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European Journal of Pharmacology 379 (1999) 141–149

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Opioidergic and adrenergic modulation of formalin-evoked spinal c-*fos* mRNA expression and nocifensive behavior in the rat

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Received 21 June 1999; accepted 25 June 1999

Abstract

Fos protein expression has been used to reflect neuronal activation in pain processing pathways although analgesics may uncouple behavioral and Fos responses. We determine whether formalin-induced spinal c-*fos* mRNA expression (Northern blotting) correlates with nocifensive behavior following pretreatment with morphine, the α_2-adrenoceptor agonist dexmedetomidine, or their respective antagonists naloxone and atipamezole. Both opiate and α_2-adrenoceptor agonists reduced formalin-induced c-*fos* gene transcription and nocifensive behavior via their cognate receptors. Unexpectedly, blockade of either the opiate or α_2-adrenergic receptors, alone, caused an increase in formalin-evoked c-*fos* mRNA; while blocking the opiate receptor had no effect on formalin-induced behavior, α_2-adrenoceptor block had an analgesic effect, indicating discordance between c-*fos* message transcription and nocifensive behavior. We concluded that the formalin-induced spinal c-*fos* signal was a poor predictor of the behavioral response to pharmacological manipulation of pain processing pathways.

Keywords: Spinal cord; c-*fos*; Formalin test; Opioid receptor; α_2-Adrenoceptor

1. Introduction

Formalin injection causes a biphasic inflammatory response in peripheral tissues, leading to the rapid and transient induction of c-*fos* and other immediate early genes in the spinal cord (Morgan and Curran, 1991; Doyle et al., 1997; Harris, 1998). After formalin injection the c-*fos* gene is induced in the nuclei of superficial dorsal horn neurons receiving synaptic contacts from the substance P containing terminals of small diameter nociceptive primary afferents (Tao and Zhao, 1997). Substance P is the endogenous ligand of the tachykinin NK_1 receptor, which contributes to the formalin-evoked c-*fos* expression (Tao et al., 1996). When neurons are stimulated by excitatory neurotransmitters such as glutamate or substance P, intracellular signaling activates transcriptional expression of c-*fos* mRNA within 5 min, which subsequently translates Fos protein expression (Szekely et al., 1989; Morgan and Curran, 1991; Chapman et al., 1995; Elliot et al., 1995; Johnson et al., 1997). Therefore, c-*fos* expression in the spinal cord is regarded as an early marker of total neuronal activation (Munglani and Hunt, 1995; Dickenson et al., 1997).

Fos immunohistochemistry has been used to quantitatively localize this protein in neural circuits at the level of individual cells in rats subjected to peripheral noxious stimulation (Hunt et al., 1987). A quantitative correlation has been observed between formalin-induced nocifensive behavior and spinal Fos protein expression (Gogas et al., 1996). Many investigators have also attempted to use Fos to quantitate neural responses to sustained noxious stimulation and thereby to assess the efficacy of analgesics in awake, intact, unrestrained animals. Unfortunately, noxious stimulus-evoked Fos protein expression may be a poor predictor of analgesic behavioral response (Gogas et

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00463-X

al., 1991, 1996; Hammond et al., 1992; Hylden et al., 1992; Sun et al., 1996; Abbadie et al., 1997; Rohde et al., 1997; Harris, 1998; Yashpal et al., 1998). The discordance between analgesic effects on Fos protein and behavior may reflect the semiquantitative nature of Fos protein measurement. Fos protein studies use an all-or-none cell counting method, which does not consider the variability in staining intensity with immunohistochemistry. We hypothesized that the Northern blot assay for mRNA would provide a more sensitive and quantitative measure of spinal c-*fos* expression than Fos protein, and would enable us to detect with great sensitivity pharmacological effects on gene transcription. The Northern blot method utilizes laser densitometry quantitation of the extracted neural c-*fos* mRNA rather than assessing individual cells which have achieved a threshold for Fos expression. Furthermore, some of the expressed c-*fos* mRNA may not translate into Fos protein, therefore, c-*fos* signal may be a more sensitive measure of neuronal activity.

Both morphine, a mu opiate receptor agonist, and dexmedetomidine, a selective α_2-adrenoceptor agonist, exert their analgesic actions via spinal and supraspinal sites within the endogenous opioidergic and adrenergic analgesic pathways. Although several previous studies have examined the effects of these drugs and their antagonists on noxious stimulus-evoked spinal Fos protein (Gogas et al., 1991, 1996; Hammond et al., 1992; Pertovaara et al., 1993), none have used quantitative c-*fos* mRNA assays. Only one prior correlative study has examined both spinal c-*fos* mRNA expression and nocifensive behavior. These investigators found that dextromethorphan, a presumed NMDA receptor antagonist, completely blocked both formalin-induced spinal c-*fos* signal and behavior in mice (Elliot et al., 1995). The aim of our study was to quantify the modulation of formalin-induced c-*fos* mRNA expression after analgesic treatment and correlate this to pain behavior, to test our hypothesis that c-*fos* signal can accurately predict the behavioral analgesic response.

2. Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee at the Veterans Affairs Palo Alto Health Care System and conformed to the ''Guide for the Care and Use of Laboratory Animals'' (NIH publication 85-23, revised 1985). A total of 171 Sprague–Dawley male rats (200 to 230 g) were obtained from B&K Universal (Fremont, CA) for use in this study. Animals were housed in pairs with a 12-h light/dark cycle and ad libitum water and food.

2.1. Formalin injection

The plantar surface of the right hindpaw was subcutaneously injected just under the skin with either a 50-μl volume of 5% formalin (diluted in saline) or a 50-μl volume of saline.

2.2. Behavioral testing

The testing room was dimly lit and kept at a temperature between 23 and 24°C. The rats were individually placed in Plexiglas cylinders (20.4 cm diameter, 40.6 cm high) on a glass top table with a video surveillance camera mounted below. After 30-min acclimation, the right hindpaw was injected and the animals were videotaped for the next 65 min. Based on pilot data and consistent with the literature, the total time spent licking the right hindpaw over the next 5 min was defined as the early phase (phase I). The total licking time recorded during the 15–50-min interval after hindpaw injection was defined as the late phase (phase II). The behavioral assays and the Northern blotting assays were not performed in the same groups of rats.

