar co-injection of carrageenan plus vehicle (control) and the effects of intraplantar co-injection of carrageenan plus racemic flurbiprofen (1, 10 and 30 μg in 50 μl of vehicle) on the number of c-Fos-LI neurons, 3 h after carrageenan. Each drawing includes all c-Fos-LI neurons in one 40-μm section of segments L4–L5; each dot represents one c-Fos-LI neuron. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. Scale bar = 500 μm.

Table 4 The carrageenan-evoked spinal c-Fos protein expression and peripheral oedema in the control carrageenan group and groups receiving intraplantar co-injection of carrageenan plus racemic flurbiprofen (1, 10 and 30 μg in 50 μl of vehicle).

Group	*Dose (μg/ intrapl.)*	*c-Fos-LI neurons in L4–L5 segments*			*Peripheral oedema*	
		Total	*Laminae I–II*	*Laminae V–VI*	*Paw Diameter*	*Ankle Diameter*
Controls	–	133±9	61±4	46±4	1.11±0.04	1.00±0.03
Racemic flurbiprofen	1	96±4 (28±3)**	47±3 (23±4)**	33±2 (29±5)**	1.00±0.03 (17±4)*	0.98±0.01 (8±5)
	10	78±8 (42±6)***	42±3 (30±6)**	24±4 (47±9)***	0.94±0.03 (27±5)***	0.90±0.02 (40±9)**
	30	53±4 (60±3)***	29±3 (52±5)***	18±1 (62±2)***	0.92±0.02 (30±3)***	0.84±0.02 (67±7)***

Results for spinal c-Fos expression are expressed as mean number (±s.e.m.) of c-Fos-LI neurons per L4–L5 segments (Total), and per laminar region (laminae I–II and laminae V–VI). Results for peripheral oedema are expressed as mean value (±s.e.m.; in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Results expressed as % reduction of control value of studied parameters are presented in brackets. Note that in non-stimulated rats, the value for paw and ankle diameter are 0.48±0.02 and 0.75±0.03 cm respectively. For the calculation of the % reduction for paw and ankle diameters see Methods. Significance compared to control carrageenan group was performed using ANOVA and Fisher's PLSD test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

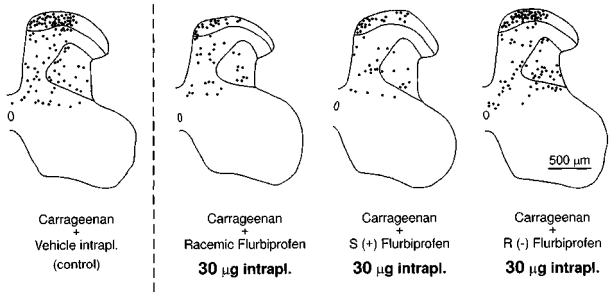

Figure 7 Camera lucida drawings, of individual representative example of segments L4–L5 of rat spinal cord, illustrating the c-Fos protein-like immunoreactivity evoked by intraplantar co-injection of carrageenan plus vehicle (control) and the effects of intraplantar co-injection of carrageenan plus racemic-, S(+) or R(−)-flurbiprofen (30 μg for each substance) on the number of c-Fos-LI neurons, 3 h after carrageenan. Each drawing includes all c-Fos-LI neurons in one 40-μm section of segments L4–L5; each dot represents one c-Fos-LI neuron. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. Scale bar = 500 μm.

