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The contribution of NMDA receptor activation to spinal c-Fos expression in a model of inflammatory pain

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1 Intraplantar carrageenin (6 mg 150 μl^{-1}) evoked a high level of spinal c-Fos expression in the dorsal horn, of segments L4–L5 of the spinal cord, and an extensive peripheral oedema; both parameters were assessed 3 h after carrageenin.

2 Two series of experiments were performed, with the mean total number of Fos like-immunoreactive neurones (Fos-LI), after carrageenin, not being significantly different for the two series of experiments (266 ± 17 and 332 ± 31 Fos-LI neurones). For both series of experiments Fos-LI neurones were predominantly located in the superficial and deep laminae, only 10% of the total number of Fos-LI neurones were located in the nucleus proprius and 10% were located in the ventral horn.

3 Pre-administration of the N-methyl-D-aspartate (NMDA) receptor antagonist, (+)-HA966 (0.5 mg kg^{-1} and 2.5 mg kg^{-1}, s.c.), 30 min before carrageenin, did not significantly influence the total number of Fos-LI neurones, as compared to control carrageenin expression.

4 Pre-administration of the highest dose of (+)-HA966 (10 mg kg^{-1}) significantly reduced the number of deep laminae Fos-LI neurones ($28 \pm 3\%$ reduction of control number of Fos-LI neurones after carrageenin, $P \leqslant 0.05$), without influencing the number of superficial Fos-LI neurones. There was a tendency towards a reduction of the number of Fos-LI neurones in the nucleus proprius by the highest concentration of pre-administered (+)-HA966, ($31 \pm 8\%$ reduction), but this effect did not reach significance.

5 Pre-plus post-administered (+)-HA966 (0.5 mg kg^{-1}), 30 min before and again 45 min after intraplantar carrageenin, did not significantly influence the total number of Fos-LI neurones, as compared to control carrageenin expression.

6 Pre- plus post-administration of 2.5 mg kg^{-1} (+)-HA966 significantly reduced the total number of Fos-LI neurones, as compared to control carrageenin expression. This effect was reflected by a significant reduction in the number of Fos-LI neurones in the nucleus proprius ($36 \pm 7\%$ reduction of control carrageenin c-Fos expression respectively, $P \leqslant 0.05$).

7 Pre-plus post-administration of 10 mg kg^{-1} of (+)-HA966 significantly reduced the number of Fos-LI neurones in the superficial laminae, nucleus proprius, deep laminae and ventral horn ($33 \pm 0.5\%$, $55 \pm 6\%$, $40 \pm 4\%$ and $51 \pm 4\%$ reduction of control carrageenin c-Fos expression, respectively, $P \leqslant 0.05$, for all areas).

8 A single post-administration of (+)-HA966 (10 mg kg^{-1}), 45 min after intraplantar carrageenin, did not significantly influence the number of Fos-LI neurones in the superficial, deep laminae or ventral horn, but significantly reduced the number of Fos-LI neurones in the nucleus proprius, as compared to control carrageenin expression ($39 \pm 8\%$ reduction of control carrageenin c-Fos expression, $P \leqslant 0.05$).

9 None of the concentrations of (+)-HA966 studied, irrespective of the timing of administration, influenced the peripheral carrageenin oedema. Our results illustrate a contribution of central NMDA receptor activation to carrageenin-evoked spinal c-Fos expression. These results extend previous studies demonstrating the contribution of the NMDA receptor to central hyperalgesia and the expression of c-Fos.

Keywords: Carrageenin; c-Fos; NMDA receptor antagonist; rat spinal cord

Introduction

At the spinal level, nociceptive processing can be modulated by both inhibitory and excitatory systems; this plasticity, which can occur over a short time course, may contribute to central hypersensitivity (see references in Dubner & Ruda, 1992; Dickenson, 1994). There is strong evidence for a contribution of N-methyl-D-aspartate (NMDA) receptor-mediated events to the generation of central hypersensitivity, associated with sustained nociceptive inputs, in animal models of inflammatory pain, in particular the second phase of the formalin response (Haley *et al.*, 1990; Dickenson & Aydar, 1991; Coderre & Melzack, 1992; Yamamoto & Yaksh, 1992; Kristensen *et al.*, 1993; Vaccarino *et al.*, 1993; Hunter & Singh, 1994; Millan & Seguin, 1993; 1994). In addition, previous studies have provided evidence for a contribution of the NMDA receptor to the central hyperalgesia associated with the carrageenin model of inflammatory pain (Ren *et al.*, 1992; Eisenberg *et al.*, 1994; Laird *et al.*, 1994).