2.3. Drugs

Dexmedetomidine, a selective α_2-adrenoceptor agonist, and atipamezole, a selective α_2-adrenoceptor antagonist, were supplied by Orion (Turku, Finland). Morphine sulfate and naloxone hydrochloride were obtained from Sigma (St Louis, MO). All drugs were dissolved in saline and given by intraperitoneal (i.p.) injection in a volume of 1 ml/kg body weight. All drug injections were performed 30 min prior to the hindpaw injection.

2.4. Spinal cord preparation

The animal was decapitated immediately after a rapid induction of anesthesia with 70% nitrous oxide and 5% isoflurane. The entire spinal cord was expelled from the spinal canal by forcefully injecting cold saline at the sacral vertebral level (Draisci and Iadarola, 1989). The lumbosacral nerve roots were removed from the spinal cord and a 1-cm segment of the cord was taken from between 5 and 15 mm rostral to the caudal termination of the cord. Specimens were frozen immediately on dry ice and stored at −80°C. Spinal cord harvesting was completed within 3 min after decapitation.

In one set of experiments, the segmental distribution of the spinal c-*fos* expression was investigated by removing three contiguous 1 cm sections of rat spinal cord, beginning 5 mm rostral to the conus medullaris.

2.5. RNA isolation and Northern blotting assays

Total RNA was extracted by an acid guanidine phenol chloroform method (RNA STAT-60®; TEL-TEST ''B'', Friendswood, TX) as originally described by Chomcznyski and Sacchi (1987) and was quantified by absorbance at 260 nm. Total RNA (5 μg) was fractionated by elec-

trophoresis on a 1.0% agarose-formaldehyde gel and blotted on a nylon membrane (S&S Nytran®, Schleicher & Schuell, Keene, NH) by downward capillary transfer (Turboblotter®, Schleicher & Schuell).

Membranes were prehybridized for 30 min at 68°C in QuikHyb® Hybridization Solution (Stratagene, La Jolla, CA) and hybridized for 1 h in the same solution added with 10 μg herring sperm DNA and ^{32}P-labeled cDNA probe for rat c-*fos* (897-bp fragment) or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 316-bp fragment) (pTRI-GAPDH-Rat; Ambion, Austin, TX). An 897-bp fragment of rat c-*fos* cDNA was produced by PCR with the following forward and backward primers, respectively; 5′-GATGTTCTCGGGTTTCAACGA-3′ and 5′-GCATAGAAGGAACCAGACAGGTCC-3′. The cDNA probes had been labeled with [α-^{32}P] dCTP by random hexamer priming (Multiprime DNA labelling systems; Amersham, Arlington Heights, IL). After hybridization, the membranes were washed in 2 × sodium chloride–sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at room temperature twice for 15 min and 0.1 × SSC and 0.1% SDS at 60°C for 30 min and were then exposed to Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 12–16 h.

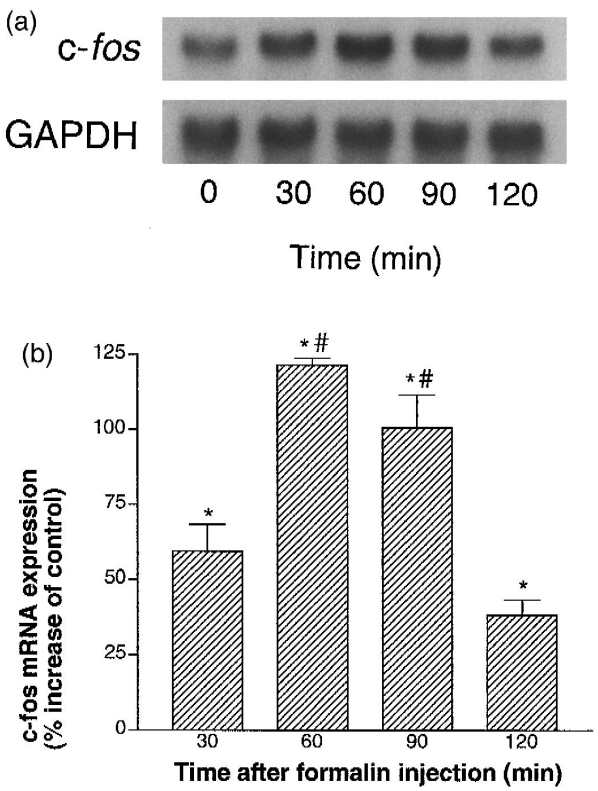

Fig. 1. (a) RNA blots probed for c-*fos* and GAPDH mRNA. Total RNA was extracted from lumbar enlargement 30, 60, 90 and 120 min after formalin injection. Blotted membrane was hybridized with ^{32}P-labeled c-*fos* and GAPDH probes successively. Densitometry by Phosphoimager from a typical experiment shows a rapid increase and recovery in c-*fos* mRNA expression when compared with relatively constant GAPDH mRNA expression. (b) Time course of formalin induced spinal c-*fos* mRNA expression. c-*fos* mRNA expression following s.c. formalin injection was normalized with GAPDH expression. Data are expressed as a percent increase of control group rats undergoing no injection. Peak increase in c-*fos* mRNA was reached around 60 min after formalin injection. $n = 3$ in each group, $^{*}P < 0.05$ compared with control, $\#P < 0.05$ compared with 30 min group.

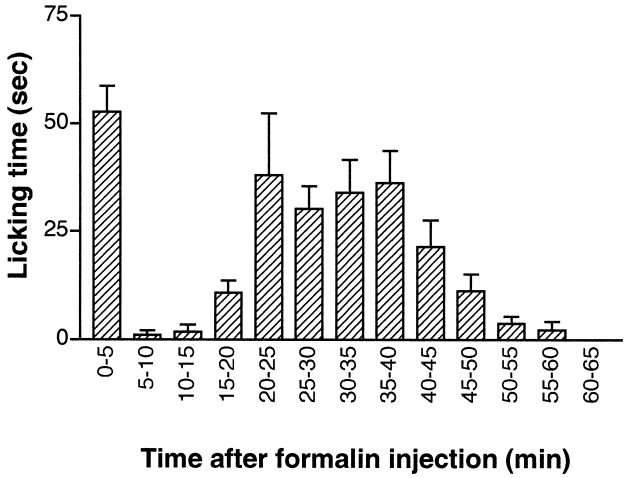

Fig. 2. Time course of licking behavior after formalin injection. There were two peaks of nocifensive behavior after intraplantar formalin, the first phase (0–5 min) with a mean licking time of 53 s, and the second phase (15–50 min) with a mean time of 181 s ($n = 12$).

