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Effects of the high-efficacy 5-HT_{1A} receptor agonist, F 13640 in the formalin pain model: A c-Fos study

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Abstract

We studied the effects of the high-efficacy 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonist, F 13640 on both formalin-induced spinal cord c-Fos protein expression and pain behaviours in the rat. Replicating earlier data, F 13640 (0.63 mg/kg, i.p.; $t_{-15\ min}$) completely inhibited the elevation and licking of the formalin-injected paw. In the same animals, and in spite of the agent as in earlier data increasing the number of c-Fos labelled nuclei when it was administered alone, F 13640 markedly reduced the number of formalin-induced c-Fos labelled nuclei. This was found in both the superficial (I–II) and deep (V–VI) dorsal horn laminae (2 h post-injection: 72±2% and 92±1% of reduction, respectively; $P<0.001$ in either case), spinal areas that contain neurons responsive to nociceptive stimulation. Co-operation occurred so that after the co-administration of F 13640 and formalin, c-Fos expression was inferior to that induced when either stimulation was administered alone. The data provide initial evidence for the agent's inhibitory effects on noxiously evoked c-Fos expression. The results indicate that co-operation between 5-HT_{1A} receptor activation and nociceptive stimulation powerfully inhibits responses to severe, tonic nociception.

Keywords: 5-HT_{1A} receptor; c-Fos protein; Formalin; F 13640; Morphine; Rat spinal cord

1. Introduction

The high-efficacy 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonist, F 13640 produces exceptionally powerful analgesia in rodent models of chronic (Colpaert et al., 2002; Deseure et al., 2002, 2003; Wu et al., 2003) and acute (Colpaert et al., 2002; Bardin et al., 2003) pains of nociceptive or neurophatic origin. This analgesia results from two novel neuro-adaptive mechanisms (Colpaert et al., 2002); one consists of inverse tolerance: upon chronic administration of F 13640, analgesia grows rather than decays as is the case with opioids. A second mechanism is referred to as co-operation; the analgesic effect of F 13640 increases with the intensity and duration of nociceptive stimulation. Consistent with a theory that signal transduction in pain processing generates dual, paradoxical effects (Colpaert, 1996), available evidence suggests that high-efficacy 5-HT_{1A} receptor activation by F 13640 induces these neuroadaptive mechanisms by mimicking the central effects of nociceptive stimulation; indeed, in pain-free rats, F 13640 lowers the mechanical threshold for vocalization (Colpaert et al., 2002) and induces c-Fos protein expression in spinal cord neurons (Buritova et al., 2003).

Demonstrating co-operation, and at a dose and time at which it lowers the vocalization threshold in pain-free rats, F 13640 profoundly inhibits pain behaviours in the formalin model (Tjolsen et al., 1992; Abbott et al., 1999) of tonic nociceptive pain (Colpaert et al., 2002; Bardin et al., 2003). The extent to which 5-HT_{1A} receptor ligands inhibit formalin-induced behaviours correlates with the extent to which the ligands activate 5-HT_{1A} receptors (Colpaert et al., 2002). As the agent selectively activates 5-HT_{1A} receptors to an exceptional extent, F 13640's inhibition of formalin-

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induced behaviours is paralleled only by the opioid, morphine and surpasses that found with any other known molecular mechanism of analgesia (Bardin et al., 2003).

The expression of the immediate-early gene, c-*fos* and of c-Fos protein, in the spinal cord dorsal horn, constitutes a marker of the neuronal activity that can be induced by noxious stimuli (Hunt et al., 1987; Munglani and Hunt, 1995; Chapman and Besson, 1997; Harris, 1998; Herdegen and Leah, 1998); it in particular allows to quantify the effects of analgesic agents and identify their neuroanatomical localization (Presley et al., 1990; Chapman and Besson, 1997). Intraplantar formalin injection evokes c-Fos protein expression principally in superficial (I–II) and deep (V–VI) dorsal horn laminae (Presley et al., 1990; Peterson et al., 1997; Jinks et al., 2002), spinal areas that contain neurons responsive to noxious stimuli. This formalin-induced c-Fos protein expression can be reduced by opioids and other analgesics (Presley et al., 1990; Abbadie et al., 1997; Chapman and Besson, 1997).

The present study aimed to elucidate the neurobiological substrate of the co-operative mechanism whereby high-efficacy 5-HT_{1A} receptor activation can produce analgesia. The study determined whether, at a dose at which F 13640 itself induces c-Fos protein expression (Buritova et al., 2003), the agent would, paradoxically, inhibit formalin-induced c-Fos protein expression. Any such inhibition was compared with that produced by morphine. Part of the data has been presented in abstract form elsewhere (Larrue et al., 2003).

2. Methods

2.1. Animals

Experiments were performed in 206 male Sprague–Dawley rats (Ico: OFA SD (I.O.P.S.) Iffa Credo, France) weighing 220±20 g. Rats were housed in plastic breeding cages in an animal room (21±1 °C; relative humidity: 55±5%) with a 12 h alternating light/dark cycle (lights on at 7:00 AM) and with filtered water and food (standard rat chow; A04, SAFE, Epinay sur Orge, France) available ad libitum. A 5-day acclimatisation period was allowed before animals were used in experiments. Guidelines of the Ethics Committee of the International Association for the study of Pain (Zimmermann, 1983) were followed and the protocol approved by the institutional Ethical Review Committee.