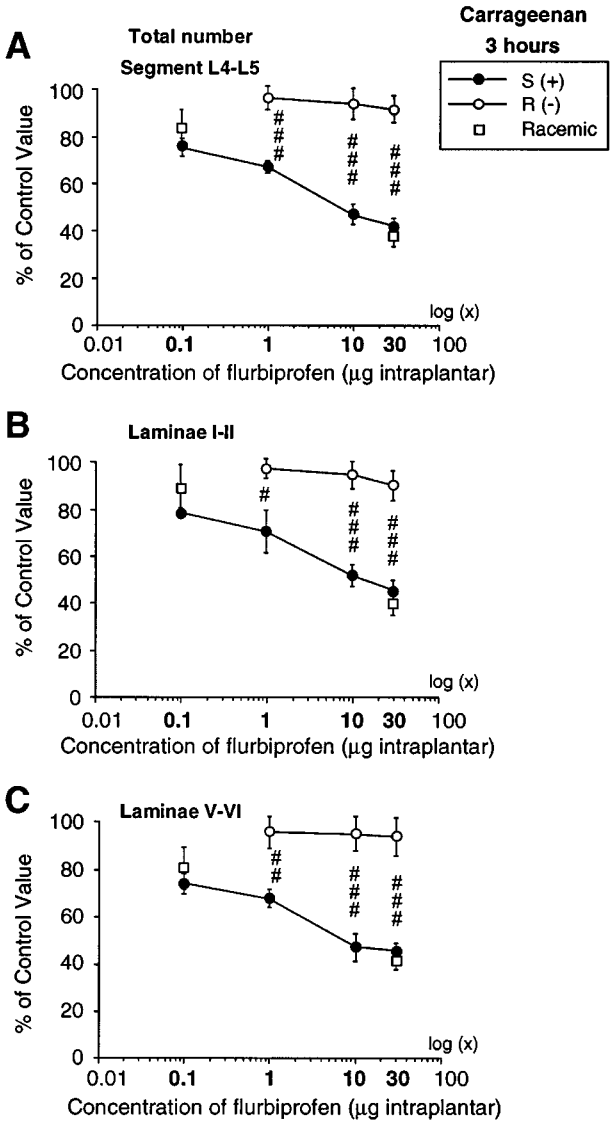

Figure 8 Effects of intraplantar racemic-flurbiprofen (0.1 and 30 μg; $n=5$ for both groups; □), S(+)-flurbiprofen (0.1, 1, 10 and 30 μg; $n=5$ for each group; ●) and R(−)-flurbiprofen (1, 10 and 30 μg; $n=5$ for each group; ○) on the total number of c-Fos-LI neurons (Total number; A), the number of superficial (Laminae I–II; B) and deep (laminae V–VI; C) c-Fos-LI neurons in the L4–L5 segments, 3 h after carrageenan. Results are expressed as % of the control value for the total number of c-Fos-LI neurons and their number in each region (± s.e.m.). Statistical comparisons between the effects of S(+)-flurbiprofen versus R(−)-flurbiprofen were performed using ANOVA and Fisher's PLSD test (#$P<0.05$, ##$P<0.01$, ###$P<0.001$).

in the present study (see Table 1). The aim of the study was to compare the effects of racemic-, S(+)- and R(−)-flurbiprofen administered either intravenously or intraplantarly, under conditions of carrageenan inflammation.

The intravenous pre-administration of racemic- or S(+)-flurbiprofen produced similar dose-related reductions of the number of carrageenan-evoked c-Fos-LI neurons at the spinal cord level. The effects of these compounds on spinal c-Fos expression were correlated with their effects on the peripheral oedema after carrageenan. A similar relationship has already been shown when considering the effects of systemic administration of various NSAIDs in the same experimental paradigm (see references in Buritova *et al*., 1996a; Buritova & Besson, 1998). In the present study, the relatively low dose of 0.3 mg kg^{-1} of racemic- or S(+)-flurbiprofen was sufficient to produce marked effects on the carrageenan-evoked spinal c-Fos protein expression (43±2 and 43±6 % reduction of the total number of c-Fos-LI neurons, respectively), which were comparable to the effects of the same dose of lornoxicam, a new oxicam (45±3% reduction of the total number of c-Fos-LI neurons; Buritova & Besson, 1998), and those of 1 mg kg of ketoprofen, another propionic acid (47±5% reduction of the total number of c-Fos-LI neurons; Buritova *et al*., 1996a). For higher doses of racemic- or S(+)-flurbiprofen (3 and 9 mg kg^{-1}), the effects on carrageenan-evoked c-Fos protein expression were comparable to those previously obtained with

Table 5 The carrageenan-evoked spinal c-Fos protein expression and peripheral oedema in the control carrageenan group and groups receiving intraplantar co-injection of carrageenan plus racemic flurbiprofen (1, 10 and 30 μg; $n=5$), or S(+)-flurbiprofen (0.1, 1, 10 and 30 μg; $n=5$ for each group) or R(−)-flurbiprofen (1, 10 and 30 μg; for each group).

