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Carrageenin-evoked c-Fos expression in rat lumbar spinal cord: the effects of indomethacin

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Abstract

This study evaluated the effects of systemic indomethacin on carrageenin evoked c-Fos expression in rat lumbar spinal cord neurons. Fos-like immunoreactivity was not observed after the intraplantar injection of the control vehicle saline. 2 h after administration of carrageenin (6 mg/150 μl) into the hind limb, Fos-like immunoreactive neurons were observed in the lumbar spinal cord (64 labelled neurons per L4–L5 sections) and were numerous in the superficial laminae (I–II), whereas at 3–4 h both superficial and deeper laminae (V, VI and ventral horn) were labelled. 3 h after carrageenin administration, maximal Fos-like immunoreactivity was observed (104 labelled neurons per L4–L5 sections). At later time points Fos-like immunoreactivity was observed predominantly in the deeper laminae. Fos-like immunoreactivity was rarely observed within laminae III–IV at any of the time points. At 24 h, the number of Fos-like immunoreactive neurons decreased (36 labelled neurons per L4–L5 sections). With increasing doses of carrageenin, an increase in the number of Fos-like immunoreactive neurons was observed. The number of Fos-like immunoreactive neurons induced by the carrageenin stimulation (6 mg, at 3 h) was clearly reduced by oral pretreatment with indomethacin (20 mg/kg). In addition, i.v. indomethacin (1, 2.5 or 5 mg/kg) dose dependently reduced the number of Fos-like immunoreactive neurons and the inflammation of the paw and the ankle of the injected foot. A strong relationship between the effect of indomethacin on c-Fos expression and its effect on inflammatory processes was observed. These results suggest that Fos-like immunoreactivity induced by carrageenin inflammation may be a very useful tool to study the effects of anti-inflammatory drugs, at both peripheral and central levels of inflammation.

Keywords: c-Fos expression; Indomethacin; Nociception; Carrageenin, inflammation

1. Introduction

The focal point of nociceptive transmission is the dorsal horn of the spinal cord (see Woolf, 1994). Noxiously evoked neuronal responses are subject to plasticity, the capacity for change within neuronal systems, which may result in enhanced or altered responses of these dorsal horn neurons. Therefore a considerable number of studies have focused on the noxiously evoked responses of the dorsal horn neurons.

Physiological stimulation of rat primary sensory neurons has been shown to result in the expression of Fos-like protein immunoreactivity in the nuclei of postsynaptic dorsal horn neurons of the spinal cord (Hunt et al., 1987). After several years of investigations, there is accumulating evidence that various nociceptive peripheral stimuli result in c-Fos expression at the spinal level (see references in Abbadie et al., 1994a). Electrical stimulation of the sciatic nerve at Aδ- and C-fibre intensity, but not at Aα- and Aβ-fibre intensity, has been shown to induce c-Fos expression in the superficial laminae and in the neck of the lumbar dorsal horn (Herdegen et al., 1991), corresponding to the population of neurons which receive noxious inputs (see references in Besson and Chaouch, 1987). Furthermore, it must be emphasised that morphine, which reduces noxiously evoked responses of dorsal horn neurons without affecting innocuously evoked responses (Duggan et al., 1977; LeBars et al., 1976), dose dependently blocks c-Fos expression in a naloxone reversible manner (Abbadie and Besson, 1993a; Abbadie et al., 1994a,b; Hammond et al., 1992; Presley et al., 1990;

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Tölle et al., 1990, 1994a,b). Acute noxious inflammatory states are also associated with the spinal expression of c-Fos protein, such as subcutaneous injection of formalin (Abbadie et al., 1992; Gogas et al., 1991; Leah et al., 1992; Presley et al., 1990; Strassman and Vos, 1993; Williams et al., 1989, 1990) and Freund's adjuvant (Hylden et al., 1992; Menétrey et al., 1989; Naranjo et al., 1991). In addition, chronic inflammation, such as poly and mono chronic arthritis, is also associated with such an expression of c-Fos protein (Abbadie and Besson, 1992; Lantéri-Minet et al., 1993). Overall, from these studies it seems reasonable to assume that the relay of noxious inputs into the dorsal horn may result in the expression of c-Fos protein in a specific laminar distribution (laminae I–II–V–VI) whereas innocuous inputs, induced for example by non-noxious brushing (Hunt et al., 1987), result in fewer neurons expressing the c-Fos protein and in a different laminar distribution.

At the site of inflammation, algogenic substances derived from tissue damage and from fine sensory afferent endings, activated by local C-fibre axon reflexes (see references in Levine et al., 1993; Yaksh, 1993), are associated with significant increases in the excitability of polymodal nociceptors and high threshold mecanoreceptors (see references in Handwerker and Kobal, 1993; Schaible and Schmidt, 1988). Carrageenin inflammation induces the release and/or generation of mediators such as serotonin, histamine, kinins, leukotriene B_4, thromboxane B_2 and prostaglandins, which can directly stimulate or sensitise free nerve endings (see references in Levine et al., 1993).

Subcutaneous injection of carrageenin produces an acute restricted inflammation associated with ipsilateral oedema (Winter et al., 1962). Behavioural studies have shown that peripheral carrageenin results in both heat and mechanical hyperalgesia (Hargreaves et al., 1988; Iadarola et al., 1988; Joris et al., 1990; Kayser and Guilbaud, 1987). The hyperalgesia is dose dependently related to the dose of carrageenin, and is maximal at 4 h, corresponding to the time of maximal oedema (Hargreaves et al., 1988). The inflamed paw is kept elevated (persistant flexion) and guarded from any disturbance during this hyperalgesic period (Iadarola et al., 1988; Kayser and Guilbaud, 1987). Electrophysiological studies have shown that subcutaneous carrageenin increases the ventrobasal thalamic complex neuronal responses (Guilbaud et al., 1986) and is associated with changes in dorsal horn neuronal responses, which are related to the degree of excitability (Stanfa et al., 1992). Carrageenin-induced hyperalgesia has been shown to be reduced by systemic opioids (Joris et al., 1990; Kayser et al., 1991). Both behavioural (Kayser and Guilbaud, 1990) and electrophysiological (Stanfa et al., 1992) studies have shown rats with inflammation to be more sensitive to morphine than normal rats.

The aim of the present study is to gauge the effect of indomethacin, a widely used non-steroidal anti-inflammatory drug (NSAID), on carrageenin evoked c-Fos expression. NSAIDs, such as indomethacin, inhibit the cyclo-oxygenase enzyme and therefore inhibit the production of prostaglandins (Vane, 1971; see references in Samuelsson et al., 1978). The inhibition of prostaglandin production by NSAIDs has been shown to prevent the sensitisation of the afferent endings and consequently decreases hyperalgesia (Ferreira et al., 1973; Moncada et al., 1975).

In order to determine the optimal parameters of stimulation for the pharmacological studies, the time course of c-Fos expression (i.e. various delays post carrageenin injection) was studied to determine the time course for the maximal expression of c-Fos. In addition, we quantitatively studied the dose-response relationship between increasing doses of carrageenin and c-Fos expression in the lumbar spinal cord. Finally, the effect of intravenous and oral administration of indomethacin (1, 2.5 or 5 mg/kg i.v.; 20 mg/kg p.o.) on carrageenin-induced Fos-like immunoreactivity was studied.