The relative amount of mRNA expression in each blot was determined by laser densitometry using PhosphorImager (Molecular Dynamics). Densitometric scores of c-*fos* mRNA expression were normalized to that of GAPDH.

2.6. Statistical analysis

Results were analyzed using factorial analysis of variance, and expressed as a mean ± standard error of the mean. When indicated, a Bonferroni correction was used. For c-*fos* mRNA only those parameters blotted and hybridized on the same membrane were compared. All formalin-induced increases of c-*fos* mRNA levels above baseline are listed as a percentage of the baseline c-*fos* mRNA levels observed after saline hindpaw injection, i.e., ([formalin s.c. − saline s.c.]/saline s.c.) × 100%.

3. Results

3.1. Time course

The c-*fos* mRNA levels in the spinal cord showed a transient increase from 30 to 120 min after s.c. formalin, peaking at 60 min (Fig. 1A,B). After determining the time course of the c-*fos* mRNA response to formalin, all subsequent spinal cord sections were removed 60 min after hindpaw injection. Consistent with previous reports, the time course of formalin-induced hindpaw licking behavior demonstrated two phases, an early phase I peak between 0

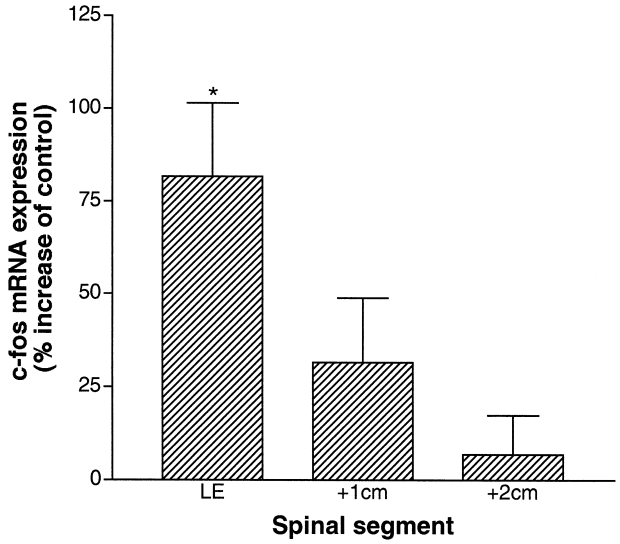

Fig. 3. Segmental distribution of formalin-induced spinal c-*fos* mRNA expression. Three consecutive spinal cord segments of 1 cm were dissected from each rat 1 h after formalin injection. In control rats, lumbar enlargement segment was dissected after saline injection in the hindpaw. Data are expressed as a percent increase of control. Note that the formalin injection resulted in significant c-*fos* mRNA increase only in the lumbar enlargement. LE: lumbar enlargement, +1: 1 cm rostral to LE, +2: 2 cm rostral to LE. $n = 6$ for each group, $^{*}P < 0.05$ compared with control.

and 5 min and a late phase II peak between 15 and 50 min after formalin injection (Fig. 2).

3.2. Segmental distribution

Formalin injection in the rat hindpaw caused an 82% increase in the spinal c-*fos* expression at the level of the lumbar enlargement (Fig. 3), compared with saline injection ($P < 0.001$). There was no significant increase in the two segments rostral to the lumbar segment. To improve the sensitivity of c-*fos* mRNA expression only the lumbar enlargement segments were used for subsequent experiments.

3.3. Effects of morphine and naloxone

Intraperitoneal morphine pretreatment resulted in suppression of the formalin-induced spinal c-*fos* (Fig. 4A). High dose morphine (10 mg/kg) reduced the c-*fos* expression by 75% as compared with the i.p. saline pretreatment ($P < 0.01$). Similarly, morphine suppressed both phase I and II licking behavior (Fig. 4B,C), with high dose morphine group exhibiting 90% reduction in licking behavior ($P < 0.001$ and $P < 0.02$ for phase I and II, respectively).

Naloxone (5 mg/kg) pretreatment completely reversed the inhibitory effect of a high dose of morphine (Fig. 4) on formalin-evoked c-*fos* expression ($P < 0.001$) and both phases of the licking behavior ($P < 0.001$ and $P < 0.01$ for phase I and II, respectively). Pretreatment with naloxone alone increased the formalin-induced spinal c-*fos* by