The intracellular changes associated with NMDA receptor activation are, presumably, essential to the development of NMDA receptor-mediated central hypersensitivity. Primarily, an influx of calcium into neurones, via the channel associated with the NMDA receptor, results in increased intracellular levels of calcium (Mayer & Westbrook, 1987). Such events are functionally relevant to nociceptive transmission, since spinal administration of calcium channel antagonists strongly reduces the second phase, with a less profound effect on the first phase, of the formalin response (Malmberg & Yaksh, 1994). Thus calcium channel blockade mimics, at least in part, the effect of NMDA receptor antagonism on the formalin re-

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sponse. In addition, increased levels of intracellular calcium are known to stimulate the expression of the immediate early gene, c-fos, and reciprocally blockade of voltage sensitive calcium channels inhibits this induction. Furthermore, many of the circumstances under which c-fos is induced in the nervous system involves glutamate receptor, including NMDA receptor, activation (see references in Morgan, 1991). However, as far as spinal nociceptive transmission is considered, the effect of NMDA receptor antagonism on c-Fos expression is not clear. Intrathecal administration of MK801 (Kehl *et al.*, 1991) and subcutaneous administration of dextromethorphan (Elliott *et al.*, 1994), both NMDA receptor antagonists, have been shown to reduce spinal c-Fos expression evoked by formalin. In contrast, systemic ketamine, also an NMDA receptor antagonist, has been shown not to reduce noxious heat evoked c-Fos expression (Tolle *et al.*, 1991). The lack of concordance between these studies may reflect the differential role of the NMDA receptor in different nociceptive models; NMDA receptor activation is predominantly associated with states of prolonged afferent activation and the development of central hypersensitivity, both of which are associated with the formalin response.

The aim of our study was to evaluate the effect of subcutaneous (+)-(1-hydroxy-3-aminopyrrolidine-2-one) ((+)-HA966), a partial agonist at the modulatory strychnine-insensitive glycine site of the NMDA receptor complex (see references in Kemp & Leeson, 1993), on carrageenin evoked expression of spinal c-Fos (Draisci & Iadarola, 1989; Noguchi *et al.*, 1991; 1992; Honoré *et al.*, 1995a,b). Intraplantar carrageenin (Winter *et al.*, 1962) is associated with a classical inflammation (see references in Kocher *et al.*, 1987) and a heat and mechanical hyperalgesia which peaks 3 h after carrageenin (Joris *et al.*, 1990; see references in Dubner & Ruda, 1992; Ferreira *et al.*, 1993). Pharmacological manipulation of the glycine site of the NMDA receptor, either with antagonists or partial agonists, produces effective NMDA receptor antagonism with the benefit that there is a larger window between the desirable and undesirable effects (see references in Kemp & Leeson, 1993). Subcutaneous (+)-HA966, which crosses the blood brain barrier (see references in Kemp & Leeson, 1993), dose-dependently reduces the second phase of the formalin response, in the absence of motor deficits (Millan & Seguin, 1993; 1994). The time course of action of (+)-HA966 is not well established; however, it has an onset time of 30 min and a duration of action in the order of at least 60 min (Millan & Sequin, 1993). Due to the long time course of the carrageenin inflammation relative to the shorter duration of action of (+)-HA966 and the importance of the NMDA receptor to the induction and maintenance of central hypersensitivity, we have studied the effect of pre-administration of (+)-HA966, as compared to pre- plus post-administration of (+)-HA966, on carrageenin-evoked expression of spinal c-Fos. In addition the effect of post-administration of (+)-HA966 on carrageenin-evoked expression of spinal c-Fos was studied.

Methods

Experimental animals

Experiments were performed on 43 adult male albino Sprague-Dawley rats (Charles River, France), weighing 225–250 g. Two series of experiments were performed with statistical comparisons made within each series of experiments but not between the two series of experiments.

In the first series of experiments, the effects of subcutaneous (+)-HA966 (0.5, 2.5 and 10 mg kg^{-1}, dissolved in distilled water, Tocris Cookson) on spinal c-Fos expression, 3 h after intraplantar carrageenin, was studied ($n=5$ for all doses). (+)-HA966 was injected 30 min prior to intraplantar carrageenin (6 mg 150 μl^{-1}, in saline). A control group of rats received an equal volume of subcutaneous distilled water and intraplantar carrageenin (6 mg 150 μl^{-1}, in saline, $n=4$).