2. Materials and methods

2.1. Experimental animals

Experiments were performed on 126 adult male albino Sprague-Dawley rats (Charles River, France), weighing 225–250 g. Guidelines on ethical standards for investigations of experimental pain in conscious animals were followed (Zimmermann, 1983). The rats were kept in an animal room at a constant temperature of 22°C, with a 12-h alternating light-dark cycle. Food and water were available ad libitum; the food was directly available on the sawdust in the cages, to minimise the need for the rats to make potentially painful movements to obtain food. Four sets of experiments were performed. Since immunochemistry may vary between experiments, the immunohistochemistry for all of the experiments was performed simultaneously.

In the first series of experiments, the time course of c-Fos expression was studied. Intraplantar carrageenin (6 mg diluted in 150 μl of saline, λ-carrageenin, Sigma) was injected into the right foot of the rat and the animals were perfused 2 h ($n = 5$), 3 h ($n = 11$), 4 h ($n = 6$), 6 h ($n = 6$), 8 h ($n = 6$), 16 h ($n = 6$) or 24 h ($n = 6$) post carrageenin injection. Control rats received an intraplantar injection of 150 μl of saline ($n = 5$) and were perfused 3 h post saline injection. The first time point, 2 h, was chosen since the time course of the expression of the c-*fos* mRNA has been shown to start 30 min after the stimulation and to be predominant 1 h after the stimulation (Draisci and Iadarola, 1989).

In the second series of experiments, the effects of various dose of carrageenin (1 mg, 3 mg or 6 mg, $n = 7$, $n = 7$ and $n = 4$ respectively) on c-Fos expression were studied. The rats were perfused 3 h after the intraplantar injection of carrageenin.

In the third series of experiments, the effects of various doses of indomethacin (indomethacin, injectable solution, Indocid 50 mg, Merck Sharp & Dohme-Chibret; diluted in normal saline) on carrageenin (6 mg)-evoked c-Fos expression were studied. Indomethacin (1, 2.5 or 5 mg/kg i.v., $n = 9$, $n = 9$ and $n = 10$ respectively) was intravenously injected 25 min prior to carrageenin administration and the rats were perfused 3 h post carrageenin injection. A control group of rats received an equal volume of intravenous saline ($n = 9$).

In the fourth series of experiments, the effect of oral indomethacin (a total of 20 mg/kg p.o.) on carrageenin (6 mg)-evoked c-Fos expression at 3 h was studied. Since oral indomethacin has a short half-life, 10 mg/kg indomethacin was given orally 45 min prior to carrageenin and a second dose (10 mg/kg) was given 60 min after the carrageenin injection ($n = 5$). A control group of rats received the same volume of saline ($n = 5$) in the same administration regimen.

In the fifth series of experiments, the effect of the highest dose of intravenous indomethacin injected alone without any stimulation was studied. The rats received intravenously either indomethacin (5 mg/kg) or saline and were perfused 3 h and 25 min after this injection ($n = 5$ for each group).

2.2. Immunohistochemistry

At different times after the carrageenin injection, the animals were deeply anaesthetised with pentobarbital (55 mg/kg i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate-buffered saline 0.1 M followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cord was then removed and postfixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in phosphate buffer. Frontal frozen sections, 40 μm thick, were cut and collected in phosphate buffer to be processed immunohistochemically as free floating sections.

The serial sections from the lumbar segment were immunostained for Fos-like protein according to the avidin-biotin-peroxidase method (Hsu et al., 1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in phosphate-buffered saline 0.1 M with 0.3% Triton-X and were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein (Oncogene Science). The Fos antibody, a rabbit polyclonal antibody directed against residues 4–17 of the N-terminal region of the peptide, was used at 1:4000. The incubated sections were washed 3 times in 1% normal goat serum in phosphate-buffered saline 0.1 M with 0.3% Triton-X and incubated in biotinylated rabbit anti-sheep IgG for 1 h at room temperature, then washed twice in 1% normal goat serum in phosphate-buffered saline 0.1 M with 0.3% Triton-X and incubated for 1 h in avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed 3 times in phosphate-buffered saline 0.1 M and developed in 1-naphthol ammonium carbonate solution (89.5 ml 0.1 M phosphate buffer, 10 ml ammonium carbonate (1% in distilled water), 0.5 ml 1-naphthol (N-199-2 Aldrich, 10% in absolute alcohol) and 0.1 ml hydrogen peroxide) for 5 min, and were washed 3 times in phosphate buffer to stop the staining reaction. The sections were mounted on gelatine-coated slides and air dried for the stain to be intensified and made alcohol resistant through basic dye enhancement in 0.025% crystal violet (42555 Aldrich) in phosphate buffer for 3 min. After 2 short phosphate buffer rinses to take off the excess stain, sections were differentiated in 70% alcohol and the differentiation time was evaluated under the microscope. After being air dried, the slides were covered with a coverslip. To test the specificity of the primary antibody, controls were performed: preabsorption with the corresponding synthetic peptide or omission of any stage of the protocol abolished the staining.

2.3. Counting of Fos labelled cells

Fos-like immunoreactivity was studied through the L2 to L6 spinal segments. Tissue sections were first examined using darkfield microscopy to determine the segmental level according to Molander et al. (1984), as well as the grey matter landmarks. The sections were then examined under lightfield microscopy at $\times 10$ to localise Fos positive cells. Labelled nuclei were counted using a camera lucida attachment.

For the studies of the different experimental parameters (various delays post carrageenin injection and various carrageenin doses), for each rat, three calculations were made: (1) the total number of Fos-like immunoreactive neurons in the grey matter for 15 sections through L2–L6 segments (three sections per segment), (2) the number of Fos-like immunoreactive neurons per segmental level, and (3) the number of Fos-like immunoreactive neurons per specific laminar region of the spinal grey matter in segments L4 and L5. For this purpose, four regions were defined: superficial dorsal horn, i.e. laminae I and II; nucleus proprius, i.e. laminae III and IV; neck of the dorsal horn, i.e. laminae V and VI; and the 'ventral horn', i.e. laminae VII, VIII and X (ventral).

For the study of the effects of indomethacin, for each rat, two counts were made: (1) the total number

of Fos-like immunoreactive neurons in the grey matter for 10 sections through L4–L5 segments, and (2) the number of Fos-like immunoreactive neurons per specific laminar region of the spinal grey matter in these 10 sections.

2.4. Pathological observations

In order to assess the development of inflammation, we considered two pathological parameters at the time the animals were killed. We measured the diameter of both the ipsilateral and contralateral ankle and paw. Since the studies of Fos-like immunoreactivity and the assessment of pathological parameters were performed in the same animals, it was possible to look for an eventual correlation between inflammatory parameters and Fos-like immunoreactivity.