2.2. Compounds

F 13640 [(3-chloro-4-fluoro-phenyl)-[4-fluoro-4-{[(5-methyl-pyperidin-2-ylmethyl)-amino]-methyl}piperidin-1-yl]-methanone, fumaric acid salt] (synthesised in-house) and morphine (morphine chlorhydrate, Cooper Rhone Poulenc Rorer, Coopération Pharmaceutique Française, Melun, France) were dissolved in bi-distilled water. These drugs and saline were intraperitoneally (i.p.) injected in a volume of 10 ml/kg. Doses refer to free base weight.

2.3. Formalin test

The formalin test was carried out as described previously (Bardin et al., 2003). Each rat was placed in the observation chamber (with a mirror placed under the floor at a 45° angle to allow an unobstructed view of the paw) for a 15-min habituation period. Thereafter, the rat received a 50-μl subcutaneous injection of either saline or diluted formalin (formaldehyde 2.5%; Sigma Aldrich, L'isle d'Abeau Chesnes, France) into the plantar surface of the right hindpaw (intraplantar injection; i.pl.). Following this, the rat was returned to the chamber. The behavioural responses were observed during two 5-min periods: 0–5 min (early phase) and 22.5–27.5 min (late phase) after injection when the formalin-induced pain behaviours were most apparent (Abbott et al., 1999). During each of these two observation periods, every 30 s, rats were observed for the presence or absence of spontaneous pain behaviours, i.e. 1) the injected hindpaw is elevated and not in contact with any surface, and 2) the injected hindpaw is licked. The 30 s observation cycle was repeated 10 times during the 5 min period; thus, the incidence of pain-related behaviours was free to vary from 0 to 10 for each of the two observation periods and with each of the two behaviours. The observer was blind to the treatment conditions.

2.4. Experimental design

In the same animals, we determined the effects of either F 13640 or morphine on both formalin-induced pain behaviours and spinal c-Fos protein expression. The six experimental groups were as follows: saline i.p.+saline i.pl. (n=12); F 13640 i.p.+saline i.pl. (n=6); morphine i.p.+saline i.pl. (n=6); saline i.p.+formalin i.pl. (n=12); F 13640 i.p.+formalin i.pl. (n=6); morphine i.p.+formalin i.pl. (n=6). Either 0.63 mg/kg of F 13640, 20 mg/kg of morphine or saline was injected 15 min prior to the i.pl. injection of formalin or saline. The behavioural responses were observed as specified above, and rats were perfused either 1, 2 or 4 h after the i.pl. injection. In addition, non-stimulated rats (n=6) were included and perfused under the same experimental conditions as stimulated rats, but received no pharmacological treatment or i.pl. injection.

The dose and time of pretreatment with F 13640 were chosen in accordance with data demonstrating that a single injection of the agent (0.63 mg/kg, i.p.) blocks formalin-induced pain behaviours (Bardin et al., 2003) and induces spinal c-Fos protein expression (Buritova et al., 2003) in the rat. Note that the 0.63 mg/kg dose of F 13640 also produces such signs of the behavioural 5-HT syndrome as forepaw treading, flat body posture and lower lip retraction (Bardin et al., 2003). Nevertheless, these signs do not account for F 13640's effects on formalin-induced pain behaviours; as

demonstrated previously, in the formalin pain model, the analgesic effects of 5-HT_{1A} receptor activation are behaviourally specific (Bardin et al., 2001; see also: Bruins Slot et al., 2003; Deseure et al., 2003).

The dose and time of morphine pretreatment were chosen in view of previous behavioural (Bardin et al., 2003) and c-Fos (Presley et al., 1990) studies performed in the formalin pain model. Because, unlike 0.63 mg/kg of F 13640, 10 mg/kg (i.p.) of morphine was insufficient to completely suppress formalin-induced pain behaviours (Bardin et al., 2003), the higher, 20 mg/kg dose was chosen so as to obtain a behavioural effect that would be similar to that of F 13640.

2.5. Tissue preparation and immunohistochemistry

Rats were anaesthetised with pentobarbital (55 mg/kg, i.p.; Ceva Santé Animale, Libourne, France) and perfused intracardially with 300 ml of phosphate buffered saline (PBS; 0.1 M phosphate buffer+saline 0.9%) followed by 500 ml of 4% paraformaldehyde (Sigma Aldrich, L'isle d'Abeau Chesnes, France). The spinal cord was then removed and postfixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose. The lumbar spinal cords were cut in 40-μm frozen serial sections and collected in PBS. The sections were sampled at 120-μm intervals to be processed immunohistochemically as free floating sections.

As previously described (Buritova et al., 2003), immunohistochemistry was performed with polyclonal antiserum, generated in rabbits and directed against the c-Fos protein (Santa Cruz Biotechnology, Santa Cruz, USA; SC52 solution, 0.2 mg/ml; diluted at 1:20,000), using the method of Hsu et al. (1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 5% normal goat serum in PBS with 0.3% Triton-X and were then incubated for 16 h at room temperature in the primary antiserum directed against the c-Fos protein. The incubated sections were washed 3 times in PBS and incubated in biotinylated goat anti-rabbit antiserum (Vector Laboratories, Burlingame, USA; BA-1000) for 1 h at room temperature, then washed twice in PBS and incubated for 1 h in Avidin–Biotin–Peroxidase complex (Vector Laboratories, Burlingame, USA; PK-6100). Finally, the sections were washed 3 times in PBS and developed in chromogen solution (Vector Laboratories, Burlingame, USA; Peroxidase Substrate Kit SK-4600) for 10 min, and were washed again 3 times in PBS to stop the staining reaction. The sections were mounted on gelatine-subbed slides. After being air-dried, sections were differentiated in 70%, 95% and 100% alcohol (differentiation time was evaluated under the microscope) and coverslipped. Sections from control and treated animals were processed in parallel under identical experimental conditions.