	Dose (μg/	*c-Fos-LI neurons in L4–L5 segments*			*Peripheral oedema*	
Group	intrapl.)	*Total*	*Laminae I–II*	*Laminae V–VI*	*Paw Diameter*	*Ankle Diameter*
Controls	–	127±9	53±7	47±3	0.98±0.02	1.00±0.02
Racemic	0.1	106±10 (16±8)*	46±6 (12±11)	38±4 (19±9)*	0.95±0.01 (6±3)	0.97±0.01 (2±5)
flurbiprofen	30	49±6 (61±5)***	21±3 (49±5)***	20±2 (57±5)***	0.80±0.03 (36±5)***	0.84±0.02 (64±7)***
S(+)	0.1	96±5 (25±4)**	41±1 (21±2)*	35±2 (26±4)**	0.89±0.03 (18±7)*	0.92±0.02 (32±8)**
flurbiprofen	1	85±3 (33±2)***	37±5 (29±9)**	32±2 (32±4)***	0.88±0.03 (20±5)**	0.91±0.02 (36±7)**
	10	60±6 (53±4)***	27±2 (48±5)***	22±3 (53±6)***	0.79±0.04 (38±8)***	0.88±0.03 (48±10)***
	30	54±4 (58±3)***	24±2 (54±4)***	22±1 (54±3)***	0.76±0.02 (44±5)	0.84±0.02 (64±7)***
R(—)	1	123±6 (3±5)	51±2 (2±4)	46±3 (4±7)	1.01±0.01 (0±2)	1.01±0.02 (0±7)
flurbiprofen	10	119±8 (6±7)	50±3 (5±6)	45±3 (4±8)	0.96±0.02 (4±5)	0.97±0.03 (12±10)
	30	116±8 (8±6)	47±3 (10±6)	45±4 (5±8)	0.96±0.02 (4±4)	0.98±0.01 (8±5)

Results for spinal c-Fos expression are expressed as mean number (±s.e.m) of c-Fos-LI neurons per L4–L5 segments (Total), and per laminar region (laminae I–II and V–VI). Results for peripheral oedema are expressed as mean value (±s.e.m.; in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Results expressed as % reduction of control value of studied parameters are presented in brackets. Note that in non-stimulated rats, the value for paw and ankle diameter are 0.48±0.02 and 0.75±0.03 cm respectively. For the calculation of the % reduction for paw and ankle diameters see Methods. Significance compared to control carrageenan group was performed using ANOVA and Fisher's PLSD test (*$P\geqslant0.05$, **<0.01, ***$P<0.001$).