2.5. Statistical tests

Statistical analysis was made to compare the total number of labelled cells, using 1-way analysis of variance for the different groups of animals, and 2-way analysis of variance for the different groups of animals and the spinal level, and 3-way analysis of variance for the different groups of animals, the spinal level, and the laminar region; to compare the ankle or the paw diameters we used 1-way analysis of variance for the different groups of animals. For multiple comparisons, the Fisher's PLSD (protected least significant difference) test was used. Dose-dependent effects were demonstrated by linear regression. The investigator responsible for plotting and counting the Fos-like immunoreactive neurons was blind to the experimental situation of each animal.

3. Results

In non-stimulated freely moving rats, Fos-like immunoreactivity was almost absent (< 5 labelled neurons by section) in the lumbar spinal grey matter whereas in carrageenin stimulated animals, Fos-like immunoreactive nuclei appeared as round structures, stained to a variable degree ipsilateral to the stimulation. Cells in the contralateral side were labelled extremely weakly and our analysis was focused on the ipsilateral side. To quantify the number of Fos-like immunoreactive nuclei, we took into account all labelled nuclei without considering the intensity of the staining.

3.1. Time course of the c-Fos expression during the 24 h following the carrageenin injection

Two hours after carrageenin injection, numerous Fos-like immunoreactive neurons were observed within the L2–L6 segments (Fig. 1A). A maximal number of labelled cells was observed at 3 and 4 h and thereafter there was a clear decrease at 6 h but still a substantial number of labelled cells at 24 h.

At 3 h, most Fos-like immunoreactive neurons were essentially located in the superficial dorsal horn (laminae I–II, 44% of the total number of labelled cells) and in the neck of the dorsal horn (laminae

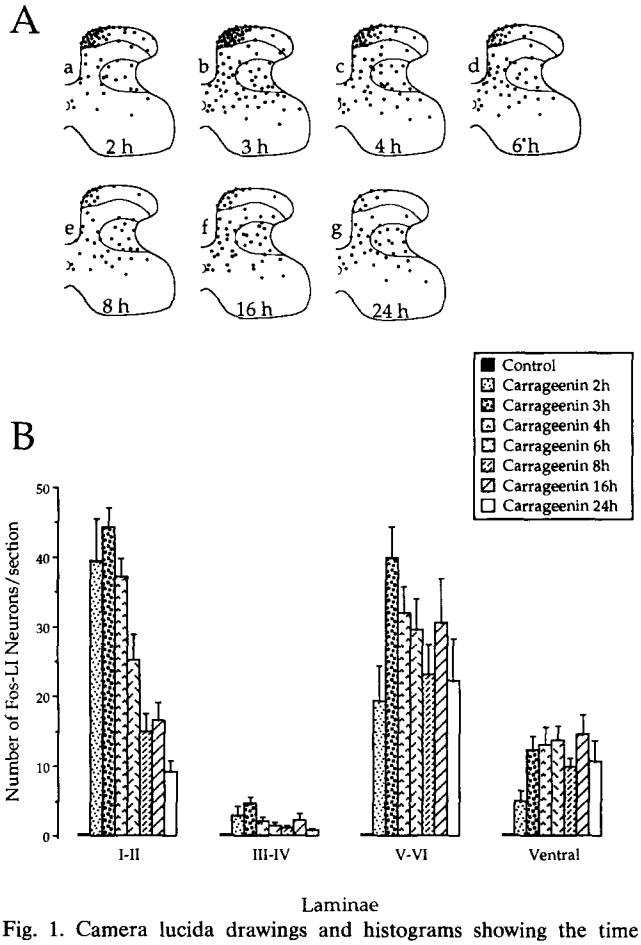

Fig. 1. Camera lucida drawings and histograms showing the time course of c-Fos expression after an intraplantar injection of carrageenin (6 mg) into the rat's right foot. Eight experimental situations are represented: rats received an intraplantar injection of either saline (and were perfused 3 h after; control group) or carrageenin (6 mg) and were perfused 2 h, 3 h, 4 h, 6 h, 8 h, 16 h or 24 h after. A: Each schema includes all labelled cells in one 40-μm section; each dot represents one labelled cell. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. B: Results are expressed as mean number of Fos-like immunoreactive neurons ± S.E.M. per laminar region of L4–L5 segments. Note that (1) intraplantar injection of saline induced no Fos-like immunoreactive neuron, (2) the number of Fos-like immunoreactive neurons increased when the duration of the stimulation increased to a maximal value at 3 h and then decreased, (3) Fos-like immunoreactivity predominated in laminae I–II and V–VI of L4–L5 segments, (4) the greatest number of Fos-like immunoreactive neurons was located in the superficial laminae at 2, 3 and 4 h, (5) for later post-injection perfusion time, most of the Fos-like immunoreactive neurons were located in the V–VI laminae.

V–VI, 40%). The number of Fos-like immunoreactive neurons in the ventral horn was moderate (11.5%) while very few neurons expressed the Fos protein in laminae III and IV (4.55%). An extremely low number of Fos positive cells in laminae III–IV was observed throughout the experiment (Fig. 1B).

The number of labelled cells in the superficial layers was maximal at 2, 3 and 4 h after carrageenin (Fig. 1B). A significant decrease (38%, $P < 0.0001$) at 6 h and a strong reduction at 24 h (77%, $P < 0.0001$) was observed. A different time course was observed in the neck of the dorsal horn. At this level, a significant increase in the number of Fos-labelled cells was observed between 2 and 3 h ($P < 0.01$) and sustained labelling was observed until 24 h. However, the decrease in the number of labelled cells between 3 and 24 h was significant ($P < 0.01$). Since a maximal number of labelled cells was observed in both superficial laminae and in the neck of the dorsal horn 3 h after carrageenin, such a delay was selected in order to gauge the effects of the intraplantar injection of various doses of carrageenin on c-Fos expression.

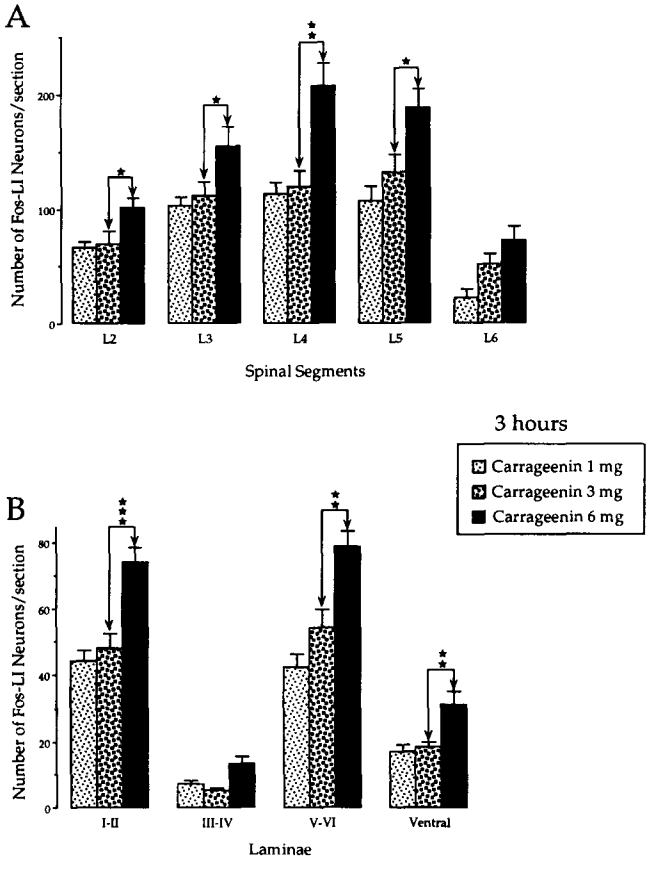

Fig. 2. Histograms showing the number of Fos-like immunoreactive neurons 3 h after carrageenin injection into the rat's right hind paw. Three experimental situations are represented: the rat hind paw was injected with either 1, 3 or 6 mg of carrageenin. Results are expressed as mean number of Fos-like immunoreactive neurons ± S.E.M. per segmental distribution (A) and per laminar region in L4–L5 segments (B). Significance is expressed comparing the groups to each others ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Note that (1) the number of Fos-like immunoreactive neurons increased with the doses of injected carrageenin, (2) the number of Fos-like immunoreactive neurons was greatest in L3–L5 segments in laminae I–II and V–VI.