2.6. Counting of c-Fos protein immunoreactive nuclei

Considerable evidence indicates that noxiously evoked c-Fos protein expression is largest in the lumbar spinal cord (Hunt et al., 1987; Presley et al., 1990). In particular, the number of c-Fos labeled neurons evoked by an intraplantar formalin injection is maximal at the L3–L5 segment levels, corresponding to the segmental innervation of the rat plantar hindpaw (Presley et al., 1990). We here therefore studied formalin-induced and, also, F 13640-induced c-Fos expression at the L3–L5 segment levels. The expression was quantified by determining the number of c-Fos labelled nuclei in these segments. Tissue sections were first examined using lightfield microscopy at 4× to determine the segmental level according to Molander et al. (1984). The sections were then examined under lightfield microscopy at 10× to localise c-Fos labelled nuclei. c-Fos labelled nuclei were plotted and counted using a *camera lucida* attachment. The investigator who plotted and counted the c-Fos labelled nuclei was blind to the treatment conditions.

To study the laminar distribution of c-Fos labelled nuclei, four regions were arbitrarily defined in the spinal grey matter of the L3–L5 segments; this was carried out in

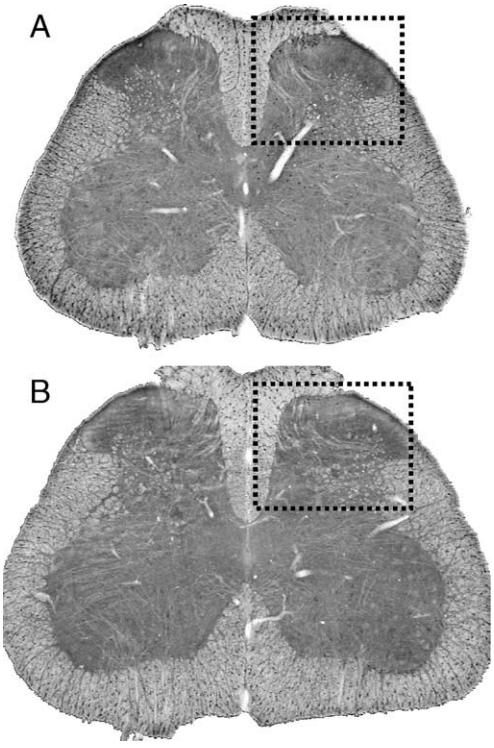

Fig. 1. Photomicrographs illustrating the effects of F 13640, a high-efficacy 5-HT1A receptor agonist, on formalin-induced c-Fos protein expression in the rat spinal cord. Each photomicrograph is a representative example of 40 μm sections at the level of L4 segment showing c-Fos labelled nuclei in the grey matter (black dots). Two experimental groups are represented: saline i.p.+formalin i.pl. (A), and F 13640 0.63 mg/kg i.p.+formalin i.pl. (B). In saline-pretreated animals (A), 2 h after i.pl. injection of formalin, c-Fos labelled nuclei were numerous in the lumbar spinal cord ipsilaterally to the formalin-injected hindpaw (right hindpaw). c-Fos labelled nuclei were virtually absent (<4 c-Fos labelled nuclei per section) in the contralateral spinal cord. Framed regions of ipsilateral dorsal horn laminae in (A) and (B) are shown with a higher magnification (20×) in Fig. 2A and B, respectively.

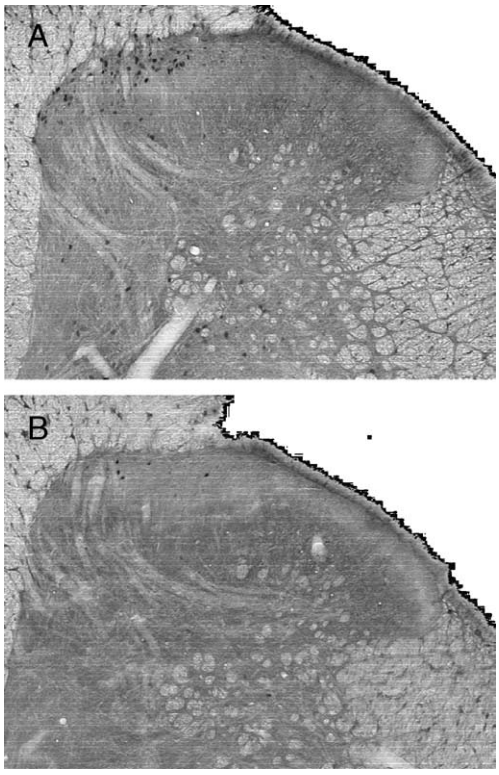

Fig. 2. Photomicrographs illustrating the effects of F 13640 on formalin-induced c-Fos protein expression in the spinal dorsal horn. Each photomicrograph is, in corresponding details, the magnification (20×) of regions (ipsilateral dorsal horn laminae) framed in Fig. 1A and B, respectively. Two experimental groups are represented: saline i.p.+formalin i.pl. (A), and F 13640 0.63 mg/kg i.p.+formalin i.pl. (B). In saline-pretreated animals (A), there are numerous c-Fos labelled nuclei in the superficial (I–II) and deep (V–VI) laminae of the dorsal horn, ipsilateral to the noxious stimulus due to formalin injection. Pretreatment with 0.63 mg/kg of F 13640 (B) markedly reduced the number of formalin-induced c-Fos labelled nuclei in all laminae.

accordance with the cytoarchitectonic organisation of the spinal cord (Molander et al., 1984; Molander and Grant, 1986). These regions were: superficial laminae of the dorsal horn (corresponding to laminae I–II), the nucleus proprius (laminae III–IV), deep laminae of the dorsal horn (laminae V–VI) and the ventral horn (laminae VII–X) of the spinal cord (for more details see Fig. 1 in Buritova et al., 2003; Presley et al., 1990). For analysis of c-Fos labelled nuclei location, the ten sections with the greatest degree of c-Fos protein expression were selected through L3–L5 segments in each animal. For each animal, two counts were made in the grey matter of these 10 sections: (1) the total number (mean±S.E.M.) of c-Fos labelled nuclei per section, and (2) the number (mean±S.E.M.) of c-Fos labelled nuclei per section in four defined regions: laminae I–II, III–IV, V–VI and VII–X (see above).