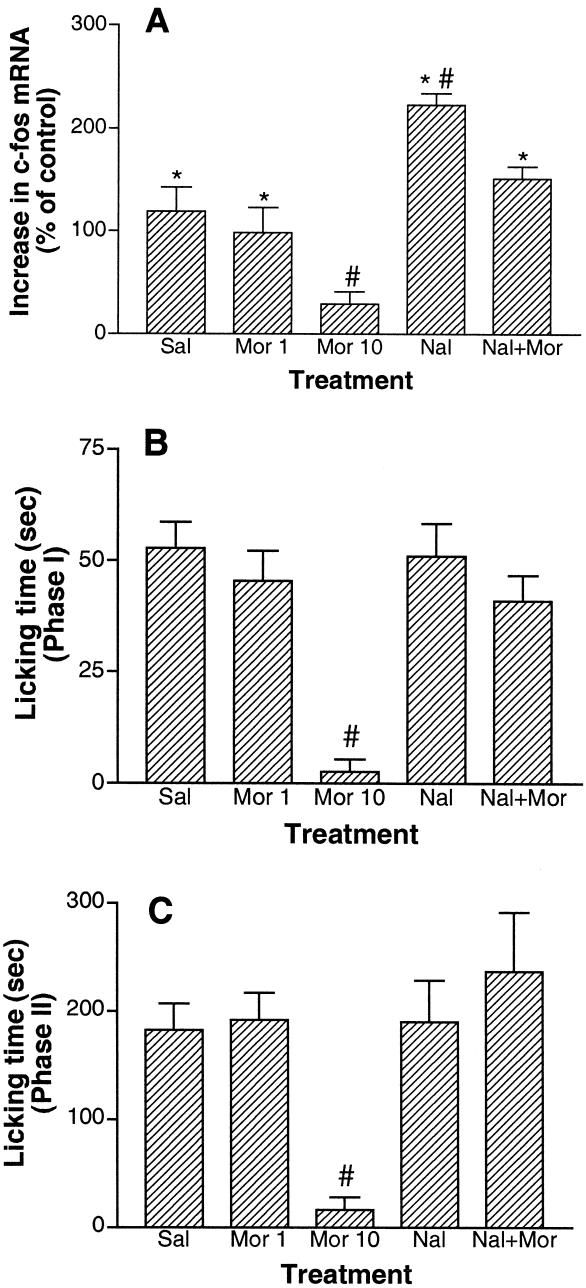

Fig. 4. Effect of morphine and naloxone on the formalin-induced spinal c-*fos* mRNA expression and licking behavior. Each rat was pretreated i.p. 30 min before formalin as follows. Sal; saline, Mor 1; 1 mg/kg morphine, Mor 10; 10 mg/kg morphine, Nal; 5 mg/kg naloxone, Mor + Nal; 10 mg/kg morphine + 5 mg/kg naloxone. Control rats were pretreated i.p. with saline and injected with hindpaw saline. (A) Morphine dose-dependently suppressed the formalin-induced c-*fos* mRNA expression, which was completely reversed with naloxone pretreatment combined with morphine. Note that pretreatment with naloxone alone enhanced the formalin-induced c-*fos* mRNA increase as compared with saline pretreatment. $n = 5$ in each group. (B, C) Systemic morphine dose-dependently reduced both phases of formalin behavior, but naloxone alone had no effect. The analgesic effects of the high dose morphine were completely blocked with naloxone. Sal ($n = 12$); Mor 1 ($n = 6$); Mor 10 ($n = 6$); Nal ($n = 11$), Mor + Nal ($n = 12$), $^{*}P < 0.05$ compared with control, $\#P < 0.05$ compared with Sal.

88% compared with the saline pretreatment ($P < 0.001$, Fig. 4A). When naloxone pretreatment was given to rats that received intraplantar saline instead of formalin, there was no change in spinal c-*fos* ($n = 5$, data not shown). In contrast to naloxone's enhancing effects on the formalin-evoked c-*fos* response, no effect of naloxone was observed for either the first or second phase of licking behavior (Fig. 4B,C).

3.4. Effects of dexmedetomidine and atipamezole

Dexmedetomidine pretreatment resulted in suppression of the formalin-induced spinal c-*fos* (Fig. 5A). High dose dexmedetomidine (100 μg/kg) reduced the c-*fos* expression by 49% as compared with the i.p. saline pretreatment ($P < 0.01$). Dexmedetomidine was more effective in reducing nocifensive behavior (Fig. 5B,C), with high dose dexmedetomidine completely preventing any formalin-evoked licking ($P < 0.001$).

Similar to the effects of naloxone, pretreatment with atipamezole alone enhanced the formalin-induced spinal c-*fos* expression by 47% compared with the saline pretreatment ($P < 0.01$, Fig. 5A). In contrast to atipamezole's enhancing effects on c-*fos* response, atipamezole reduced both the first and second phases of licking behavior following formalin injection ($P < 0.05$, Fig. 5B,C). When atipamezole pretreatment was given to rats that received intraplantar saline instead of formalin, there was no change in spinal c-*fos* ($n = 5$, data not shown).

Not only did atipamezole pretreatment reversed the inhibitory effect of a high dose dexmedetomidine on formalin-evoked c-*fos* ($P < 0.001$) and nocifensive behavior ($P < 0.001$), but the combination of atipamezole and dexmedetomidine increased nocifensive behavior above that seen in animals given saline-pretreatment only ($P < 0.01$ and $P < 0.001$ for phase I and II, respectively).