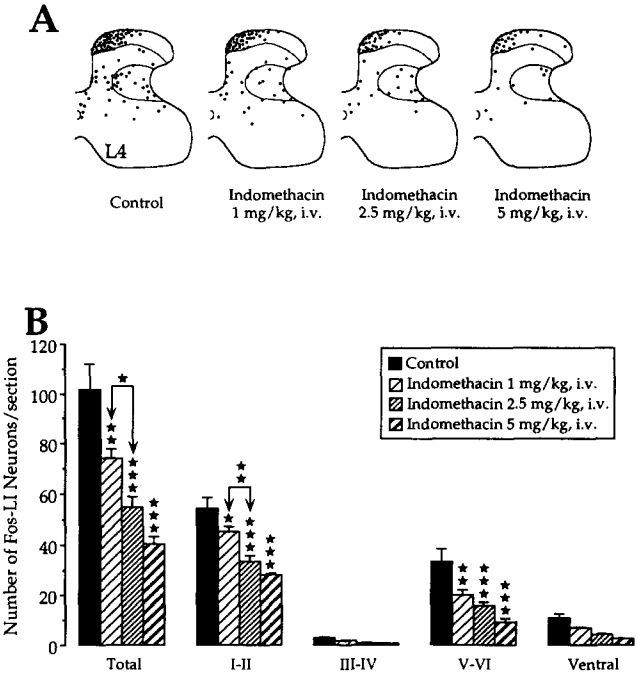

Fig. 3. Camera lucida drawings and histograms showing the effects of indomethacin on Fos-like immunoreactivity, 3 h after carrageenin injection (6 mg) into the rat's right foot. Four experimental situations are represented: rats were pretreated 25 min prior to stimulation with either saline (control) or indomethacin (1, 2.5 or 5 mg/kg i.v.). A: Each schema includes all labelled cells in one 40-μm section; each dot represents one labelled cell. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. B: Results are expressed as mean number of Fos-like immunoreactive neurons ± S.E.M. in all laminae of the L4–L5 segments (Total) and per laminar region of L4–L5 segments. Significance is expressed taking as reference the control group, using the PLSD Fisher's test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Note that (1) Fos-like immunoreactivity predominated in laminae I–II and V–VI, (2) indomethacin dose dependently decreased the number of Fos-like immunoreactive nuclei induced by carrageenin stimulation, and (3) the effect of indomethacin was significant in the superficial and profound laminae.

3.2. Effects of the dose of carrageenin

An increase in the number of Fos-like immunoreactive neurons was observed with increasing doses of carrageenin (Fig. 2A, B). Both 3 mg and 6 mg resulted in an increase in the total number of Fos-like immunoreactive neurons (23.5% increase and 76% increase; $P < 0.01$ respectively), as compared to that observed with 1 mg of carrageenin. The experimental groups were significantly different for the total number of

Fos-like immunoreactive neurons in the L2–L6 segment ($F(2,15) = 7.8$, $P < 0.005$), the rostrocaudal Fos-like immunoreactivity distribution ($F(2,75) = 28.3$, $P < 0.0001$), and the laminar Fos-like immunoreactivity distribution ($F(2,132) = 34.5$, $P < 0.0001$) (Fig. 2). There was a tendency towards an increase in the number of Fos-like immunoreactive neurons observed after 3 mg of carrageenin as compared to 1 mg, but this was not significant. However, a significantly greater number of neurons were observed with 6 mg of carrageenin as compared to 3 mg ($P < 0.01$).

For all the groups, Fos-like immunoreactivity was clearly present in the L2–L6 segments (Fig. 2A). In adjacent segments (rostral to L2 and caudal to L6), Fos-like immunoreactive nuclei were sparse (approximately 1–4 per section) and located predominantly in the deep laminae of the dorsal horn. The rostrocaudal distribution of Fos-like immunoreactive neurons indicates that there was dense labelling in L4–L5 segments and to a lesser extent in L3.

3.3. Effects of indomethacin

3.3.1. Indomethacin i.v.

In the first part of our experiment we showed that 3 h post injection and 6 mg of carrageenin are optimal conditions for maximal c-Fos expression in L4–L5 segments and therefore the effects of indomethacin on c-Fos expression were studied under these conditions.

Fos-like immunoreactivity was almost absent (< 5 labelled neurons by section) after systemic administration of 5 mg/kg indomethacin or saline without any other intentional stimulation.

Pretreatment with indomethacin (1, 2.5 or 5 mg/kg i.v.) significantly decreased the number of Fos-like