2.7. Statistical analysis

Statistical analysis of c-Fos data was performed using the repeated measurement analysis of variance: for any given animal, the number of c-Fos labelled nuclei was counted in 10 sections of the spinal cord. This approach allowed the direct analysis of raw data rather than of their central tendency (mean or median). In case of significance of the treatment group effect or of the interaction between the treatment group and post-injection time, the contrast method based on the Fisher statistic was used in post-hoc analyses (Myers and Well, 1995). The analyses was conducted with the Mixed procedure of SAS 8.2 software for PC (Littell et al., 2000).

As in earlier studies (Bardin et al., 2003), behavioural data were analysed by Kruskal–Wallis one way analysis of variance (ANOVA) on ranks. ANOVAs were performed separately on data obtained during the early and late phases of the formalin-induced behaviours. The Dunn's test was used for multiple comparisons. $P<0.05$ was considered to be statistically significant.

3. Results

Spinal c-Fos protein expression was virtually absent (<4 c-Fos labelled nuclei per section in the L3–L5 segments) in non-stimulated rats (i.e., in rats that received no experimental treatment whatsoever; $n=6$).

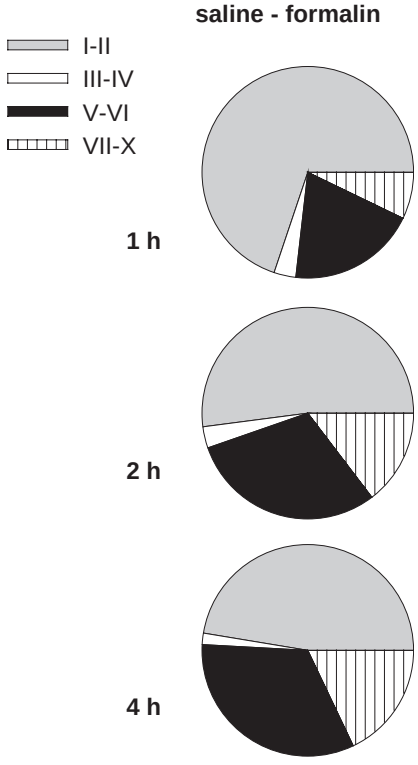

Fig. 3. Laminar distribution of formalin-induced c-Fos protein expression (i.e. number of c-Fos labelled nuclei in laminar regions I–II, III–IV, V–VI and VII–X, expressed as a percentage of the total number observed in the section) in saline-pretreated animals (group: saline i.p.+formalin i.pl.) at 1, 2 and 4 h after i.pl. injection of formalin.

3.1. Spinal c-Fos protein expression induced by noxious formalin stimulation

The number of c-Fos labelled nuclei and their laminar distribution were determined at post-injection times ranging from 1 to 4 h after i.pl. injection of either formalin or saline (Figs. 1–5). Following saline injection (group: saline i.p.+saline i.pl.), spinal c-Fos protein expression was virtually absent (<4 c-Fos labelled nuclei per section) at all post-injection times studied (Fig. 4). In contrast, formalin (group: saline i.p.+formalin i.pl.) induced spinal c-Fos protein expression in a time-dependent manner, the maximal number of c-Fos labelled nuclei occurring 2 h after formalin injection (Figs. 1A, 2A and 4). Formalin-induced c-Fos labelled nuclei were numerous in the spinal cord ipsilaterally to the formalin-injected hindpaw, while they were virtually absent (<4 c-Fos labelled nuclei per section) on the contralateral side (Figs. 1A and 2A). Formalin-induced c-Fos labelled nuclei were located mainly in the superficial dorsal horn (laminae I–II); their relative incidence in deep dorsal horn laminae (V–VI) increased with the post-injection time (Fig. 3). In particular, 2 h after formalin injection, the distribution of c-Fos labelled nuclei in superficial (I–II) and deep (V–VI) dorsal horn laminae corresponds to 52% and 30% of the total number of c-Fos labelled nuclei per section (Figs. 3 and 5).