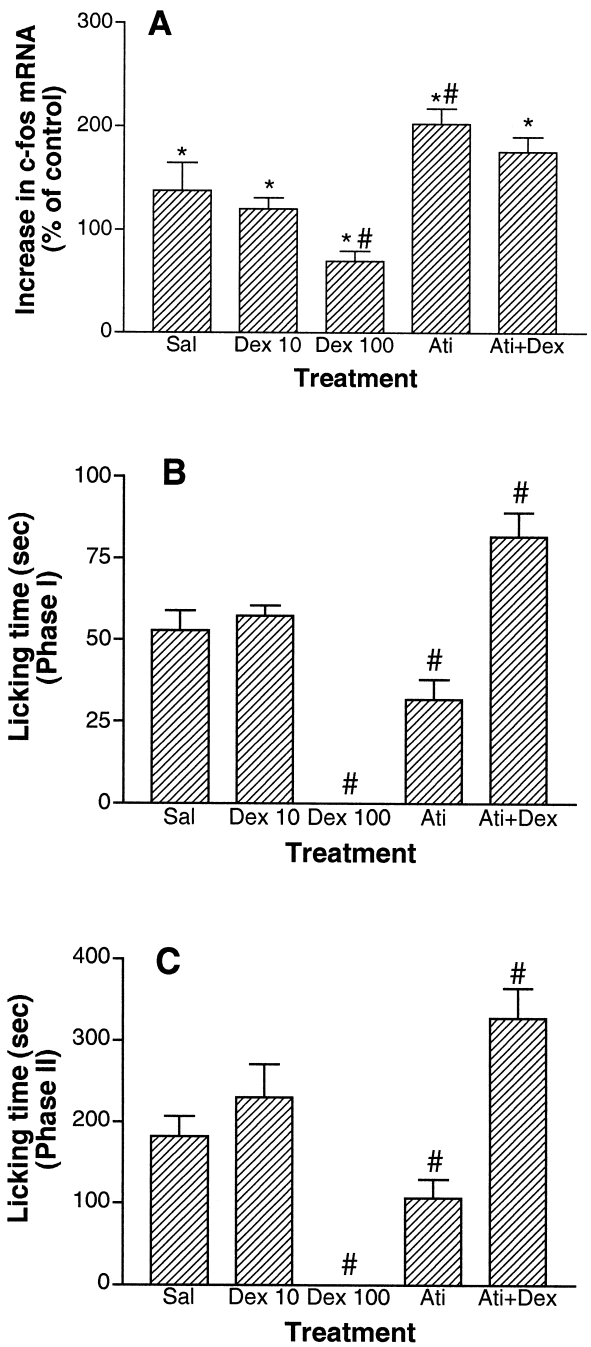

Fig. 5. Effect of dexmedetomidine and atipamezole on the formalin-induced spinal c-*fos* mRNA expression and licking behavior. Each group was pretreated i.p. as follows. Sal; saline, Dex 10; 10 μg/kg dexmedetomidine, Dex 100; 100 μg/kg dexmedetomidine, Ati; 1.5 mg/kg atipamezole, Dex + Ati; 100 μg/kg dexmedetomidine + 1.5 mg/kg atipamezole. Control rats were pretreated with i.p. saline and injected with hindpaw saline. (A) Dexmedetomidine dose-dependently suppressed the formalin-induced c-*fos* expression, which was completely reversed with atipamezole pretreatment combined with dexmedetomidine. Note that pretreatment with atipamezole alone enhanced the formalin-induced c-*fos* increase as compared with saline pretreatment. $n = 5$ in each group. (B, C) Systemic dexmedetomidine dose-dependently reduced both phases of formalin behavior, but its antagonist atipamezole also had an analgesic effect on both phases of nociceptive behavior. The analgesic effects of high dose dexmedetomidine were not only blocked by atipamezole, but licking behavior was paradoxically increased with the combination of atipamezole and dexmedetomidine. Sal ($n = 12$); Dex 10 ($n = 6$); Dex 100 ($n = 6$); Ati ($n = 18$), Dex + Ati ($n = 12$), $^*P < 0.05$ compared with control, $\#P < 0.05$ compared with Sal.

4. Discussion

Using Northern blot analysis to quantify formalin-induced spinal c-*fos* mRNA transcription, the analgesic drugs morphine and dexmedetomidine dose-dependently reduced both c-*fos* expression and licking behavior. These suppressive effects were completely reversed, respectively, by the opioid receptor antagonist naloxone and by the α_2-adrenoceptor antagonist atipamezole. In addition, when given alone, these antagonists enhanced formalin-induced spinal c-*fos* expression, but had either no (naloxone) or an inhibitory (i.e., antinocifensive for atipamezole) effect on formalin behavior.

The c-*fos* mRNA increased 30 min after formalin injection, reached its peak within 60 min, began dropping by 90 min and returned nearly to baseline by 120 min (Fig. 1). The peak of the phase II behavioral response preceded the c-*fos* peak expression by about 30 min. This time course is consistent with evidence that both the first and second phase of the formalin assay are associated with nociceptor firing and spinal Fos expression (Puig and Sorkin, 1995; Tokunaga et al., 1995; Peterson et al., 1997), and that the peak expression of c-*fos* occurs 30–45 min after stimulation (Morgan and Curran, 1991). Spinal Fos protein first appears in the dorsal horn 60 min after formalin injection and the peak response is observed 2 to 4 h after formalin (Presley et al., 1990), at a time when the nocifensive behavior has long terminated. The time course of c-*fos* mRNA precedes that seen for spinal Fos, as would be expected for transcription vs. translation of protein synthesis (Morgan and Curran, 1991).

In a previous study using immunohistochemistry methods, spinal Fos protein expression was not detected after saline hindpaw injection (Gogas et al., 1996); by contrast, we are able to detect spinal c-*fos* mRNA after hindpaw saline injection which was further increased by 82% following formalin. In agreement with our data, other studies using either the RNA protection assay (Elliot et al., 1995) or Northern blot assay (Hunter et al., 1995) demonstrated significant spinal c-*fos* mRNA after saline hindpaw injection, which increased after formalin injection.

In the present study, morphine dose-dependently suppressed the formalin-evoked spinal c-*fos* mRNA (Fig. 4), which is in good agreement with immunohistochemical studies showing that systemic (Presley et al., 1990) and intracerebroventricular (i.c.v.) (Gogas et al., 1991, 1996) opioids reduce formalin-evoked spinal Fos protein. We noted that the inhibitory effect of morphine on early and late formalin-induced nocifensive behavior parallels its action on c-*fos* expression. Prior studies have shown similar reductions in formalin behavior with i.c.v. (Calcagnetti et al., 1988; Gogas et al., 1991, 1996), intrathecal (Malmberg and Yaksh, 1993), or systemic (North, 1978; Dennis et al., 1980; Skingle et al., 1982; Drower et al., 1987; Wilson et al., 1991; Wheeler-Aceto and Cowan, 1993) administration of opioids.

The inhibitory effect of morphine on spinal c-*fos* and formalin behavior was completely reversed by naloxone, indicating that the effects of morphine were mediated by opiate receptors. This is consistent with previous studies demonstrating that naloxone completely reverses the suppressive effect of opioids on formalin-induced spinal Fos protein (Presley et al., 1990; Gogas et al., 1991, 1996) and on both phases of formalin behavior (Dennis et al., 1980; Malmberg and Yaksh, 1993; Wheeler-Aceto and Cowan, 1993). Yet, pretreatment with naloxone, alone, caused an 88% increase in formalin-induced c-*fos* mRNA, which contrasts with prior studies indicating no significant effect of naloxone on inflammation-induced spinal Fos protein (Presley et al., 1990; Gogas et al., 1991; Abbadie and Besson, 1993). Interestingly, other investigators have observed naloxone eliciting an increase in spinal Fos protein when extremely noxious repetitive thermal stimuli were applied (Abbadie et al., 1994; Tolle et al., 1994), suggesting naloxone may evoke a larger and more readily detectable Fos response with a stronger nocifensive stimulus. The difference between our results addressing c-*fos* mRNA expression and these others which examined Fos protein expression after inflammation may be explained by the fact the c-*fos* mRNA assay is more sensitive than Fos protein measurements. Assuming spinal c-*fos* expression can be regarded as an indirect marker of neuronal activation, our data suggest that the endogenous opioidergic system inhibits formalin elicited spinal neuron activity. Our interpretation is corroborated by data which demonstrate that electrical or exogenous opioidergic activation of the descending analgesic system inhibit spinal Fos expression induced by prolonged noxious stimulation (Jones and Light, 1990; Gogas et al., 1991), and that dorsal lateral funiculus transection (which interrupts descending inhibitory pathways) can enhance formalin-induced spinal Fos expression (Zhang et al., 1994; but see Gogas et al., 1991). Naloxone alone had no effect on spinal c-*fos* mRNA expression after saline injection, indicating that the endogenous opioidergic system does not inhibit c-*fos* expression after a brief noxious stimulus.