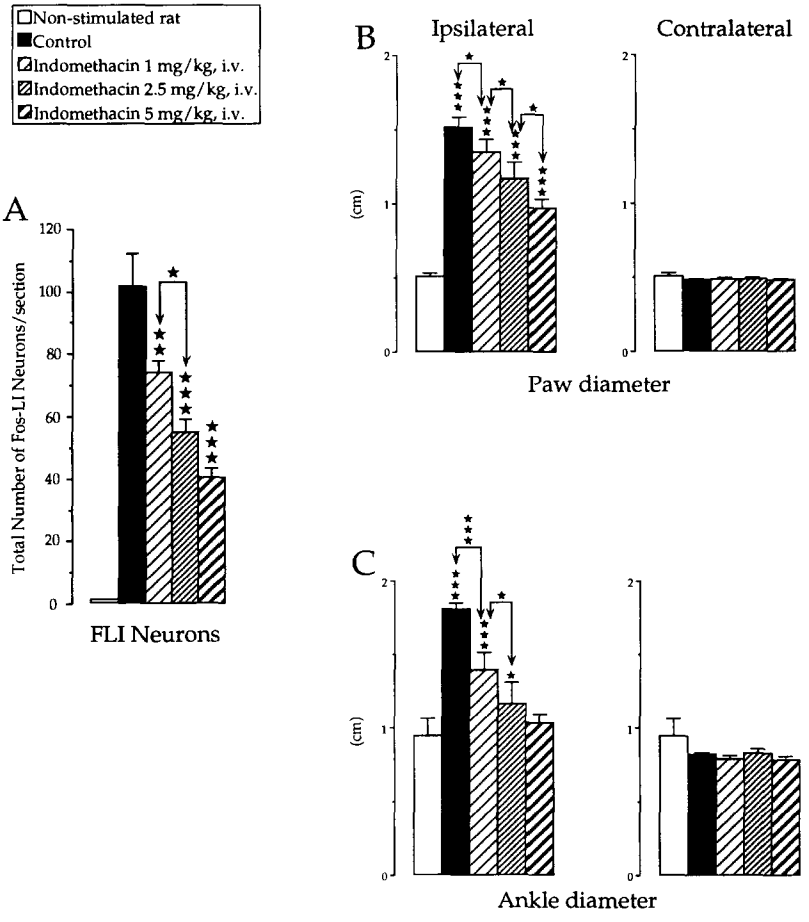

Fig. 4. Histograms showing the effects of indomethacin 3 h after carrageenin injection (6 mg) into the rat's right foot. Five experimental situations are represented: non-stimulated rats or stimulated rats pretreated 25 min prior to stimulation with either saline (control) or indomethacin (1, 2.5 or 5 mg/kg i.v.). Results are expressed as mean number of Fos-like immunoreactive neurons ± S.E.M. in the segments L4–L5 (A), as mean paw diameter ± S.E.M. (B), as mean ankle diameter ± S.E.M. (C). A: Significance is expressed taking as reference the control group, using the PLSD Fisher's test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). B and C: Significance is expressed taking as reference the group of non-stimulated rats using the PLSD Fisher's test ($^{*}P < 0.05$, $^{***}P < 0.001$). Note that (1) no inflammation occurred in the contralateral foot of the rat, (2) indomethacin dose relatedly decreased the number of Fos-like immunoreactive nuclei, the inflammation of the paw and the ankle induced by carrageenin inflammation.

immunoreactive nuclei induced by 6 mg of carrageenin as compared to control values (Fig. 3). The total number of Fos-like immunoreactive nuclei within the L4–L5 segments was dose dependently ($r^2 = 0.56$; $P < 0.0001$) reduced by indomethacin (27%, 46% and 60% reduction considering all the laminae, $F(3,33) = 18.1$ $P < 0.0001$). In addition, indomethacin (1, 2.5 and 5 mg/kg i.v.) decreased the number of Fos-like immunoreactive neurons in the superficial laminae (reductions of 17%, 38% and 49%) and in the neck of the dorsal horn of the spinal cord (reductions of 40%, 53% and 73%).

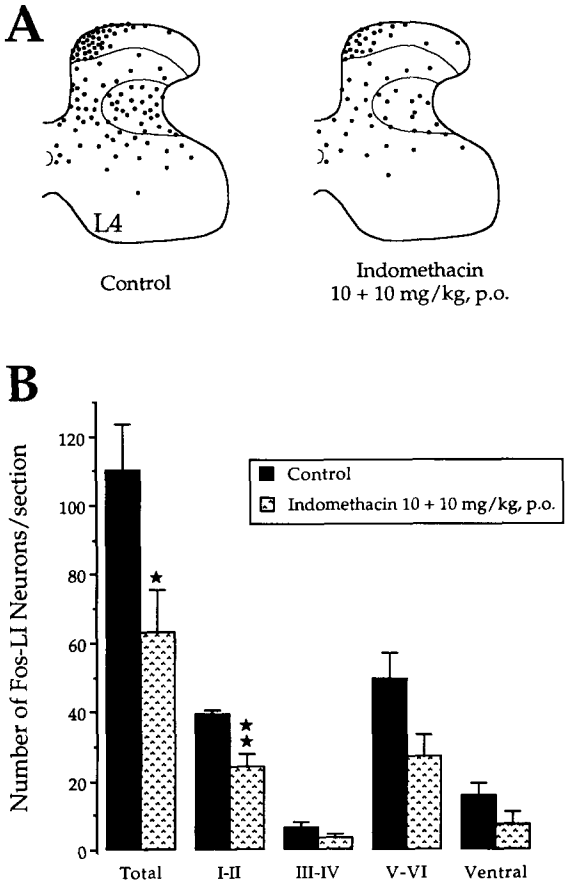

Fig. 5. Camera lucida drawings and histograms showing the effects of indomethacin on Fos-like immunoreactivity, 3 h after carrageenin injection (6 mg) into the rat's right foot. Two experimental situations are represented: rats were pretreated 45 min prior to stimulation and 60 min after stimulation with either saline (control) or indomethacin (10 mg/kg p.o.; for a total dose of 20 mg/kg p.o.). A: Each schema includes all labelled cells in one 40-μm section; each dot represents one labelled cell. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. B: Results are expressed as mean number of Fos-like immunoreactive neurons ± SEM in all laminae of the L4–L5 segments (Total) and per laminar region of L4–L5 segments. Significance is expressed taking as reference the control group, using the PLSD Fisher's test ($^*P < 0.05$, $^{**}P < 0.01$). Note that (1) Fos-like immunoreactivity predominated in laminae I–II and V–VI, (2) indomethacin significantly decreased the number of Fos-like immunoreactive nuclei induced by carrageenin stimulation, and (3) the effect of indomethacin was significant in the superficial laminae.

Again, this effect was significant when considering the laminar distribution ($F(3,132) = 41.1$, $P < 0.0001$) (Fig. 3B).

Indomethacin (1, 2.5 and 5 mg/kg i.v.) strongly reduced the signs of inflammation induced by carrageenin. Indomethacin dose dependently reduced the increase, induced by inflammation, in the ankle diameters (48%, 75% and 90% reduction respectively), $F(8,77) = 21$ $P < 0.0001$ (Fig. 4C). This was paralleled by a decrease in the paw diameter (16%, 34% and 54% reduction respectively), $F(8,77) = 51.6$, $P < 0.0001$ (Fig. 4B). Inflammation was not observed on the contralateral side. There was a clear relationship between the effects of i.v. indomethacin on the total number of Fos-like immunoreactive neurons and on signs (paw and ankle diameters) of inflammation induced by the intraplantar injection of carrageenin (Fig. 4).

3.3.2. *Indomethacin p.o.*

Under the same experimental conditions described above, pretreatment with indomethacin (10 + 10 mg/kg p.o.) significantly decreased (43% reduction, $P < 0.05$) the total number of Fos-like immunoreactive nuclei in the L4–L5 segments as compared to control values ($F(1,8) = 6.7$, $P < 0.05$; Fig. 5). Indomethacin (p.o.) decreased the number of Fos-like immunoreactive neurons in the superficial laminae (39% reduction, $P < 0.01$) and in the neck of the dorsal horn of the spinal cord (46% reduction, $P < 0.06$; see Fig. 5 for details). Again, the effects were significant when considering the laminar distribution ($F(1,31) = 16.1$, $P < 0.001$; Fig. 5B). Indomethacin (p.o.) blocked the development of inflammation of the ankle $F(4,19) = 11.9$, $P < 0.0001$ and significantly decreased the inflammation of the paw (reduction of 30%), $F(4,19) = 49.4$, $P < 0.0001$.