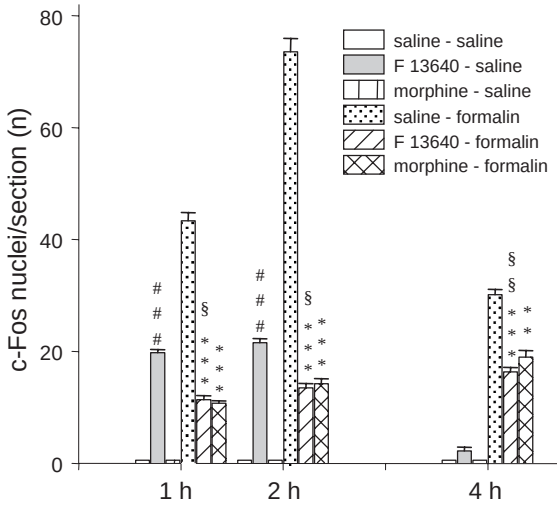

Fig. 4. Time course of effects of F 13640 and morphine on the saline- or formalin-induced c-Fos protein expression in the rat spinal cord. Six experimental groups are represented: saline i.p.+saline i.pl. ($n=12$); F 13640 0.63 mg/kg i.p.+saline i.pl. ($n=6$); morphine 20 mg/kg i.p.+saline i.pl. ($n=6$); saline i.p.+formalin i.pl. ($n=12$); F 13640 0.63 mg/kg i.p.+formalin i.pl. ($n=6$); morphine 20 mg/kg i.p.+formalin i.pl. ($n=6$). Data represent the mean±S.E.M. of the total number of c-Fos labelled nuclei per section in segments L3–L5 of the rat spinal cord at 1, 2 and 4 h after i.pl. injection of either saline or formalin. Fisher's test: $^{**}P<0.01$ and $^{***}P<0.001$ for difference between the group: saline i.p.+formalin i.pl. and F 13640 or morphine pre-treated groups: F 13640 i.p.+formalin i.pl., morphine i.p.+formalin i.pl.; $^{\#\#\#}P<0.001$ for difference between the group: F 13640 i.p.+saline i.pl. and the group: saline i.p.+saline i.pl.; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ for difference between the group: F 13640 i.p.+formalin i.pl. and the group: F 13640 i.p.+saline i.pl.

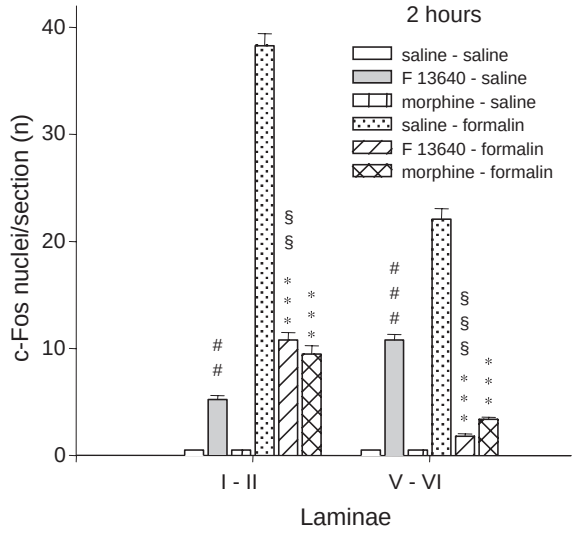

Fig. 5. Effects of F 13640 and morphine on c-Fos protein expression in dorsal horn laminae I–II and V–VI of the spinal cord, 2 h after i.pl. injection of either saline or formalin. Six experimental groups are represented: see legend to Fig. 4. Data represent the mean±S.E.M. number of c-Fos labelled nuclei per section in superficial (I–II) and deep (V–VI) dorsal horn laminae of the L3–L5 segments, 2 h after i.pl. injection of either saline or formalin. Fisher's test: $^{***}P<0.001$ for difference between the group: saline i.p.+formalin i.pl. and F 13640 or morphine pre-treated groups: F 13640 i.p.+formalin i.pl., morphine i.p.+formalin i.pl.; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ for difference between the group: F 13640 i.p.+saline i.pl. and the group: saline i.p.+saline i.pl.; $^{\S\S}P<0.01$ and $^{\S\S\S}P<0.001$ for difference between the group: F 13640 i.p.+formalin i.pl. and the group: F 13640 i.p.+saline i.pl.

3.2. Effects of F 13640 and morphine on formalin-induced spinal c-Fos protein expression

The effects of F 13640 (0.63 mg/kg, i.p.) and morphine (20 mg/kg, i.p.) on the number of saline- or formalin-induced c-Fos labelled nuclei at various post-injection times are shown in Figs. 1, 2, 4 and 5. Analysis indicated a significant effect of group ($F[5,126]=346.19$; $P<0.001$) and time ($F[2,126]=31.24$; $P<0.001$), as well as a significant group × time interaction ($F[10,126]=30.92$; $P<0.001$). c-Fos protein expression was virtually absent (<4 c-Fos labelled nuclei per section) after treatment with either saline (group: saline i.p.+saline i.pl.) or morphine (group: morphine i.p.+saline i.pl.) at all post-injection times (Fig. 4). F 13640-induced c-Fos protein expression (group: F 13640 i.p.+saline i.pl.) appeared bilaterally within 1 to 4 h, with substantial diminution at 4 h (1, 2 and 4 h post-injection times: $P<0.001$, $P<0.001$ and $P>0.05$ as compared to the group: saline i.p.+saline i.pl., respectively; Fig. 4). The same dose of F 13640 (group: F 13640 i.p.+formalin i.pl.) strongly reduced the formalin-induced c-Fos protein expression at all post-injection times (74±2%, 82±1% and 46±3% of reduction of the total number of c-Fos labelled nuclei per section at 1, 2 and 4 h after formalin

injection, respectively; $P<0.001$ in each case as compared to the group: saline i.p.+formalin i.pl.; Fig. 4). In particular, the total number of c-Fos labelled nuclei in the group: F 13640 i.p.+formalin i.pl. was significantly inferior to that induced by F 13640 alone (group: F 13640 i.p.+saline i.pl., $P<0.05$ at 1 and 2 h, respectively; Fig. 4) except at the time interval of 4 h when F 13640 itself did not induce c-Fos expression (Fig. 4). This inhibition of total c-Fos protein expression was mainly due to that occurring in deep (V–VI) laminae of the spinal dorsal horn (for data at 2 h, see Fig. 5). F 13640's effects were significant in both the superficial (I–II) and deep (V–VI) dorsal horn laminae (1 h post-injection time: 73±2% and 68±3% of reduction of the number of formalin-induced c-Fos labelled nuclei as compared to the group: saline i.p.+formalin i.pl., respectively; 2 h: 72±2% and 92±1% of reduction, respectively; 4 h: 31±4% and 52±4% of reduction, respectively; $P<0.001$ in each case); for data at 2 h, see Fig. 5.