In the second series of experiments, the effect of a pre- plus an additional post-administration of subcutaneous (+)-HA966 (0.5+0.5, 2.5+2.5 and 10+10 mg kg^{-1}) on spinal c-Fos expression, 3 h after intraplantar carrageenin, was studied. Pre-administered HA966 was given 30 min before, and post-administered HA966 was given 45 min after carrageenin. In addition, the effect of the highest dose of HA966 (10 mg kg^{-1}), given only as a post-administration (45 min after carrageenin), on spinal c-Fos expression, 3 h after carrageenin, was studied. As before, a control group of rats received equal volumes of subcutaneous distilled water, at the appropriate time points, and intraplantar carrageenin ($n=5$). As with our previous studies (Honoré *et al.*, 1995a,b), all rats were perfused 3 h after carrageenin.

Immunohistochemistry

Three hours after the carrageenin injection, the animals were deeply anaesthetized with pentobarbitone (55 mg kg^{-1}, i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate buffered saline 0.1 M (PBS) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord was then removed and post-fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen sections, 40 μm thick, were cut and collected in PB 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 PBS with 0.3% Triton-X (NGST). The sections were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein (Oncogene Science Inc.). The Fos antibody a rabbit polyclonal antibody directed against residues 4–17 of the N-terminal region of the peptide, was used at 1:4,000. The incubated sections were washed 3 times in 1% NGST and incubated in biotinylated rabbit anti-sheep IgG for 1 h at room temperature, washed twice in 1% NGST and incubated for 1 h in Avidin-Biotin-Peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed 3 times in PBS and developed in 1-naphtol ammonium carbonate solution (89.5 ml 0.1 M PB, 10 ml ammonium carbonate (1% in distilled water), 0.5 ml 1-naphtol (N-199-2 Aldrich, 10% in absolute ethanol) and 0.1 ml hydrogen peroxide) for 5 min, and were then washed 3 times in PB to stop the staining reaction. The sections were mounted on gelatine-subbed 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 PB for 3 min. After 2 short PB rinses to take off the excess stain, sections were differentiated in 70% ethanol and the differentiation time was evaluated under the microscope. After being air dried, the slides were coverslipped. To test the specificity of the primary antibody, controls were performed; pre-absorption with the corresponding synthetic peptide or omission of any stage in the protocol abolished the staining.

Counting of c-Fos positive neurones

Tissue sections were first examined by darkfield microscopy to determine the segmental level according to Molander *et al.* (1984), as well as the gray matter landmarks. The sections were then examined under lightfield microscopy at X10 to localize Fos-like immunoreactive neurones (Fos-LI neurones). Labelled nuclei were counted with a camera lucida attachment. To study the laminar distribution, 4 regions were defined: superficial dorsal horn (laminae I–II; superficial), nucleus proprius (laminae III–IV; nucleus proprius), neck of the dorsal horn (laminae V–VI; deep laminae) and the ventral gray (laminae VII–X; ventral).

We have previously shown that the most numerous Fos-LI neurones were localized in the L4–L5 segments, two counts per slice were made: (1) the total number of Fos-LI neurones in the