4. Discussion

This study demonstrates that the injection of carrageenin into the hind paw of the rat induces the expression of the c-Fos protein in numerous neurons in the spinal cord of the rat. These effects were strongly reduced by pretreatment with the NSAID, indomethacin. In addition, we have shown that there is a clear relationship between these later effects and those of the drug on the inflammatory signs.

Our study is the first to quantitatively evaluate the number of Fos-like immunoreactive neurons, and the laminar and segmental distribution following various experimental parameters (doses of carrageenin and delays between carrageenin injection and perfusion). The first part of our investigation confirms and extends a previous study which showed an increase in c-*fos* mRNA in laminae I–II and V–VI associated with an increase in preprodynorphin and preproenkephalin

mRNA in the same areas after carrageenin inflammation (Draisci and Iadarola, 1989). In this previous study an increase in c-*fos* mRNA began at 30 min and reached a maximum 2 h after the carrageenin injection.

The rostrocaudal Fos-like immunoreactivity induced by intraplantar injection of carrageenin (6 mg) extended over several segments (L2–L6). The maximal number of Fos-like immunoreactive nuclei was localised in L4–L5 segments. This result is in good agreement with the well-established spinal projections of afferent fibres innervating the hind paw (LaMotte et al., 1991; Molander and Grant, 1986; Molander et al., 1984; Swett and Woolf, 1985). More precisely, Fos-like immunoreactivity was almost exclusively localised in the superficial laminae (laminae I–II) and in the neck of the dorsal horn (laminae V–VI). These results confirm those obtained in previous studies with the same stimulation (Draisci an Iadarola, 1989; Noguchi et al., 1991, 1992), noxious heat (Abbadie et al., 1994b; Hunt et al., 1987; Tölle et al., 1991, 1994a,b; Williams et al., 1989), noxious cold (Abbadie et al., 1994a), formalin injection (Abbadie et al., 1992; Presley et al., 1990), Freund's adjuvant (Lantéri-Minet et al., 1993; Menétrey et al., 1989) and mechanical stimulation of the arthritic rat's hindpaw (Abbadie and Besson, 1993b).

In the present study, 2 h after the stimulation, numerous Fos-like immunoreactive neurons were seen, essentially in the superficial laminae, with this time course paralleling that of the development of the inflammation induced by carrageenin in freely moving rats (DiRosa et al., 1971). The number of Fos-like immunoreactive neurons was maximal at 3–4 h post carrageenin, which follows the peak increase in c-*fos* mRNA observed 2–3 h after the carrageenin injection (Draisci and Iadarola, 1989). Furthermore, this time point of peak c-Fos expression is similar to the time of peak behavioural hyperalgesia (Kayser and Guilbaud, 1987; Hargreaves et al., 1988; Hylden et al., 1991) and oedema and erythema (Di Rosa et al., 1971; Rao et al., 1991). At 3 to 4 h, Fos-like immunoreactive neurons were localised both in superficial and deep laminae. This finding is in good agreement with previous studies which have shown that increasing the duration between stimulation and perfusion increases the number of Fos-like immunoreactive neurons in deeper laminae (Bullitt et al., 1992; Williams et al., 1989). At later time points, the number of Fos-like immunoreactive neurons decreased in all areas of the grey matter of the lumbar enlargement, with these effects being more pronounced in the superficial laminae. Again, this decrease follows the same time course as described by Draisci and Iadarola (1989). In the present study, with later time points (> 4 h), the majority of Fos-like immunoreactive neurons were localised in laminae V–VI. At 24 h, Fos-like immunoreactive neurons were essentially localised in the neck of the dorsal horn. A similar time course and localisation of the c-Fos protein have been previously described after an injection of formalin (Presley et al., 1990). However, it has previously been reported that numerous Fos-like immunoreactive neurons are present in both the superficial and deep laminae 3 days after a carrageenin injection (Noguchi et al., 1991, 1992). This difference might be due to differences in the specificity of the primary antibody used for the immunohistochemistry.

A clear dose-response relationship between the doses of carrageenin and the number of Fos-like immunoreactive neurons was not observed. However, the highest dose of carrageenin (6 mg) resulted in a significantly greater number of Fos-like immunoreactive neurons as compared to 1 or 3 mg of carrageenin. Our results confirm that the number of Fos-like immunoreactive neurons is influenced, to an extent, by the 'intensity' of the stimulation. Despite the fact that we were unable to quantify the intensity of the staining of each individual neuron, we observed that the intensity of the staining was more pronounced with the higher dose of carrageenin. This finding is in agreement with previous studies of c-Fos evoked by cold stimulation or heat stimulation (Abbadie et al., 1994a,b).

In our study, the neurons which expressed carrageenin-evoked c-Fos were located similarly to those neurons which are driven by noxious stimuli, within the superficial laminae and the neck of the dorsal horn (see references in Besson and Chaouch, 1987; Willis and Coggeshall, 1991). Since the majority of small-diameter myelinated and unmyelinated afferents terminate in the superficial dorsal horn, it is reasonable to hypothesise that many of the neurons in the superficial dorsal horn which express c-Fos are driven monosynaptically by small-diameter, presumably nociceptive primary afferents from the inflamed paw. Since the deeper dorsal horn neurons receive an indirect nociceptive input from the superficial laminae, and do not express c-Fos when activated by innocuous stimuli, it seems highly probable that the neurons in the neck of the dorsal horn which express c-Fos are also driven by nociceptive inputs from the inflamed paw. As discussed in the Introduction, the expression of c-Fos has been shown to be reduced by systemic opiates, providing further evidence for c-Fos expression being predominantly associated with nociceptive transmission. A systematic study of the behaviour of the animal after the carrageenin injection was not performed; however, all rats exhibited freezing behaviour and avoided putting the inflamed paw on the floor of the cage, and some rats licked the inflamed paw but this effect was not consistently observed. The functional significance of the labelling we obtained after carrageenin injection and the relationship between nociception and inflammation remains to be elucidated.

Using the optimal experimental parameters deter-

mined in the first part of the study, we assessed the effect of indomethacin on the carrageenin-evoked expression of c-Fos. Indomethacin (i.v.) produced a dose-dependent reduction in the number of carrageenin evoked c-Fos positive neurons. Indomethacin was also active after an oral pretreatment. With both routes of administration, indomethacin reduced the c-Fos expression in both the superficial and the deep laminae of the dorsal horn. This effect was associated with a parallel reduction in the paw and ankle diameters of the injected foot, suggesting a strong relationship between the effects of indomethacin on inflammatory processes and on c-Fos expression. The effects of indomethacin on c-Fos expression seem to be a consequence of reduced peripheral inflammation, presumably due to the inhibition of prostaglandin production. Overall, from our results it seems feasible to hypothesise that with a reduced development of inflammation there is a parallel reduction in nociceptive inputs to the spinal cord and consequently a reduced expression of c-Fos. Our results are in agreement with those of behavioural studies with carrageenin, in which the carrageenin-evoked hyperalgesia was dose dependently related to the dose of carrageenin (Hargreaves et al., 1988) and was reduced by indomethacin (Hargreaves et al., 1988; Mahdy et al., 1990; Rao et al., 1991; Reiter et al., 1985). However, a central effect of NSAIDs (Chapman and Dickenson, 1992; Jurna and Brune, 1990; Jurna et al., 1992; Malmberg and Yaksh, 1992) may also contribute to this effect.