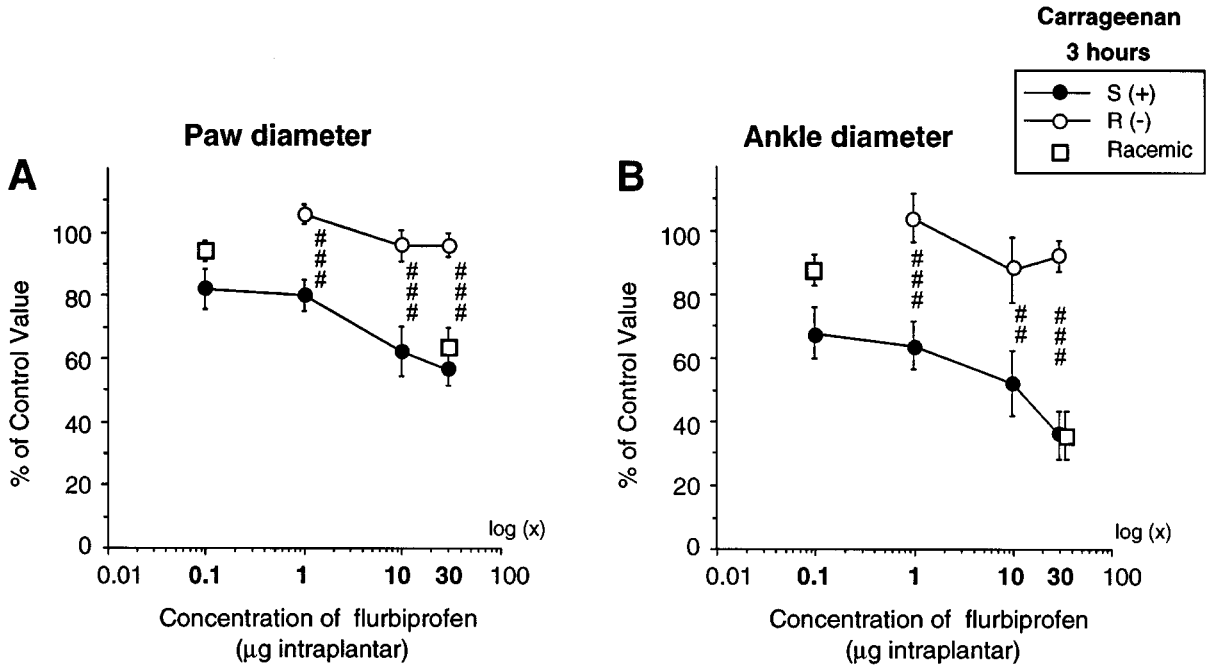

Figure 9 Effects of intraplantar racemic-flurbiprofen (0.1 and 30 μg; $n=5$ for both groups; □), S(+)-flurbiprofen (0.1, 1, 10 and 30 μg; $n=5$ for each group; ●) and R(−)-flurbiprofen (1, 10 and 30 μg; $n=5$ for each group; ○) on the carrageenan-enhanced paw (A) and ankle (B) diameters, 3 h after intraplantar carrageenan. Results are expressed as % of the control value of the carrageenan-enhanced paw and ankle diameter (± s.e.m.). Statistical comparisons between the effects of S(+)-flurbiprofen versus R(−)-flurbiprofen were performed using ANOVA and Fisher's PLSD test (#$P<0.05$, ##$P<0.01$, ###$P<0.001$).

the doses in the same range of other intravenously pre-administered NSAIDs under carrageenan inflammation (50–70% reduction of the total number of spinal c-Fos-LI neurons; see references in Buritova & Besson, 1997a, 1998).

The correlations between the effects of intravenous racemic- or S(+)-flurbiprofen on the spinal c-Fos protein expression and the peripheral oedema suggest a predominant peripheral site of action of these compounds with a subsequent reduction of nociceptive inputs to the spinal cord and thus a decrease in carrageenan-evoked spinal c-Fos protein expression. To confirm this assertion we have evaluated the effects of intraplantar injection of the racemic- or S(+)-flurbiprofen. Even with a low dose of intraplantar racemic-flurbiprofen (1 μg), significant effects were seen about 28% reduction of the total number of c-Fos-LI neurons, and about 17% reduction of diameter at the paw level. Similarly, significant effects of intraplantar S(+)-flurbiprofen were observed for an extremely low dose of 0.1 μg; about 25% reduction of the total number of c-Fos-LI neurons, and about 18 and 32% reduction of paw and ankle diameters respectively. The effects of intraplantar racemic- and S(+)-flurbiprofen on both the carrageenan-evoked spinal c-Fos protein expression and peripheral oedema increased with the dose and are of the same order for the 1, 10 and 30 μg of both drugs. Furthermore, the effects of intraplantar racemic- or S(+)-flurbiprofen on spinal c-Fos expression were correlated with those on peripheral oedema.

Table 6 The carrageenan-evoked spinal c-Fos protein expression and peripheral oedema in the control carrageenan group and groups receiving intraplantar co-injection of carrageenan plus racemic-, or S(+)- or R(−)-flurbiprofen (100 μg for each substance; $n=5$ for each group).

Group	*Dose (μg/ intrapl.)*	*c-Fos-LI neurons in L4–L5 segments*			*Peripheral oedema*	
		Total	*Laminae I–II*	*Laminae V–VI*	*Paw Diameter*	*Ankle Diameter*
Controls	–	135±6	52±2	52±3	1.03±0.02	1.10±0.02
Racemic flurbiprofen	100	44±5 (67±4)***	23±4 (56±8)***	15±2 (71±3)***	0.82±0.04 (38±7)***	0.91±0.03 (54±8)***
S(+) flurbiprofen	100	51±6 (62±5)***	25±3 (52±6)***	19±3 (64±5)***	0.78±0.01 (45±2)***	0.87±0.04 (66±10)***
R(–) flurbiprofen	100	109±12 (19±9)*	45±3 (13±7)	42±5 (19±9)*	1.04±0.02 (0±3)	1.01±0.02 (26±5)*

Results for spinal c-Fos expression are expressed as mean number (±s.e.m.) of c-Fos-LI neurons per L4–L5 segments (Total), and per laminar region (laminae I–II and V–VI). Results for peripheral oedema are expressed as mean value (±s.e.m.; in centimetres) of the diameter at paw and ankle levels (Paw, Ankle). Results expressed as % reduction of control value of studied parameters are presented in brackets. Note that in non-stimulated rats, the value for paw and ankle diameter are 0.48±0.02 and 0.75±0.03 cm respectively. For the calculation of the % reduction for paw and ankle diameters see Methods. Significance compared to control carrageenan group was performed using ANOVA and Fisher's PLSD test (*$P<0.05$, **<0.01, ***$P<0.001$).