Formalin-induced c-Fos expression was also significantly decreased by 20 mg/kg of morphine (group: morphine i.p.+formalin i.pl.) at all post-injection times (75±1%, 81±1% and 37±4% of reduction of the total number of c-Fos labelled nuclei per section; $P<0.001$, $P<0.001$ and $P<0.01$ as compared to the group: saline i.p.+formalin i.pl. at 1, 2 and 4 h after formalin injection, respectively; Fig. 4). There was no significant difference between the effects of morphine (20 mg/kg) and those of F 13640 (0.63 mg/kg) on the total number of formalin-induced c-Fos labelled nuclei at any post-injection time (1, 2 and 4 h after formalin injection; $P>0.05$ in each case). The reducing effects of morphine were significant in both the superficial (I–II) and deep (V–VI) dorsal horn laminae except those in deep laminae at 4 h after formalin injection (1 h post-injection time: 81±1% and 49±3% of reduction of the number of formalin-induced c-Fos labelled nuclei as compared to the group: saline i.p.+formalin i.pl., $P<0.001$ and $P<0.05$, respectively; 2 h: 75±2% and 85±1% of reduction, respectively, $P<0.001$ for both; 4 h: 50±4% and 20±6% of reduction, $P<0.001$ and $P>0.05$, respectively); for data at 2 h, see Fig. 5.

3.3. Effects of F 13640 and morphine on formalin-induced pain behaviours

The 0.63 mg/kg dose of F 13640 (group: F 13640 i.p.+formalin i.pl.) produced a complete suppression of all

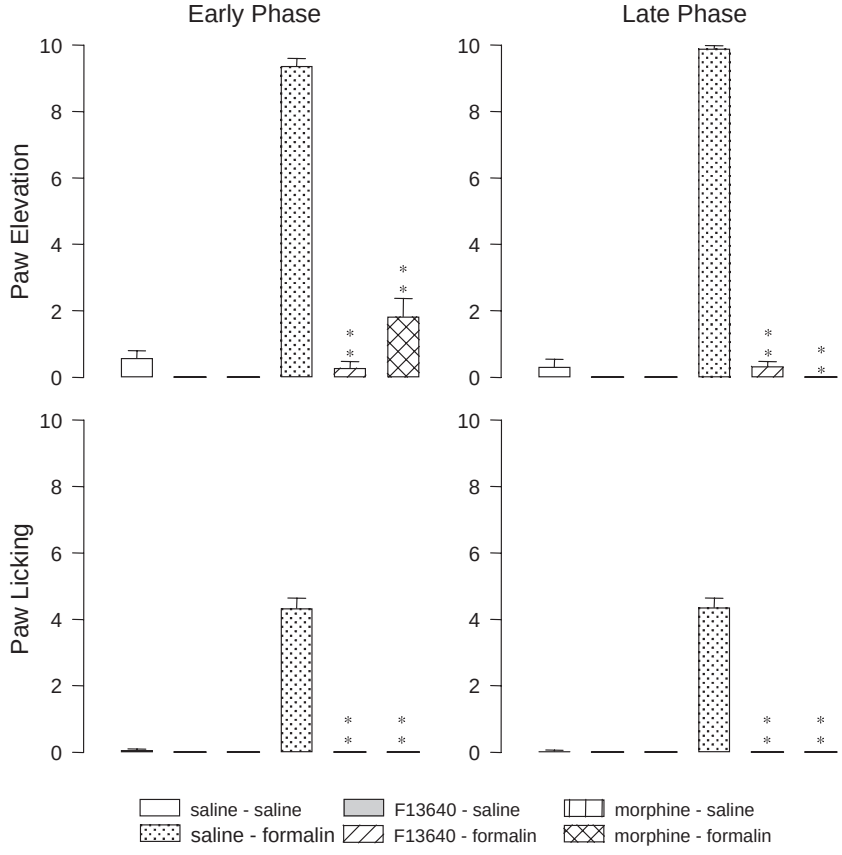

Fig. 6. Effects of F 13640 and morphine on elevation and licking of the formalin-injected paw during the early (0–5 min) and late (22.5–27.5 min) phases after i.pl. injection of formalin in the rat. Six experimental groups are represented: saline i.p.+saline i.pl. ($n=36$); F 13640 0.63 mg/kg i.p.+saline i.pl. ($n=18$); morphine 20 mg/kg i.p.+saline i.pl. ($n=18$); saline i.p.+formalin i.pl. ($n=36$); F 13640 0.63 mg/kg i.p.+formalin i.pl. ($n=18$); morphine 20 mg/kg i.p.+formalin i.pl. ($n=18$). Values represent the mean±S.E.M. scores (maximal score=10). Dunn's test: $^{**}P<0.01$ for difference between the group: saline i.p.+formalin i.pl. and F 13640 or morphine pre-treated groups: F 13640 i.p.+formalin i.pl., morphine i.p.+formalin i.pl.

four parameters of pain-related behaviours in formalin-injected rats, i.e. both the paw elevation and licking during both the early and late phases after formalin injection (Fig. 6). F 13640's effects were significant with both early- and late-phase paw licking (100% of reduction as compared to the group: saline i.p.+formalin i.pl.; $P<0.01$ in either case) and with both early- and late-phase paw elevation (97±2% and 96±1% of reduction as compared to the group: saline i.p.+formalin i.pl., respectively; $P<0.01$ in either case).