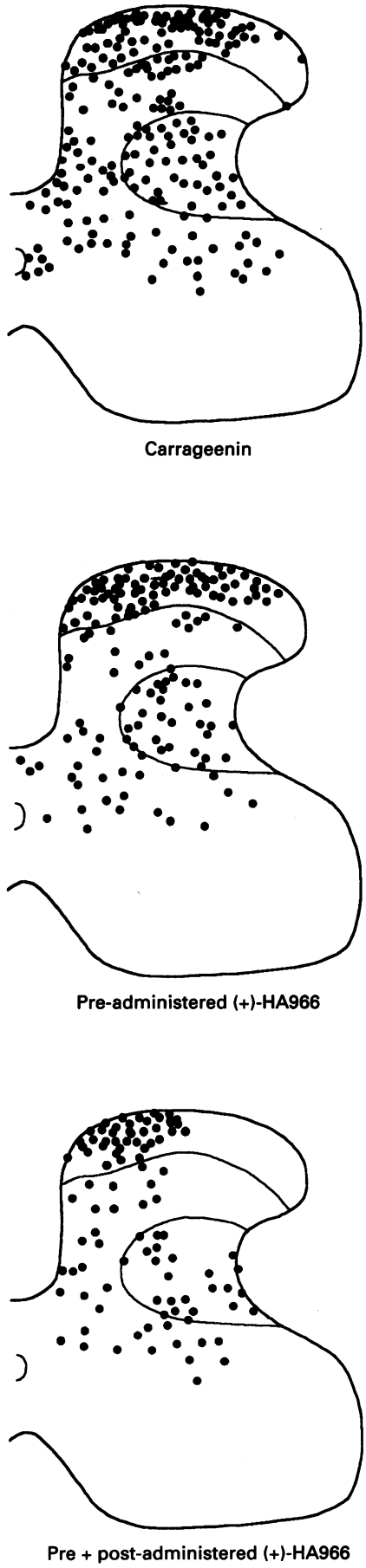

Figure 1 Examples of camera lucida drawings illustrating the effect of pre-administration, as compared to pre- plus post-administration, of subcutaneous (+)-HA966 (10 mg kg^{-1}) on Fos-LI neurones, 3 h after intraplantar carrageenin. The control carrageenin group received subcutaneous distilled water. Each drawing is an example of a 40 μm section of the L4–L5 segment of the spinal cord, and includes all FLI-neurones, each dot representing one FLI-neurone. Drawings are representative of the laminar distribution of FLI-neurones, the four regions studied were laminae I–II (superficial laminae), laminae III-IV (nucleus proprius), laminae V–VI (deep laminae) and the ventral horn. The boundaries of the superficial laminae, and of the reticular part of the neck of the dorsal horn, are outlined.

gray matter for 10 sections through L4–L5 segments, and (2) the number of Fos-LI neurones per specific laminar region of the spinal gray matter in these 10 sections.

Inflammatory observations

In order to assess the development of the inflammation, we considered one of the signs of inflammation, the peripheral oedema, at the time the animals were killed. The diameters of the ankle and of the paw, both ipsilateral and contralateral to the stimulation were measured with calibrated calipers. Both the study of Fos-LI neurones and the assessment of inflammatory parameters were performed in the same rats.

Statistical tests

Statistical analysis of variance (ANOVA) was made to compare the total number of labelled cells, using 1-way ANOVA for the different groups of animals, and 2-way ANOVA for the different groups of animals and the laminar region. For comparisons of the ankle or the paw diameters we used 1-way ANOVA for the different groups of animals. Fisher's PLSD test was used for multiple comparisons and simple regressions were performed to establish dose-dependent effects. The investigator responsible for plotting and counting the Fos-LI neurones was blind to the experimental situation of each animal.

Results

For the two series of experiments, the mean total number of Fos-LI neurones after intraplantar carrageenin were 266±17 and 332±31 Fos-LI neurones per section, in segments L4–L5 of the spinal cord, respectively (Figure 1). There was no significant difference between the total number of Fos-LI neurones for the two experiments. For both series of experiments the Fos-LI neurones were equally distributed between the superficial (106±3 and 102±10 Fos-LI neurones respectively) and deep laminae (108±9 and 133±16 Fos-LI neurones respectively). Again, for both series of experiments, the remaining Fos-LI neurones were distributed in the nucleus proprius (22±2 and 38±6 Fos-LI neurones respectively) and the ventral horn (29±2 and 48±9 Fos-LI neurones respectively). We have previously shown that in the absence of carrageenin injection the basal number of Fos-LI neurones is less than 5 per section (Honoré *et al.*, 1995a).

The effect of pre-administered (+)-HA966 on C-Fos expression

Pre-administration of the lower doses of (+)-HA966 (0.5 and 2.5 mg kg^{-1}) did not significantly influence the total number of Fos-LI neurones (94±8% and 84±4% of control number of Fos-LI neurones after carrageenin, respectively), neither the number of superficial nor deep Fos-LI neurones was influenced by these doses of (+)-HA966 (Table 1). Pre-administration of the highest dose of (+)-HA966 (10 mg kg^{-1}) significantly reduced the total number of Fos-LI neurones (24±3% reduction of control number of Fos-LI neurones after carrageenin, $P<0.05$, Figure 1). In accordance with this effect, the number

of Fos-LI neurones in the deep laminae was significantly reduced by 10 mg kg^{-1} of (+)-HA966 (Table 1). Furthermore, there was a tendency towards a reduction of the number of Fos-LI neurones in the nucleus proprius by 10 mg kg^{-1} of (+)-HA966; however, this effect did not reach significance. In contrast, the number of Fos-LI neurones in the superficial laminae and ventral horn was not significantly influenced by the highest concentration of pre-administered (+)-HA966 (Table 1).