Thus, the present study demonstrates, surprisingly for the first time, the marked anti-inflammatory effects of intraplantar racemic- or S(+)-flurbiprofen. The marked effects of low doses (1 and 10 μg) of intraplantar injection of racemic- and S(+)-flurbiprofen argues for a peripheral site of action (directly in the inflammatory site) of intravenous administration of these compounds. However, for the higher dose (30 μg) of intraplantar drugs, vascular reabsorption and thus a central site of action of these compounds cannot be excluded. Indeed, various central sites of action (spinal and/or supraspinal) have been proposed (for review see Jurna, 1997). For example, behavioural studies using the rat paw formalin test and intrathecal administration have provided evidence for a spinal site of action of racemic-flurbiprofen (Malmberg & Yaksh, 1992a) and also its S(+) and R(−) enantiomers (Malmberg & Yaksh, 1994b).

In contrast to the marked effects of intraplantar racemic- or S(+)-flurbiprofen, R(−)-flurbiprofen was totally ineffective following intraplantar administration (doses up to 30 μg). Interestingly, it must be underlined that in our comparative study, the effects of intravenous R(−)-flurbiprofen were always significantly less pronounced than those of intravenous S(+)-flurbiprofen whatever the dose administered. The present results showing the effects of intravenous R(−)-flurbiprofen on carrageenan-evoked c-Fos expression are in agreement with, and extent, behavioural studies in the formalin model demonstrating its antinociceptive effects after systemic (intraperitoneal; Geisslinger *et al*., 1994) or intrathecal (Malmberg & Yaksh 1994b) administrations. Although it is difficult to compare these two studies to the present one, it appears that whatever the route of administration (i.p., i.t., or i.v.) S(+)-flurbiprofen is more efficacious than R(−)-flurbiprofen.

In the present study, the absence of effect for intraplantar R(−)-flurbiprofen on both carrageenan-evoked spinal c-Fos expression and peripheral oedema is reminiscent with the previous behavioural results demonstrating that local administration of R(−)-flurbiprofen into the inflamed paw was unable to block prostaglandin E_2-induced hyperalgesia (Geisslinger *et al*., 1994). In addition, our c-Fos protein study extend and strongly confirm an electrophysiological study (Neugebauer *et al*., 1995) in which it has been demonstrated that both the intravenous and intraplantar injection of S(+)-flurbiprofen, but only intravenous R(−)-flurbiprofen, reduced the responses of dorsal horn neurons to pressure applied to the inflamed knee joint induced by carrageenan and kaolin. Thus, this could be involved in an subsequent reduction of the number of spinal c-Fos-LI neurons observed for S(+) and R(−)-flurbiprofen in the present study. Interestingly, the results of the electrophysiological study (Neugebauer *et al*., 1995) are totally in accordance with those we observed with the same doses (0.3, 3 and 9 mg kg^{-1} i.v.) of S(+) and R(−)-flurbiprofen when considering carrageenan-evoked spinal c-Fos protein expression: (i) the dose of 0.3 mg kg^{-1} of intravenous S(+)-flurbiprofen has strong depressive effects on neuronal responsiveness (decrease about 45%) which closely parallel with the depressive effect we observed by counting the number of spinal c-Fos-LI neurons (decrease of 43±6%); (ii) the dose of 0.3 mg kg^{-1} of intravenous R(−)-flurbiprofen was totally inefficacious on both the neuronal activity and total number of c-Fos-LI neurons and (iii) for the highest studied dose (9 mg kg^{-1} i.v.), the effects of S(+)-flurbiprofen were significantly more marked than those of R(−)-flurbiprofen on both the spinal c-Fos protein expression ($P<0.001$ for total number of c-Fos-LI neurons) and peripheral oedema ($P<0.01$ for ankle diameter). Despite the lack of detailed statistical analysis in the electrophysiological study (Neugebauer *et al*., 1995), the effects of S(+)- and R(−) enantiomers for high doses (3 and 9 mg kg^{-1} i.v.) seem to be very similar (about 80% reduction of neuronal activity for both compounds). More precisely, the strong depressive effects of intravenous S(+) and R(−)-flurbiprofen were similar for neuronal responses to both noxious and innocuous stimuli applied on either ipsilateral inflamed or contralateral non-inflamed ankle. These results are surprising since from a general point of view several behavioural studies have shown that the effects of NSAIDs are more marked on increased pain threshold seen under inflammatory conditions (Attal *et al*., 1988).