The 20 mg/kg dose of morphine also completely inhibited all parameters of formalin-induced pain behaviours except early-phase paw elevation (80±6% of reduction as compared to the group: saline i.p.+formalin i.pl.; $P<0.01$; Fig. 6). Nevertheless, the early-phase paw elevation in morphine pre-treated rats was not significantly different from that in rats without formalin (group: saline i.p.+saline i.pl.; $P>0.05$). The effects of morphine were not significantly different from those of F 13640 ($P>0.05$).

4. Discussion

The present data offer initial evidence that selective, high-efficacy 5-HT_{1A} receptor activation by F 13640 (Colpaert et al., 2002) inhibits noxiously evoked spinal cord c-Fos protein expression. Consistent with the central termination of rat hind-paw afferent fibres, intraplantar formalin injection induced c-Fos protein expression in the lumbar spinal cord ipsilateral to the injected hind-paw. As in earlier studies, formalin-induced c-Fos labelled nuclei were located mainly in the superficial (I–II) and deep (V–VI) dorsal horn laminae (Presley et al., 1990; Abbadie et al., 1997; Peterson et al., 1997; Jinks et al., 2002); their absolute and relative number in deep laminae increased with the time after formalin injection, reaching a maximum after 2 h following which protein expression slowly disappeared (Presley et al., 1990). Two hours after formalin, F 13640 reduced the number of c-Fos labelled nuclei in both the superficial and deep dorsal horn laminae by 72±2% and 92±1%, respectively. The dorsal horn contains neurons involved in pain-modulating systems among which are serotonergic systems (Basbaum and Fields, 1984; Besson and Chaouch, 1987; Hamon and Bourgoin, 1999). First, neurons expressing 5-HT_{1A} receptors mRNA are located within the dorsal horn (laminae III–VI; Pompeiano et al., 1992; Zhang et al., 2002), and a double-labeling study found that some 5-HT_{1A} receptor mRNA-positive cells were also gamma-amino butyric acid- (GABA) or enkephalin-immunoreactive (about 19% and 18%, respectively, Zhang et al., 2002). These morphological data suggest that F 13640 may perhaps act indirectly via spinal GABA- or enkephalinergic neurons. Note, however, that naloxone failed to affect behaviourally assessed F 13640-induced analgesia (Bardin et al., 2003). Second, dorsal horn laminae I–II and V–VI contain nociception-responsive neurons (Besson and Chaouch, 1987; Willis and Coggeshall, 1991) and are the major target of raphe-spinal axons that are thought to mediate descending controls of nociceptive processes (Basbaum and Fields, 1984; Zemlan et al., 1994). F 13640 may also act, at the supraspinal level, via 5-HT_{1A} receptors in raphe nuclei and cortex (Barnes and Sharp, 1999; Stamford et al., 2000), brain structures involved in descending nociceptive control (Kharkevich and Churukanov, 1999). Autoradiographic studies indicate that F 13640 binds to 5-HT_{1A} receptors in hippocampus and cortex (Assié et al., 2003). Regardless of the neuroanatomical site of F 13640's actions, however, the present data substantiate the agent to inhibit spinal neurons involved in nociceptive processing.

F 13640 here also, by itself, induced bilateral spinal c-Fos protein expression in the absence of nociceptive stimulation; this confirms our earlier study (Buritova et al., 2003) demonstrating, in addition, that this expression was prevented by a selective 5-HT_{1A} receptor antagonist. In both the present and earlier data, the laminar distribution of F 13640-induced c-Fos expression was not unlike that observed in rats with adjuvant arthritis (Abbadie and Besson, 1992), a model of chronic pain that is persistent and somatotopically widespread. F 13640-induced bilateral c-Fos expression contrasts with that induced ipsilaterally by nociceptive stimulation due to an intraplantar formalin injection (Presley et al., 1990), that stimulation being acute, intense and localised in the rat hindpaw rather than widespread. Note, however, that both F 13640- and formalin-induced c-Fos labelled nuclei were located mainly in the superficial (I–II) and deep (V–VI) dorsal horn laminae; spinal areas that contain nociception-responsive neurons (see above). These data suggest that F 13640-induced 5-HT_{1A} receptor activation mimicks the central effects of nociceptive stimulation (see also Colpaert et al., 2002; Buritova et al., 2003).

Most remarkably, co-operation occurred between F 13640 and formalin so that following their co-administration c-Fos protein expression was inferior to that which was observed when either stimulation was administered alone. In particular, at the 1 and 2 h intervals, total c-Fos protein expression following F 13640+formalin injection was significantly inferior to that induced by F 13640 alone (Fig. 4); this was no longer the case at 4 h, but at this time F 13640 itself no longer induced expression. The co-operative inhibition of total protein expression was largely due to this inhibition occurring in deep (V–VI) as opposed to superficial (I–II) laminae (Fig. 5). These data confirm a theory (Colpaert, 1996) as well as behavioural evidence (Colpaert et al., 2002) that co-operation occurs between 5-HT_{1A} receptor activation and nociceptive stimulation in, paradoxically, producing analgesia. The present findings also suggest that c-Fos protein expression in spinal cord dorsal horn neurons may be involved in this neuroadaptive mechanism of pain inhibition. Presumably by the mechanism of co-operation, F 13640 injection preempted formalin-induced c-Fos protein expression for the entire (1 to 4

h) period over which observations were made (Fig. 4). The mechanism thus may perhaps account for F 13640's preemptive action in models of neuropathic pain (Deseure et al., 2003; Wu et al., 2003); in spinal cord injured rats, F 13640 continued to inhibit neuropathic allodynia for 2 months after chronic F 13640 infusion had been discontinued (Wu et al., 2003).