In conclusion, this study confirms and extends previous investigations related to the effects of carrageenin inflammation on Fos-like immunoreactivity at the dorsal horn level. Our results suggest that intraplantar injection of carrageenin induces a noxious peripheral stimulation. In addition, both an antinociceptive and an anti-inflammatory action of indomethacin on carrageenin-induced inflammation have been demonstrated. The distinction between inflammation and nociception is complex, but our results suggest that a reduced inflammation is strongly associated with antinociception. Since there are numerous peripheral inflammatory mediators, the use of this objective technique to study the role of other inflammatory mediators may further extend our knowledge of inflammatory pain mechanisms.

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References

Abbadie, C. and J.-M. Besson, 1992, C-*fos* expression in rat lumbar spinal cord during the development of adjuvant-induced arthritis, Neuroscience 48, 985.

Abbadie, C. and J.-M. Besson, 1993a, Effects of morphine and naloxone on basal and evoked Fos-like immunoreactivity in lumbar spinal cord neurons of arthritic rats, Pain 52, 29.

Abbadie, C. and J.-M. Besson, 1993b, C-*fos* expression in rat lumbar spinal cord following peripheral stimulation in adjuvant-induced arthritis and in normal rats, Brain Res. 607, 195.

Abbadie, C., M.-C. Lombard, F. Morain and J.-M. Besson, 1992, Fos-like immunoreactivity in the rat superficial dorsal horn induced by formalin injection in the forepaw: effects of dorsal rhizotomies, Brain Res. 578, 17.

Abbadie, C., P. Honoré and J.-M. Besson, 1994a, Intense cold noxious stimulation of the rat hindpaw induces c-*fos* expression in lumbar spinal cord neurons, Neuroscience 59, 457.

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

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

Bullitt, E., C.L. Lee, A.R. Light and H. Willcockson, 1992, The effect of stimulus duration on noxious-stimulus induced c-*fos* expression in the rodent spinal cord, Brain Res. 580, 172.

Chapman, V. and A.H. Dickenson, 1992, The spinal and peripheral roles of bradykinin and prostaglandins in nociceptive processing in the rat, Eur. J. Pharmacol. 219, 427.

Di Rosa, M., J.P. Giroud and D.A. Willoughby, 1971, Studies of the mediators of the acute inflammatory-response induced in rats in different sites by carrageenin and turpentine, J. Pathol. 104, 15.

Draisci, G. and M.J. Iadarola, 1989, Temporal analysis of increases in c-*fos*, preprodynorphin and preproenkephalin mRNAs in rat spinal cord, Mol. Brain Res. 6, 31.

Duggan, A.W., J.G. Hall and P.M. Headley, 1977, Suppression of transmission of nociceptive impulses by morphine: selective effects of morphine administered in the region of the substancia gelatinosa, Br. J. Pharmacol. 61, 65.

Feirrera, S.H., S. Moncada and J.R. Vane, 1973, Prostaglandin and the mechanism of analgesia produced by aspirin-like drugs, Br. J. Pharmacol. 49, 86.

Gogas, K.R., R.W. Presley, J.D. Levine and A.I. Basbaum, 1991, The antinociceptive action of supraspinal opioids results from an increase in descending inhibitory control: correlation of nociceptive behavior and c-*fos* expression, Neuroscience 42(3), 617.

Guilbaud, G., V. Kayser, J.-M. Benoist and M. Gautron, 1986, Modifications in the responsiveness of rat ventrobasal thalamic neurons at different stages of carrageenin-produced inflammation, Brain Res. 385, 86.

Hammond, D.L., R.W. Presley, K.R. Gogas and A.I. Basbaum, 1992, Morphine or U-50,488 suppresses Fos protein-like immunoreactivity in the spinal cord and nucleus tractus solitarii evoked by a noxious visceral stimulus in the rat, J. Comp. Neurol. 315, 244.

Handwerker, H.O. and G. Kobal, 1993, Psychophysiology of experimentally induced pain, Physiol. Rev. 73, 639.

Hargreaves, K., R. Dubner, F. Brown, C. Flores and J. Joris, 1988, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, Pain 32, 77.

Herdegen, T., K. Kovary, J. Leah and R. Bravo, 1991, Specific temporal and spatial distribution of JUN, FOS and KROX-24 proteins in spinal neurons following noxious transsynaptic stimulation, J. Comp. Neurol. 313, 178.

Hsu, S., L. Raine and H. Fanger, 1981, Use of avidin-biotin-peroxydase complex (ABC) in immunoperoxydase techniques: a com-

parison between ABC and unlabelled antibody (PAP) procedures, J. Histochem. Cytochem. 29, 577.

Hunt, S.P., A. Pini and G. Evan, 1987, Induction of *c-fos*-like protein in spinal cord neurons following sensory stimulation, Nature 328, 632.

Hylden, J.L.K., K. Noguchi and M.A. Ruda, 1992, Neonatal capsaicin treatment attenuates spinal Fos activation and dynorphin gene expression following peripheral tissue inflammation and hyperalgesia, J. Neurosci. 12(5), 1716.

Hylden, J.L.K., D.A. Thomas, M.J. Iadarola, R.L. Nahin and R. Dubner, 1991, Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms, Eur. J. Pharmacol. 194, 135.

Iadarola, M.J., L.S. Brady, G. Draisci and R. Dubner, 1988, Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding, Pain 35, 313.

Joris, J., A. Costello, R. Dubner and K. Hargreaves, 1990, Opiates suppress carrageenan-induced oedema and hyperthermia at doses that inhibit hyperalgesia, Pain 43, 95.

Jurna, I. and K. Brune, 1990, Central effect of the non-steroid anti-inflammatory agents, indometacin, ibuprofen, and diclofenac, determined in C-fibre evoked activity in single neurones of the rat thalamus, Pain 41, 71.

Jurna, I., B. Spohrer and R. Bock, 1992, Intrathecal injection of acetylsalicylic acid, salicylic acid and indomethacin depresses C-fibre-evoked activity in the rat thalamus and spinal cord, Pain 49, 249.

Kayser, V. and G. Guilbaud, 1987, Local and remote modifications of nociceptive sensitivity during carrageenin-induced inflammation in the rat, Pain 28, 99.

Kayser, V. and G. Guilbaud, 1990, Differential effects of various doses of morphine and naloxone on two nociceptive test thresholds in arthritic and normal rats, Pain 41, 353.

Kayser, V., Y.L. Chen and G. Guilbaud, 1991, Behavioural evidence for a peripheral component in the enhanced antinociceptive effect of a low dose of systemic morphine in carrageenin-induced hyperalgesic rats, Brain Res. 560, 237.

LaMotte, C., S.E. Kapadia and C.M. Shapiro, 1991, Central projections of the sciatic, saphenous, median, and ulnar nerves of the rat demonstrated by transganglionic transport of choleragenoid-HRP (B-HRP) and wheat germ agglutinin-HRP (WGA-HRP), J. Comp. Neurol. 311, 546.