Neither phase of the nocifensive behavioral response to formalin was affected by naloxone pretreatment (Fig. 4B,C), which is consistent with prior studies reporting a similar lack of naloxone effect on the behavioral and cardiovascular responses to formalin (North, 1978; Kocher, 1988; Pertovaara et al., 1991; Taylor et al., 1997). Our results indicate a dissociation between the c-*fos* mRNA and nocifensive behavior after naloxone pretreatment. One explanation for this discordance is that blocking endogenous opioid inhibition may increase spinal neuron activity sufficiently to evoke further c-*fos* message, but not reach the threshold to elicit additional nocifensive behavior.

While a high dose of dexmedetomidine completely blocked formalin-induced nocifensive behavior, the increase in formalin-induced c-*fos* mRNA was only attenuated by 49% (Fig. 5A). These data corroborate earlier studies examining the effect of α_2-adrenoceptor agonists on formalin-induced spinal Fos (Pertovaara et al., 1993) and on formalin-induced nocifensive behavior (Dennis et al., 1980; Pertovaara et al., 1990, 1993; Tasker et al., 1992; Kanui et al., 1993; Malmberg and Yaksh, 1993). We believe that the reason why there is a divergence between formalin-induced nocifensive behavior and c-*fos* mRNA at high dose of dexmedetomidine, 100 μg/kg i.p., is because this dose produces profound sedation (Doze et al., 1989) which exaggerates its apparent analgesic action in this complex behavioral assay. Atipamezole completely reversed the inhibitory effect of dexmedetomidine on formalin-induced spinal c-*fos* and both phases of formalin behav-

ior, demonstrating that the actions of dexmedetomidine are mediated by α_2-adrenoceptors.

Similar to the action of naloxone pretreatment, atipamezole, alone, enhanced the formalin-induced increased in c-*fos* mRNA (Fig. 5A). We interpret these data to reflect the fact that formalin activates an endogenous adrenergic mediated inhibition of spinal neurons in pain-processing pathways. Atipamezole did not affect saline-evoked c-*fos* expression, which indicates that adrenergic inhibition does not influence spinal neuron activity after a brief nociceptive stimulus. A prior immunohistochemical study was unable to detect an atipamezole effect on formalin-evoked spinal Fos protein (Pertovaara et al., 1993), which again may be due to the low sensitivity of immunohistochemical techniques compared to assessment by gene expression.

Our finding that pretreatment with atipamezole, alone, had an inhibitory effect on both phases of formalin behavior (Fig. 5B,C) corroborates earlier studies (Dennis et al., 1980; Kanui et al., 1993) although this has been disputed by others (Pertovaara et al., 1990, 1993; Tasker et al., 1992; Malmberg and Yaksh, 1993). In trying to explain the apparent discrepancy between the atipamezole-induced increase in c-*fos* mRNA expression (Fig. 5A) together with a decrease in nocifensive behavior (Fig. 5B,C) following formalin injection, we speculate that these actions are produced at separate sites in the nociceptive pathway. Several investigators have demonstrated that the release of norepinephrine from activated sympathetic nerve terminals contributes to polymodal nociceptor excitation via peripheral α_2-adrenoceptor stimulation in animal models of inflammation (Hu and Zhu, 1989; Sato et al., 1993, 1994). Thus, inflammatory nociceptive behavior may be attenuated by preventing synaptic release of norepinephrine from the sympathetic nerve terminals (Coderre et al., 1984), or by direct blockade of the peripheral α-adrenergic receptors. It should also be appreciated that the increase in spinal c-*fos* mRNA may reflect activation of both *inhibitory* as well as excitatory neurons in pathways involved in nociceptive processing (Todd et al., 1994).

Despite the fact that, when given alone, either atipamezole or dexmedetomidine inhibited formalin-induced nocifensive behavior, when the two drugs were given together formalin-induced nocifensive behavior was enhanced (Fig. 5B,C). While such an ''algesic'' effect of this combination of a systemic α_2-adrenoceptor agonist and antagonist has not been previously observed in the formalin models (Dennis et al., 1980; Tasker and Melzack, 1989; Pertovaara et al., 1990; Pertovaara, 1993), there are several plausible explanations for this observation, especially in the setting of formalin-induced nocifensive behavior. Work with other inhibitory neurotransmitters (especially opiate receptor agonists) suggest that these may also cause *excitation* of nociceptive mechanisms (Maze, 1997). The weak pronociceptive actions of an analgesic agent can only be detected when its inhibitory actions are selectively attenuated, revealing its excitatory effects. Therefore, it is possible that dexmedetomidine may activate mechanisms which are excitatory in nociceptive processing following formalin and are not antagonized by atipamezole. Also, atipamezole may be expected to increase locus coeruleus activity, as suggested by studies demonstrating increased c-*fos* expression in that brain region following α_2-adrenoceptor antagonist administration (Herrera and Robertson, 1996). Stimulation of the locus coeruleus causes increased release of spinal norepinephrine which can activate unblocked α_1-adrenoceptors which may increase activity in nociceptive pathways (Kanui et al., 1993). This hypothesis would also predict that spinal c-*fos* expression could also be increased, and we observed a small, not quite significant increase in c-*fos* with the coadministration of atipamezole and dexmedetomidine (Fig. 5A).