The effect of pre- plus post-administered (+)-HA966 on c-Fos expression

Pre- plus post-administration of the lowest dose of (+)-HA966 (0.5 mg kg^{-1}) did not significantly influence the total number of Fos-LI neurones (89±6% of control number of Fos-LI neurones after carrageenin). Pre- plus post-administration of 2.5 mg kg^{-1} of (+)-HA966 and 10 mg kg^{-1} of (+)-HA966 significantly reduced the total number of Fos-LI neurones (25±6% reduction, $P \leq 0.05$, and 43±2% reduction, $P \leq 0.0001$, of control number of Fos-LI neurones after carrageenin, respectively).

Pre plus post-administration of 2.5 mg kg^{-1} of (+)-HA966 significantly reduced the number of Fos-LI neurones in the nucleus proprius; in addition, there was a tendency towards a reduction of the number of superficial and deep Fos-LI neurones (Table 2). The highest dose of pre- plus post-administered (+)-HA966 (10 mg kg^{-1}) significantly reduced the number of superficial and deep Fos-LI neurones, in addition to reducing significantly the number of Fos-LI neurones in the nucleus proprius and the ventral horn (Figure 1, Table 2).

The dose-response relationships of pre-administered as compared to pre- plus post-administered (+)-HA966 on the spinal expression of c-Fos illustrate that, in all areas studied, the effects of pre-plus post-administered (+)-HA966 were more marked than the effects of pre-administered (+)-HA966 alone (Figure 2).

The effect of post-administered (+)-HA966 on c-Fos expression

Post-administered (+)-HA966 (10 mg kg^{-1}), 45 min after carrageenin, significantly reduced the total number of Fos-LI neurones (22±2% reduction of control number of Fos-LI neurones after carrageenin, $P < 0.05$). However, the effect of post-administration of (+)-HA966 (10 mg kg^{-1}), on the total number of Fos-LI neurones, was significantly less than the effect of pre- plus post-administration of the same dose of (+)-HA966 ($P \leq 0.05$). With post-administered (+)-HA966 (10 mg kg^{-1}) there was a tendency towards a reduction of the number of Fos-LI neurones in the superficial and deep laminae of the dorsal horn and the ventral horn; these effects did not reach significance (Table 2). However, post-administered (+)-HA966 (10 mg kg^{-1}) significantly reduced the number of Fos-LI neurones in the nucleus proprius (Table 2).

(+)-HA966 does not influence carrageenin-evoked oedema

The extent of the peripheral oedema evoked by carrageenin was assessed in parallel with the c-Fos study and evaluated simultaneously in the same rats. Three hours after carrageenin, the ipsilateral paw and ankle diameters were considerably increased (248±12% and 197±14% of control non-stimulated rats, respectively). None of the doses of (+)-HA966 studied, regardless of the timing of administration, influenced the extent of the ipsilateral paw and ankle oedema. For example, the highest dose of pre- plus post-administered (+)-HA966 (10 mg kg^{-1}), which significantly reduced the number of Fos-LI neurones in all areas studied, did not influence the ipsilateral paw and ankle oedema (98±5% and 108±6% of control carrageenin paw and ankle diameters respectively). In all cases the contralateral paw and ankle diameters were not different from non-stimulated rats (98±0.1% and 98±0.2% of control non-stimulated rats, respectively).

Table 1 The effect of pre-administration of (+)-HA966 on the number of carrageenin evoked Fos-LI neurones in the laminae I-II (superficial laminae), laminae III-IV (nucleus proprius), laminae V-VI (deep laminae) and ventral horn of the L4-L5 segment of the spinal cord

Pre-administration of subcutaneous (+)-HA966	*% reduction of Fos-LI neurones*			
	Laminae I-II	*Laminae III-IV*	*Laminae V-VI*	*Ventral horn*
0.5 mg kg^{-1}	13±9%	16±15%	−3±7%	−1±10%
2.5 mg kg^{-1}	13±4%	24±4%	15±6%	20±8%
10 mg kg^{-1}	19±5%	31±8%	28±3%*	18±5%

(+)-HA966 was administered subcutaneously 30 min before intraplantar carrageenin. Results are expressed as percentage reduction of the control carrageenin evoked number of Fos-LI neurones ± s.e.mean. Statistical analysis was performed with ANOVA: *$P \leq 0.05$.