Lantéri-Minet, M., J. De Pommery, T. Herdegen, J. Weil-Fugazza, R. Bravo and D. Menétrey, 1993, Differential time-course and spatial expression of Fos, Jun and Krox-24 proteins in spinal cord of rats undergoing subacute or chronic somatic inflammation, J. Comp. Neurol. 33, 223.

Leah, J.D., J. Sandkuhler, A. Murashov and M. Zimmermann, 1992, Potentiated expression of Fos protein in the rat spinal cord following bilateral noxious cutaneous stimulation, Neuroscience 48(3), 525.

Le Bars, D., G. Guilbaud, I. Jurna and J.-M. Besson, 1976, Differential effects of morphine on responses of dorsal horn lamina V type cells elicited by A and C fibre stimulation in the spinal cat, Brain Res. 115, 518.

Levine, J.D., H.L. Fields and A.I. Basbaum, 1993, Peptides and the primary afferent nociceptor, J. Neurosci. 13, 2273.

Mahdy, S.A., A.A. Alhaider and A.A. Mahgoud, 1990, Antinociceptive and anti-inflammatory activity of a novel quipazine derivative (AAL-13): a selective inhibitor of 5-hydroxytryptamine reuptake, J. Pharm. Pharmacol. 42, 522.

Malmberg, A.B. and T.L. Yaksh, 1992, Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat, J. Pharmacol. Exp. Ther. 263, 136.

Menétrey, D., A. Gannon, J.D. Levine and A.I. Basbaum, 1989, Expression of c-*fos* protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular and visceral stimulation, J. Comp. Neurol. 285, 177.

Molander, C. and G. Grant, 1986, Laminar distribution and somatotopic organization of primary afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat, Neuroscience 19(1), 297.

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

Moncada, S., S.H. Ferreira and J.R. Vane, 1975, Inhibition of prostaglandins biosynthesis as the mechanism of analgesia of aspirin-like drugs in the dog knee joint, Eur. J. Pharmacol. 31, 250.

Naranjo, J.R., B. Mellström, M. Achaval, J.J. Lucas, J. Del Rio and P. Sassone-Corsi, 1991, Co-induction of *jun* B and c-*fos* in a subset of neurons in the spinal cord, Oncogene 6, 223.

Noguchi, K., K. Kowalski, R. Traub, A. Solodkin, M.J. Iadarola and M.A. Ruda, 1991, Dynorphin expression and Fos-like immunoreactivity following inflammation induced hyperalgesia are colocalized in spinal cord neurons, Mol. Brain Res. 10, 227.

Noguchi, K., R. Dubner and M.A. Ruda, 1992, Preproenkephalin mRNA in spinal dorsal horn neurons is induced by peripheral inflammation and is co-localized with Fos and Fos-related proteins, Neuroscience 46, 561.

Presley, R.W., D. Menétrey, J.D. Levine and A.I. Basbaum, 1990, Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord, J. Neurosci. 10(1), 323.

Rao, C.S., N. Muralidhar, M.U. Naidu, A.Y. Jurnnarkar and P.P. Singh, 1991, A rapid method for evaluation of analgesic and anti-inflammatory activity in rats, Indian J. Exp. Biol. 29, 120.

Reiter, M.J., D.H. Schwartzmiller, K.F. Swingle, G.G.I. Moore, M.B. Goldlust, K. Heghinian, D.P. DeVore, B. Choy and W.A. Weppner, 1985, Comparison of anti-inflammatory compounds in the carrageenan induced paw oedema model and the reversed passive arthus model utilizing the same animal, Life Sci. 36, 1339.

Samuelsson, B., M. Goldyne, E. Granstrom, M. Hamberg, S. Hammarstrom and C. Malmsten, 1978, Prostaglandins and thromboxanes, Annu. Rev. Biochem. 47, 997.

Schaible, H.G. and R.F. Schmidt, 1988, Excitation and sensitization of fine articular afferents from cat's knee joint by prostaglandin E_2, J. Physiol. 403, 91.

Stanfa, L.C., A.F. Sullivan and A.H. Dickenson, 1992, Alterations in neuronal excitability and the potency of spinal mu, delta and kappa opioids after carrageenan-induced inflammation, Pain 50, 345.

Strassman, A.M. and B.P. Vos, 1993, Somatotopic and laminar organization of Fos-like immunoreactivity in the medullary and upper cervical dorsal horn induced by noxious facial stimulation in the rat, J. Comp. Neurol. 331, 495.

Swett, J.E. and C.J. Woolf, 1985, The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord, J. Comp. Neurol. 231, 66.

Tölle, T.R., J.M. Castro-Lopes, A. Coimbra and W. Zieglgänsberger, 1990, Opiaites modify induction of c-*fos* proto-oncogene in the spinal cord of the rat following noxious stimulation, Neurosci. Lett. 111, 46.

Tölle, T.R., J.M. Castro-Lopes and W. Zieglgänsberger, 1991, c-*fos* induction in the spinal cord following noxious stimulation: prevention by opiates but not by NMDA antagonists, in: Proceedings of the VIth World Congress on Pain, eds. M.R. Bond, J.E. Charlton and C.J. Woolf (Elsevier, Amsterdam) p. 299.

Tölle, T.R., T. Herdegen, J. Schadrack, R. Bravo, M. Zimmermann and W. Zieglgänsberger, 1994a, Application of morphine prior to noxious stimulation differentially modulates expression of Fos, Jun and Krox-24 proteins in rat spinal cord neurons, Neuroscience 58, 305.

Tölle, T.R., J. Schadrack, J.M. Castro-Lopes, G. Evan, B.P. Roques and W. Zieglgansberger, 1994b, Effects of kelatorphan and morphine before and after noxious stimulation on immediate-early gene expression in rat spinal cord neurons, Pain 56, 103.

Vane, J.R., 1971, Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs, Nature New Biol. 231, 232.

Williams, S., A. Pini, G. Evan and S.P. Hunt, 1989, Molecular events in the spinal cord following sensory stimulation, in: Processing of Sensory Information in the Superficial Dorsal Horn of the Spinal Cord, eds. F. Cervero, J. Bennett and M. Headley (Plenum Publishing Corporation, New York) p. 273.

Williams, S., G.I. Evan and S.P. Hunt, 1990, Changing patterns of c-*fos* induction in spinal neurons following thermal cutaneous stimulation in the rat, Neuroscience 36(1), 73.

Willis, W.D. and R.E. Coggeshall, 1991, Sensory Mechanisms of the Spinal Cord (Plenum Press, New York).

Winter, C.A., E.A. Risley and G.W. Nuss, 1962, Carrageenan-induced oedema in hind paw of the rat as an assay for antiinflammatory drugs, Proc. Soc. Exp. Biol. Med. 111, 544.

Woolf, C.J., 1994, The dorsal horn: state-dependent sensory processing and generation of pain, in: Textbook of Pain, Vol. 3, eds. P.D. Wall and R. Melzack (Churchill Livingstone, London) p. 101.

Yaksh, T.L., 1993, New horizons in our understanding of the spinal physiology and pharmacology of pain processing, Semin. Oncol. 20, 6.

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