In conclusion, both morphine and dexmedetomidine suppressed the spinal c-*fos* mRNA expression and the nocifensive behavior induced by formalin injection, and this suppression was mediated by their cognate receptors. Moreover, treatment with naloxone or atipamezole alone enhanced the formalin-induced spinal c-*fos* expression, suggesting that formalin-evoked spinal neuronal activation can be inhibited by both the endogenous opioidergic and adrenergic systems. These data indicate that the Northern blot analysis is a sensitive method for quantifying the effects of pharmacological manipulation of endogenous analgesic pathways on formalin-evoked spinal c-*fos* expression. Unfortunately, spinal c-*fos* expression was not always a reliable predictor of the behavioral response to treatment. Dexmedetomidine was much more effective at reducing formalin-induced behavior than spinal c-*fos* signal. Interestingly, despite the fact that both treatments increased c-*fos* expression, naloxone alone had no effect on formalin behavior, but atipamezole had an analgesic effect. We have no simple explanation for the discordance between the enhancement of gene transcription and inhibition of formalin behavior with α_2-adrenoceptor antagonism and further investigation is required to elucidate the site or sites of α_2-adrenoceptor antagonist activity in the nociceptive pathway.

Acknowledgements

Supported by grant GM30232 from the NIH and a VA Merit Review. We thank Orion-Farmos for providing the atipamezole.

References

Abbadie, C., Besson, J.-M., 1993. Effects of morphine and naloxone on basal and evoked Fos-like immunoreactivity in lumbar spinal cord neurons of arthritic rats. Pain 52, 29–39.

Abbadie, C., Honore, P., Besson, J.M., 1994. Intense cold noxious stimulation of the rat hindpaw induces c-*fos* expression in lumbar spinal cord neurons. Neuroscience 59, 457–468.

Abbadie, C., Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Differ-

ential contribution of the two phases of the formalin test to the pattern of c-*fos* expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain 69, 101–110.

Calcagnetti, D.J., Helmstetter, F.J., Fanselow, M.S., 1988. Analgesia produced by centrally administered DAGO, DPDPE and U50488H. Eur. J. Pharmacol. 153, 117–122.

Chapman, V., Honore, P., Buritova, J., Besson, J.M., 1995. The contribution of the NMDA receptor activation to spinal c-Fos expression in a model of inflammatory pain. Br. J. Pharmacol. 116, 1628–1634.

Chomcznyski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Ann. Biochem. 162, 156–159.

Coderre, T.J., Abbott, F.V., Melzack, R., 1984. Effects of peripheral antisympathetic treatments in the tail-flick, formalin and autotomy tests. Pain 18, 13–23.

Dennis, S.G., Melzack, R., Gutman, S., Boucher, F., 1980. Pain modulation by adrenergic agents and morphine as measured by three pain tests. Life Sci. 26, 1247–1259.

Dickenson, A.H., Chapman, V., Green, G.M., 1997. The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. Gen. Pharmacol. 28, 633–638.

Doyle, C.A., Palmer, J.A., Munglani, R., Hunt, S.P., 1997. Molecular consequences of noxious stimulation. In: Borsooks, D. (Ed.), Molecular Neurobiology of Pain, Vol. 9. IASP Press, Seattle, pp. 145–169.

Doze, V.A., Chen, B.X., Maze, M., 1989. Dexmedetomidine produces a hypnotic–anesthetic action in rats via activation of central alpha-2 adrenoceptors. Anesthesiology 71, 75–79.

Draisci, G., Iadarola, M.J., 1989. Temporal analysis of increases in c-*fos*, preprodynorphin and preproenkephalin mRNAs in rat spinal cord. Mol. Brain Res. 6, 31–37.

Drower, E.J., Stapelfeld, A., Mueller, R.A., Hammond, D.L., 1987. The antinociceptive effects of prostaglandin antagonists in the rat. Eur. J. Pharmacol. 133, 249–256.

Elliot, K.J., Brodsky, M., Hynansky, A.D., Foley, K.M., Inturrisi, C.E., 1995. Dextromethorphan suppresses both formalin-induced nociceptive behavior and the formalin-induced increase in spinal cord c-*fos* mRNA. Pain 61, 401–409.

Gogas, K.R., Presley, R.W., Levine, J.D., Basbaum, A.I., 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, 617–628.

Gogas, K.R., Cho, H.J., Botchkina, G.I., Levine, J.D., Basbaum, A.I., 1996. Inhibition of noxious stimulus-evoked pain behaviors and neuronal fos-like immunoreactivity in the spinal cord of the rat by supraspinal morphine. Pain 65, 9–15.

Hammond, D.L., Presley, R., Gogas, K.R., Basbaum, A.I., 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, 244–253.

Harris, J.A., 1998. Using c-*fos* as a neural marker of pain. Brain Res. Bull. 45, 1–8.

Herrera, D.G., Robertson, H.A., 1996. Activation of c-*fos* in the brain. Prog. Neurobiol. 50, 83–107.

Hu, S., Zhu, J., 1989. Sympathetic facilitation of sustained discharges of polymodal nociceptors. Pain 38, 85–90.

Hunt, S.P., Pini, W., Evan, G., 1987. Induction of c-*fos*-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632–634.

Hunter, J.C., Woodburn, V.L., Durieux, C., Pettersson, E.K.E., Poat, J.A., Hughes, J., 1995. C-*fos* antisense oligodeoxynucleotide increases formalin-induced nociception and regulates preprodynorphin expression. Neuroscience 65, 485–492.

Hylden, J.L.K., Noguchi, K., Ruda, M.A., 1992. Neonatal capsaicin treatment attenuates spinal Fos activation and dynorphin gene expression following peripheral tissue inflammation and hyperalgesia. J. Neurosci. 12, 1716–1725.

Johnson, C.M., Hill, C.S., Chawla, S., Treisman, R., Bading, H., 1997. Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. J. Neurosci. 17, 6189–6202.

Jones, S.L., Light, A.R., 1990. Termination patterns of serotoninergic medullary raphe spinal fibers in the rat lumbar spinal cord: an anterograde immunohistochemical study. J. Comp. Neurol. 297, 267–282.