Table 2 The effect of pre-plus post-administration versus post-administration of (+)-HA966 on the number of carrageenin-evoked Fos-LI neurones in the laminae I–II (superficial laminae), laminae III–IV (nucleus proprius), laminae V–VI (deep laminae) and ventral horn of the L4-L5 segment of the spinal cord

Subcutaneous administration of (+)-HA966	*% reduction of Fos-LI neurones*			
	Laminae I-II	*Laminae III-IV*	*Laminae V-VI*	*Ventral horn*
0.5 mg kg^{-1} pre- + post-	12±6%	15±16%	7±8%	0±5%
2.5 mg kg^{-1} pre- + post-	23±10%	36±7%*	19±5%	25±7%
10 mg kg^{-1} pre- + post-	33±0.5%*	55±6%*	40±4%*	51±4%*
10 mg kg^{-1} post-	24±7%	39±8%*	23±6%	21±8%

For the pre- plus post-administration groups, (+)-HA966 was administered, subcutaneously, 30 min before and again 45 min after intraplantar carrageenin. For the post-administered group, (+)-HA966 was administered, subcutaneously, 45 min after intraplantar carrageenin. Results are expressed as percentage reduction of the control carrageenin-evoked number of Fos-LI neurones ± s.e.mean. Statistical analysis was performed with ANOVA: *$P \leq 0.05$.