In co-operation with formalin-induced nociceptive stimulation, F 13640 produced anti-nociceptive actions that were remarkably profound. The agent's inhibition of formalin-induced spinal c-Fos protein expression was similar ($P>0.05$) to that produced by a 20 mg/kg (i.p.) dose of morphine. The present data indicate that this latter dose was required for morphine to produce an inhibition of formalin-induced pain behaviours that was similar to the complete inhibition produced by 0.63 mg/kg of F 13640; morphine at 10 mg/kg in the same conditions inhibits these behaviours only partially (Bardin et al., 2003). Also, in spite of the fact that the mechanisms underlying formalin-induced early- and late-phase behaviours (and electrophysiological effects) partly differ (see Pitcher and Henry, 2002 and references therein), the effects of F 13640 thereupon were similar. With systemically administered opioids, these anti-nociceptive actions likely result from the activation of inhibitory bulbospinal pathways as well as from a coincident, direct action on local spinal cord circuits (Presley et al., 1990). With F 13640, and although 5-HT_{1A} receptors also occur in the periphery (Wu et al., 2001), these actions are likely mediated by centrally located receptors; indeed, F 13640 inhibits neuropathic allodynia following spinal cord injury (Colpaert et al., 2002; Wu et al., 2003).

In view of the widespread distribution of 5-HT_{1A} receptors in the central nervous system it may be expected that F 13640's actions involve both supraspinal and spinal pain-modulating systems (Hamon and Bourgoin, 1999). Indeed, i.c.v. injection of the 5-HT_{1A} receptor agonist, 8-OH-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) inhibits the early phase of formalin-induced pain behaviours (Fasmer et al., 1986). Behavioural and electrophysiological data demonstrate that spinal 5-HT_{1A} receptors mediate the inhibitory effect of the descending bulbospinal 5-HT system on nociceptive signal transmission in the spinal cord (El-Yassir and Fleetwood-Walker, 1990; Zemlan et al., 1994; Lin et al., 1996; Liu et al., 2002). In rats with a depletion of spinal 5-HT, intrathecal 8-OH-DPAT administration suppressed the later phase of formalin-evoked pain behaviours (Oyama et al., 1996). These results are consistent with behavioural and electrophysiological data indicating that the activation of spinal 5-HT_{1A} receptors produces analgesic effects on responses to noxious thermal, mechanical and electrical stimuli (Crisp et al., 1991; Xu et al., 1994; Gjerstad et al., 1996; Lin et al., 1996) and, also, facilitation of nociceptive responses (Zhang et al., 2001).

In the rat spinal cord, 5-HT_{1A} binding sites are located within dorsal horn laminae (Thor et al., 1993) and represent 30–50% of all 5-HT_1 binding sites (Marlier et al., 1991). Receptor autoradiography data suggest that 5-HT_{1A} receptors are located presynaptically on capsaicin-sensitive primary afferent fibres entering the spinal cord, but a greater proportion of these receptors is located postsynaptically, on intrinsic spinal neurons (Daval et al., 1987). Furthermore, in situ hybridization demonstrates the wide distribution of 5-HT_{1A} receptor mRNA in deep dorsal horn laminae (laminae III–VI; Pompeiano et al., 1992; Zhang et al., 2002). These data are in favour of an expression of 5-HT_{1A} receptors by neurons intrinsic to the dorsal horn of the spinal cord. Nevertheless, electrophysiological data indicate that 8-OH-DPAT hyperpolarizes capsaicin-sensitive dorsal root ganglion cells (Todorovic and Anderson, 1992; Del Mar et al., 1994). This hyperpolarizing action via 5-HT_{1A} receptors may be expected to produce presynaptic inhibition. A direct presynaptic inhibition of the afferent fibres is further supported by the reduction of spinal 5-HT_{1A} receptor density following neonatal capsaicin treatment or rhizotomy (Daval et al., 1987; Laporte et al., 1995). Therefore, the evidence suggests that 5-HT_{1A} receptors are involved in both pre- and post-synaptic controls on nociceptive signal transmission in the spinal cord.

In summary, the present data offer initial evidence that high-efficacy 5-HT_{1A} receptor activation, in spite of inducing such expression by itself, inhibits noxiously evoked c-Fos protein expression in dorsal horn neurons of the rat spinal cord. The inhibition by 0.63 mg/kg of F 13640 of this expression as well as of formalin-induced pain behaviours was profound, being similar to that found with a 20 mg/kg dose of morphine. Co-operation occurred so that after the co-administration of F 13640 and formalin, c-Fos protein expression was inferior to that found when either stimulation was administered alone. This suggest that spinal c-Fos protein may be involved in the novel neuroadaptive mechanism of co-operation, i.e., of the co-operation that occurs between 5-HT_{1A} receptor activation and nociceptive stimulation in, paradoxically, producing analgesia. The findings also indicate that F 13640 exerts spinal actions and suggest these to be mediated by 5-HT_{1A} receptors located in the brain and/or spinal cord. Further work is required to specify the neurobiological mechanisms and circuits mediating the powerful and paradoxical pain inhibition induced by high-efficacy 5-HT_{1A} receptor activation.

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