In the present study, the differential results obtained after intravenous and intraplantar administration of R(−)-flurbiprofen are in favour of a central site of action of this compound, i.e. at the spinal cord level as previously shown by electrophysiological investigation (see Neugebauer *et al*., 1995 and references therein). Recently, Geisslinger and Schaible (1996) suggested that in the electrophysiological study of Neugebauer *et al*. (1995), the higher potency of intravenous S(+)-flurbiprofen may result from its synergistic actions in the periphery and central nervous system (CNS), whereas intravenous R(−)-flurbiprofen is more likely act in the CNS. Our results seem to be in accordance with this hypothesis.

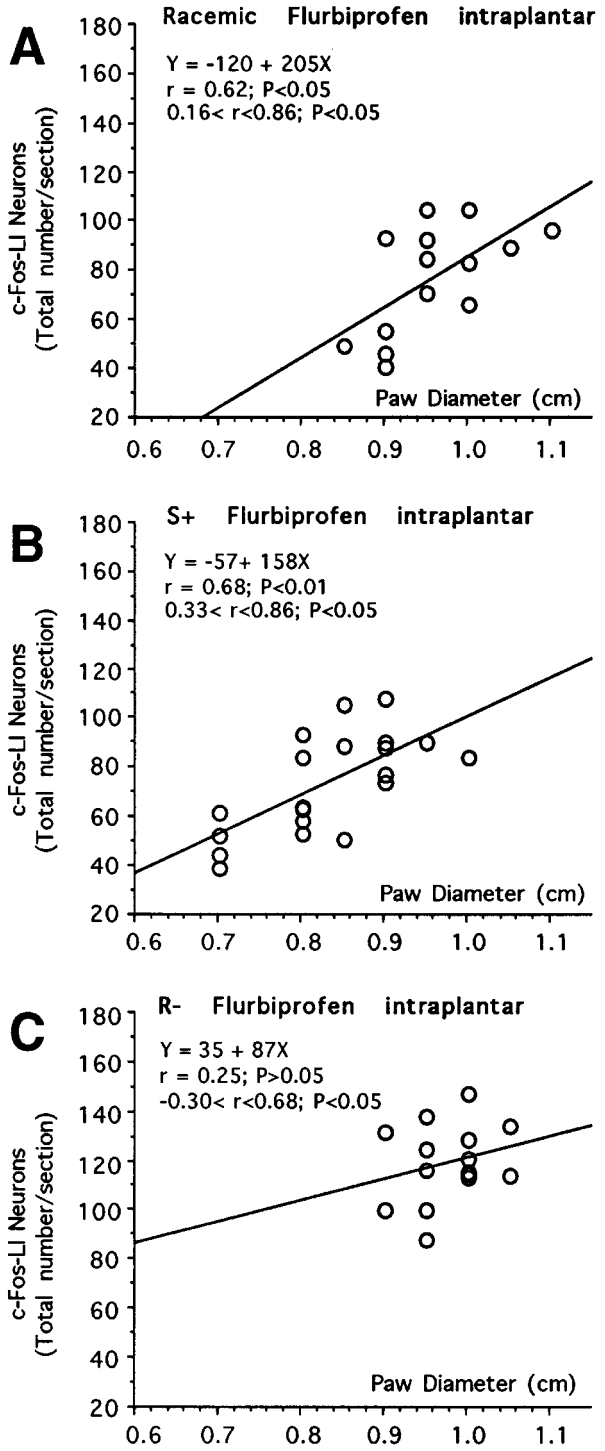

Figure 10 Correlation between the effects of racemic-flurbiprofen (1, 10 and 30 μg; $n=5$ for each group), S(+)-flurbiprofen (0.1, 1, 10 and 30 μg; $n=5$ for each group) and R(−)-flurbiprofen (1, 10 and 30 μg; $n=5$ for each group) on the carrageenan-evoked paw oedema (Paw diameter) and total number of c-Fos-LI neurons per section of L4–L5 segments (c-Fos-LI Neurons).