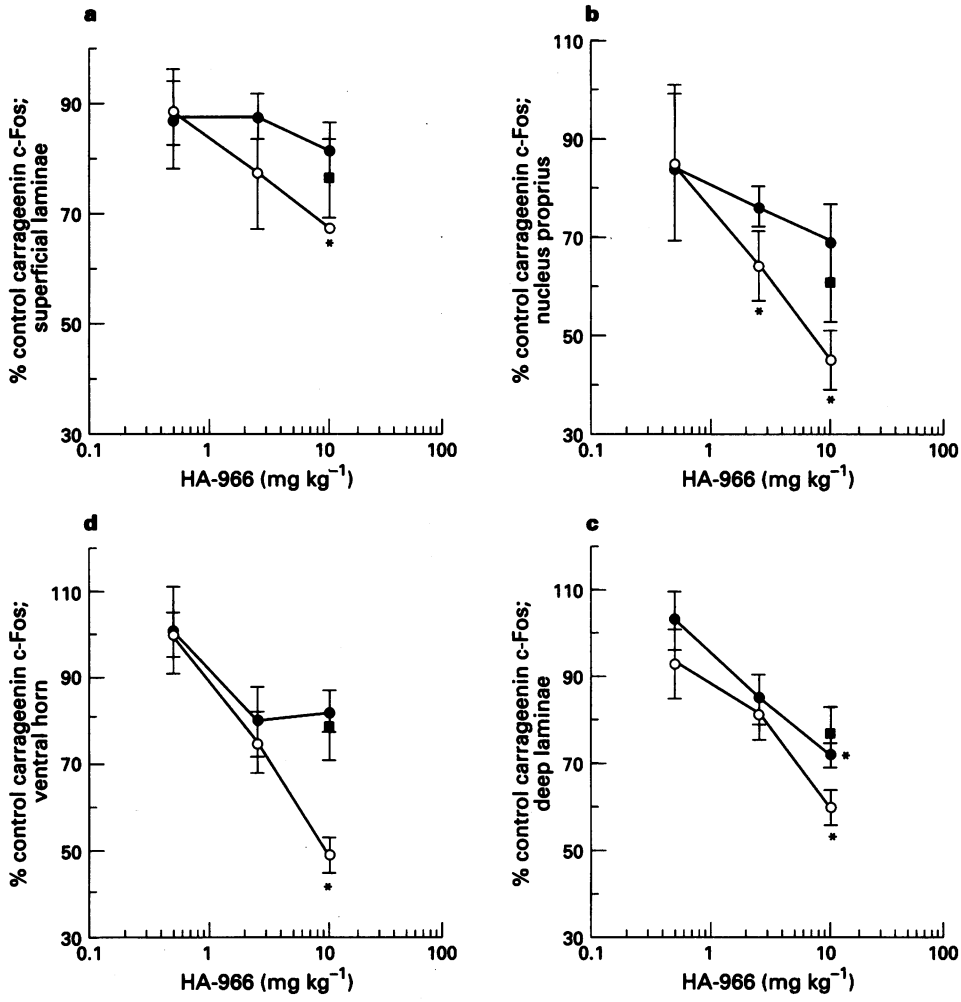

Figure 2 Comparative dose-response relationships of pre-administered (●), as compared to pre- plus post-administered (+)-HA966 (○), on the number of carrageenin-evoked Fos-LI neurones for the different areas studied, (a) laminae I–II (superficial laminae), (b) laminae III–IV (nucleus proprius), (c) laminae V–VI (deep laminae) and (d) ventral horn of the L4–L5 segment of the spinal cord. The effect of post-administration of 10 mg kg^{-1} of (+)-HA966 (■) is included for comparison. Pre-administered (+)-HA966 was given, subcutaneously, 30 min before intraplantar carrageenin, pre plus post-administered (+)-HA966 was given 30 min before and again 45 min after intraplantar carrageenin, and post-administered (+)-HA966 was given 45 min after intraplantar carrageenin. Results are expressed as percentages of the control carrageenin-evoked number of Fos-LI neurones ± s.e.mean. Statistical analysis was performed with ANOVA: *$P \leq 0.05$.

Discussion

In agreement with our previous studies (Honoré *et al.*, 1995a,b), intraplantar carrageenin was associated with a large number of spinal neurones expressing c-Fos in the L4–L5 segment of the spinal cord and an extensive peripheral inflammation. Neurones expressing c-Fos were predominantly located in the superficial (laminae I–II) and deep laminae (V–VI) of the dorsal horn. Relatively few neurones in the nucleus proprius (laminae III–VI) and ventral horn expressed c-Fos. We have previously shown that in the absence of stimulation, less than 5 Fos-LI neurones per section are present in the lumbar segment of the spinal cord (Honoré *et al.*, 1995a).

Carrageenin-evoked spinal expression of c-Fos was not influenced by the lower doses of pre-administered (+)-HA966. However, the highest dose of pre-administered (+)-HA966 significantly reduced the total number of neurones expressing c-Fos after intraplantar carrageenin; this effect was reflected by a significant reduction in the number of c-Fos neurones in the deep laminae. In contrast, the number of neurones expressing c-Fos in the superficial laminae, nucleus proprius and ventral horn were not significantly influenced by the highest dose of pre-administered (+)-HA966. Pre- plus post-administration of the highest dose of (+)-HA966 significantly reduced the expression of c-Fos both in the superficial and deep laminae, thus contrasting the selective effect of pre-administered (+)-HA966 on the number of deep neurones expressing c-Fos. Overall, there was a tendency for pre- plus post-administered (+)-HA966 to have a greater effect on the number of deep, as compared to superficial, neurones expressing c-Fos. Finally, there was a trend towards a reduction of the number of superficial and deep Fos-LI by a high dose of post-administered (+)-HA966.

Our study shows that NMDA receptor blockade, at early time points of the carrageenin response (during the first hour, pre-administration) significantly reduced c-Fos expression in the deep laminae. However, with a longer blockade of the NMDA receptor, for at least the first 2 h of the response (pre-plus post-administration), c-Fos expression in both the superficial and deep laminae was significantly reduced. (+)-HA966, is a well characterized and investigated selective functional NMDA receptor antagonist (see references in Kemp & Leeson, 1993); in addition, the concentrations of (+)-HA966 used in this study have been shown to be without motor effects (Millan & Seguin, 1993; 1994). Simplistically our results suggest that NMDA receptor activation during the early stages of the carrageenin response predominantly contribute to c-Fos expression in the deep laminae of the dorsal

horn, whereas NMDA receptor activation throughout the carrageenin response contributes to c-Fos expression in both the superficial and deep laminae of the dorsal horn. However, such an interpretation is further complicated when considering the time course of carrageenin-evoked expression of c-Fos. We have previously shown that there is a differential expression of c-Fos in the superficial laminae, which is maximal at 2 h after carrageenin, versus in the deep laminae, which is maximal at 3 h after carrageenin (Honoré *et al.*, 1995a). Overall, our results illustrate the complexities of NMDA receptor-mediated events to the expression of c-Fos in superficial, versus deep, laminae dorsal horn neurones. However, it is important to bear in mind the pharmacokinetic constraints of multiple timings of drug administration, relative to the longer time course of carrageenin-evoked c-Fos expression, which may have masked potential effects.