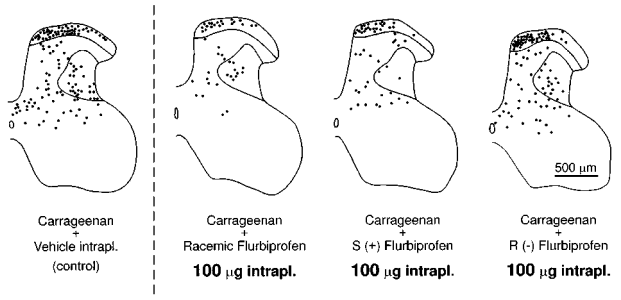

Figure 11 Camera lucida drawings, of individual representative example of segments L4–L5 of rat spinal cord, illustrating the c-Fos protein-like immunoreactivity evoked by intraplantar co-injection of carrageenan plus vehicle (control) and the effects of intraplantar co-injection of carrageenan plus racemic-, S(+)- or R(−)-flurbiprofen (100 μg for each substance) on the number of c-Fos-LI neurons, 3 h after carrageenan. Each drawing includes all c-Fos-LI neurons in one 40-μm section of segments L4–L5; each dot represents one c-Fos-LI neuron. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. Scale bar = 500 μm.

Since the dose of 30 μg of intraplantar R(−)-flurbiprofen did not have any detectable effects on both the carrageenan-evoked peripheral oedema and spinal c-Fos protein expression, we have studied the effects of a higher dose of this compound (100 μg) in comparison to the same dose of racemic- and S(+)-flurbiprofen. Interestingly, for racemic- and S(+)-flurbiprofen, the effects of 100 μg were similar to those of 30 μg, suggesting ceiling effects of these two compounds (for comparison see Tables 5 and 6). In contrast to the lack of effect for 30 μg of R(−)-flurbiprofen, the dose of 100 μg of this compound produced a weak, but significant, decrease of both the number of spinal c-Fos-LI neurons and the ankle oedema. Importantly, in the present study, the comparison of results in Tables 5 and 6 revealed that the effective dose of intraplantar R(−)-flurbiprofen (100 μg) was up to one hundred times greater than the effective dose of intraplantar racemic- or S(+)-flurbiprofen (1 μg for both). Thus, the present results are reminiscent from those of Neugebauer *et al.* (1995): indeed, their electrophysiological investigations were unable to demonstrate peripheral effects of R(−)-flurbiprofen even for very high doses of 100 and 180 μg. In the present study, the weak effects observed after 100 μg of R(−)-flurbiprofen seem to be due, in part, to the vascular reabsorption which is probably more important in awake rats than in anaesthetized rats. Overall, taking into account both the electrophysiological and c-Fos protein studies mentioned above it could be strongly suggested that the direct peripheral effects (in the inflamed tissues) of R(−)-flurbiprofen are negligible. If true, this assertion also suggests that the anti-inflammatory effects of R(−)-flurbiprofen observed after intravenous administration (3 and 9 mg kg^{-1}) are indirectly sustained by central mechanism(s) which remain to be determined.

However, there are discrepancies between our results and those of previous studies when considering the possible anti-inflammatory effects of R(−)-flurbiprofen. In our study, intravenous R(−)-flurbiprofen reduced carrageenan-evoked oedema with clearly apparent effects for the dose of 3 mg kg^{-1} (about 53% reduction of carrageenan-enhanced ankle diameter). To our knowledge, only one previous study has evaluated the anti-inflammatory effects of systemic S(+)- and R(−)-flurbiprofen in rats (Brune *et al.*, 1991). These authors claimed that, contrary to S(+)-flurbiprofen, 'the R-form had much less effect on prostaglandin synthesis and did not affect inflammation' evoked by carrageenan. However, this assertion is questionable since Brune *et al.* (1991) used an oral administration of doses up to 3 mg kg^{-1} of R(−)-flurbiprofen and even if the anti-inflammatory effects have not been significant, according to their Figure 1B, the inhibition of the carrageenan oedema is apparently around 40%. Thus,

according to our experiments, higher oral doses of R(−)-flurbiprofen would have reduced the carrageenan oedema in a significant manner.

Mechanisms of action of racemic-, S(+) and R(−)-flurbiprofen

Traditionally, it has been believed that the inhibition of synthesis of the prostaglandins via cyclooxygenase inhibition by NSAIDs (Vane, 1971; Ferreira, 1972) is the predominant basis for the therapeutic, as well as the gastrointestinal side effects of these compounds (for propionic acids see Elliott *et al*., 1988; for general review see Alhava, 1994). More recently, the varying anti-inflammatory and analgesic effects of different NSAIDs have been attributed to their different affinity for two cyclooxygenase isoenzymes COX-1 and COX-2 (Meade *et al*., 1993; Mitchell *et al*., 1993; Laneuville *et al*., 1994; Grossman *et al*., 1995). A recent *in vitro* study evaluated the effects of enantiomers of flurbiprofen on both COX-1 and COX-2 activity in different biological systems (Carabaza *et al*., 1996). These authors concluded that 'the activity for various R-enantiomers was found only at very high concentrations, from 20 to 10,000 times higher than the equipotent concentrations of the corresponding S-enantiomers'. Thus, from this study, it appears that, contrary to S(+)-flurbiprofen, the effects of R(−)-flurbiprofen cannot be explained by an inhibition of COX-2 activity. In agreement with such a hypothesis, S(+)-flurbiprofen is a potent inhibitor of prostaglandin synthesis, but R(−)-flurbiprofen is an enantiomer practically devoid of inhibitory effects on prostaglandin synthesis (Brune *et al*., 1991; for review see Geisslinger & Schaible, 1996). However, both S(+)- and R(−)-flurbiprofen inhibited prostaglandin synthesis *ex vivo* in whole blood, in the lung and in the brain (Peskar *et al*., 1991). Thus, in the central nervous system, the action of both S(+)- and R(−)-flurbiprofen could be due, in part, to prostaglandin synthesis inhibition. The possible reduction of the release of prostaglandins by NSAIDs (Malmberg & Yaksh, 1992a, 1994b, 1995), including S(+)- and R(−)-flurbiprofen, at spinal cord level has been established. Interestingly, in spinal cord slices, S(+)-flurbiprofen, and to a lesser extent R(−)-flurbiprofen, has been shown to decrease capsaicin-evoked prostaglandin E_2 release (Malmberg & Yaksh, 1994a). Unfortunately, the effects of flurbiprofen enantiomers on the prostaglandin release were not evaluated in *in vivo* studies in which an intrathecal administration of S(+)-ibuprofen, but not R(−)-ibuprofen, suppressed formalin-induced behavioural responses and spinal release of prostaglandin E_2 (Malmberg & Yaksh, 1995). From these studies, there is a need for clarification of the spinal mechanisms of action of S(+) and R(−) enantiomers. When considering the peripheral mechanisms of action, S(+)-flurbiprofen, but not R(−)-flurbiprofen, seems to act *via* prostaglandin synthesis inhibition directly in the inflamed tissue. This hypothesis is supported by a behavioural study (Geisslinger *et al*., 1994 demonstrating that following local administration into inflamed paw S(+)-flurbiprofen, but not R(−)-flurbiprofen, blocked prostaglandin E_2-induced hyperalgesia.

Other mechanisms which are independent of prostaglandin synthesis inhibition may be involved in the central effects of S(+)- and R(−)-flurbiprofen. Indeed several other mechanisms have been proposed (see references in Biela & Groppetti, 1993; McCormack, 1994; Jurna, 1997) but they remain subject to controversy and criticism. Interestingly, a more recent *in vitro* study has demonstrated an inhibition of the capsaicin-induced current by NSAIDs, such as acetylsalicylic acid, salicylic acid and diclofenac, in rat dorsal root ganglion neurons (Kress *et al*., 1996). These authors suggest that since the sensitivity to capsaicin is specific for nociceptors the inhibitory action *via* capsaicin receptors may represent a new and potentially important mechanism of the analgesic action of NSAIDs.

Finally, for R(−)-flurbiprofen, we cannot totally exclude an additional mechanism of action by chiral inversion to the S(+)-enantiomer despite the limited extent of enantiomeric bioinversion in the rat (Jamali *et al*., 1988; Menzel-Soglowek *et al*., 1992; for review see also Williams, 1990).

To conclude, this comparative study of the effects of either intravenous or intraplantar S(+)- and R(−)-flurbiprofen provides further evidence for the increase and/or the development of therapeutical approaches in two main directions: (i) The local administration of S(+)-flurbiprofen which produced a strong decrease of both the carrageenan-evoked spinal c-Fos protein expression and inflammatory oedema suggests that such modality of local administration of S(+)-flurbiprofen, and probably S(+) enantiomers of other profens, with a better availability at the site of inflammation could be a useful approach to avoid gastrointestinal side-effects; (ii) In contrast, the effects of systemic administration of R(−)-flurbiprofen despite the fact that it is less potent than S(+)-flurbiprofen on the carrageenan-evoked spinal c-Fos protein expression and inflammatory oedema, but practically devoid of the gastrointestinal site-effects (Brune *et al*., 1991, 1992a; for review see also Wechter, 1994; Davies *et al*., 1996) should be preferentially used when local administration is not possible.

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