The absolute number of neurones expressing c-Fos within the nucleus proprius was small, approximately 10% of the total number of neurones expressing c-Fos. Nevertheless there was a significant reduction in the number of Fos-LI neurones in the nucleus proprius by pre- plus post-administered (+)-HA966 and post-administered (+)-HA966, but not by pre-administered (+)-HA966. This effect of NMDA receptor antagonism on this subsection of neurones is interesting considering the nucleus proprius is the termination site for Aβ-fibres and that under normal conditions Aβ-fibre activation does not evoke nociceptive responses (see references in Meyer *et al.*, 1994) or c-Fos expression (Herdegen *et al.*, 1991). Furthermore, again under normal conditions, Aβ-fibre-evoked responses are not influenced by NMDA receptor antagonism (Dickenson & Sullivan 1990). However, there is mounting evidence that under certain conditions, including inflammation, Aβ-fibre inputs can evoke nociceptive responses (see references in Woolf & Doubell, 1994). The mechanisms of such events are not fully elucidated, and may include a loss of tonic inhibitions resulting in allodynia (Yaksh 1989), and/or a generalized increase in dorsal horn neuronal excitability. In keeping with the latter possibility, it has recently been shown, in a u.v. model of persistent hyperalgesia, that Aβ-fibre evoked responses are reduced by NMDA receptor antagonism (Chapman & Dickenson, 1994; Thompson *et al.*, 1994). Bearing in mind that our studies are relatively acute, 3 h after carrageenin, it is unlikely that the effect of NMDA receptor antagonism on c-Fos expression, at the level of the nucleus proprius, reflects a functional change in the Aβ-fibres. Furthermore, considering the contribution of peptide-mediated events to NMDA receptor activation (see references in Urban *et al.*, 1994) it is unlikely that Aβ-fibres terminating in the nucleus proprius directly activate NMDA receptors. However it is feasible that Aβ-fibre inputs are terminating on neurones which are already hyperexcitable and that this hyperexcitability is mediated, at least in part, by the NMDA receptor. Thus the reduction of the number of c-Fos neurones in the nucleus proprius by NMDA receptor antagonism, may reflect a general decrease in spinal excitability.

The peripheral odema associated with intraplantar carrageenin was not influenced by any of the concentrations of (+)-HA966 studied, at any time points of administration, agreeing with previous studies (Hunter & Singh, 1994, Laird *et al.*, 1994). Thus our results implicate a central site of action, either spinal or supraspinal, of (+)-HA966 on spinal c-Fos expression. Generally, our results support previous behavioural (Millan & Seguin, 1993; 1994) and electrophysiological (Dickenson & Aydar, 1991) studies demonstating the antinociceptive effects of antagonists of the glycine site of the NMDA receptor in the formalin model of inflammatory pain. More specifically, the effects of (+)-HA966 on the number of superficial and deep c-Fos neurones strongly supports a role of the NMDA receptor in the spinal transmission of carrageenin-evoked nociceptive inputs, thus agreeing with previous behavioural studies which have shown NMDA receptor antagonism reduces carrageenin-evoked hyperalgesia (Ren *et al.*, 1992; Eisenberg *et al.*, 1994; Laird *et al.*, 1994). Importantly, our results illustrate the contribution of NMDA receptor activation to the spinal expression of c-Fos following peripheral inflammation, thus agreeing with previous studies of the effect of NMDA receptor antagonists on c-Fos expression (Kehl *et al.*, 1991; Elliott *et al.*, 1994). Furthermore, our results demonstrate that continual NMDA receptor blockade has a greater effect on c-Fos expression, as compared to blockade during the initial time points, thus illustrating the contribution of the NMDA receptor to both the induction and maintenance of carrageenin-evoked c-Fos expression.

The activation of the immediate early gene, c-fos, and the expression of the protein product, c-Fos is associated with the encoding of transcription factors that regulate other genes involved in plasticity and adaptive changes, and the long term expression of c-Fos is considered to be a 'cellular stress response' (Morgan & Curran, 1995). Our results illustrating the contribution of NMDA receptor activation to spinal c-Fos expression, extend previous studies demonstrating the contribution of the NMDA receptor to central hyperalgesia. Interestingly, it has recently been shown that NMDA receptor antagonism attenuates the temporal summation of second pain, a psychophysical correlate of wind-up in human subjects (Price *et al.*, 1994).

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