Kanui, T.I., Tjolsen, A., Lund, A., Mjellem-Joly, N., Hole, K., 1993. Antinociceptive effects of intrathecal administration of alpha-adrenoceptor antagonists and clonidine in the formalin test in the mouse. Neuropharmacology 32, 367–371.

Kocher, L., 1988. Systemic naloxone does not affect pain-related behavior in the formalin test in rat. Physiol. Behav. 43, 265–268.

Malmberg, A.B., Yaksh, T.L., 1993. Pharmacology of the spinal action of ketorolac, morphine, ST-91, U50488H, and L-PIA on the formalin test and an isobolographic analysis of the NSAID interaction. Anesthesiology 79, 270–281.

Maze, M., 1997. ''Exciting'' aspects of opiate receptor signal transduction. Anesthesiology 87, 1032–1033.

Morgan, J.I., Curran, T., 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. Annu. Rev. Neurosci. 14, 421–451.

Munglani, R., Hunt, S.P., 1995. Molecular biology of pain. Br. J. Anaesth. 75, 186–192.

North, M.A., 1978. Naloxone reversal of morphine analgesia but failure to alter reactivity to pain in the formalin test. Life Sci. 22, 295–302.

Pertovaara, A., 1993. Antinociception induced by alpha-2-adrenoceptor agonists, with special emphasis on medetomidine studies. Prog. Neurobiol. 40, 691–709.

Pertovaara, A., Kauppila, T., Tukeva, T., 1990. The effect of medetomidine, an alpha-2 adrenoceptor agonist, in various pain tests. Eur. J. Pharmacol. 179, 323–328.

Pertovaara, A., Mecke, E., Carlson, S., 1991. Attempted reversal of cocaine-induced antinociceptive effects with naloxone, an opioid antagonist. Eur. J. Pharmacol. 192, 349–353.

Pertovaara, A., Bravo, R., Herdegen, T., 1993. Induction and suppression of immediate-early genes in the rat brain by a selective alpha-2-adrenoceptor agonist and antagonist following noxious peripheral stimulation. Neuroscience 54, 117–126.

Peterson, M.A., Basbaum, A.I., Abbadie, C., Rhode, D.S., McKay, W.R., Taylor, B.K., 1997. The differential contribution of capsaicin-sensitive afferents to behavioral and cardiovascular measures of brief and persistent nociception and to Fos expression in the formalin test. Brain Res. 755, 9–16.

Presley, R.W., Menetrey, D., Levine, J.D., Basbaum, A.I., 1990. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J. Neurosci. 10, 323–335.

Puig, S., Sorkin, L.S., 1995. Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. Pain 64, 345–355.

Rohde, D.S., Detweiler, D.J., Basbaum, A.I., 1997. Formalin-evoked Fos expression in spinal cord is enhanced in morphine-tolerant rats. Brain Res. 766, 93–100.

Sato, J., Suzudi, S., Iseki, T., Kumazawa, T., 1993. Adrenergic excitation of cutaneous nociceptors in chronically inflamed rats. Neurosci. Lett. 164, 225–228.

Sato, J., Suzuki, S., Tamura, R., Kumazawa, T., 1994. Norepinephrine excitation of cutaneous nociceptors in adjuvant-induced inflamed rats does not depend on sympathetic neurons. Neurosci. Lett. 177, 135–138.

Skingle, M., Hayes, A., Tyers, M.B., 1982. Antinociception activity of clonidine in the mouse, rat, and dog. Life Sci. 31, 1123–1132.

Sun, W.Z., Shyu, B.C., Shieh, J.Y., 1996. Nitrous oxide or halothane, or both, fail to suppress c-*fos* expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. Br. J. Anaesth. 76, 99–105.

Szekely, A.M., Barbaccia, M.L., Alho, H., Costa, E., 1989. In primary

cultures of cerebellar granule cells the activation of *N*-methyl-D-aspartate-sensitive glutamate receptors induces c-*fos* mRNA expression. Mol. Pharmacol. 35, 401–408.

Tao, Y.-X., Zhao, Z.-Q., 1997. Ultrastructure of Fos-labeled neurons relating to nociceptive primary afferent and substance P terminals in rat spinal superficial laminae. Neuropeptides 31, 327–332.

Tao, Y.-X., Wei, F., Zhao, Z.-Q., 1996. A contribution of neurokinin-1 receptor to formalin-induced c-*fos* expression in the rat spinal dorsal horn. Neurosci. Lett. 221, 105–108.

Tasker, R.A.R., Melzack, R., 1989. Different alpha-receptor subtypes are involved in clonidine produced analgesia in different pain tests. Life Sci. 44, 9–17.

Tasker, R.A.R., Connell, B.J., Yole, M.J., 1992. Systemic injections of alpha-1 adrenergic agonists produce antinociception in the formalin test. Pain 49, 383–391.

Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Continuous intravenous infusion of naloxone does not change behavioral, cardiovascular, or inflammatory responses to subcutaneous formalin in the rat. Pain 69, 171–177.

Todd, A.J., Spike, R.C., Brodbelt, A.R., Price, R.F., Shehab, S.A.S., 1994. Some inhibitory neurons in the spinal cord develop c-*fos*-immunoreactivity after noxious stimulation. Neuroscience 63, 805–816.

Tokunaga, A., Doi, M., Senba, E., 1995. Effects of local anaesthesia on formalin-induced Fos expression in the rat dorsal horn. NeuroReport 6, 2301–2304.

Tolle, T.R., Schadrack, J., Castro-Lopes, J.M., Evan, G., Roques, B.P., Zieglgansberger, W., 1994. Effects of kelatorphan and morphine before and after noxious stimulation on immediate-early gene expression in rat spinal cord neurons. Pain 56, 103–112.

Wheeler-Aceto, H., Cowan, A., 1993. Naloxone causes apparent antinociception and pronociception simultaneously in the rat paw formalin test. Eur. J. Pharmacol. 236, 193–199.

Wilson, D.G., Rees, H., Roberts, M.H.T., 1991. The antinociceptive effects of anterior pretectal stimulation in tests using thermal, mechanical and chemical noxious stimuli. Pain 44, 195–200.

Yashpal, K., Mason, P., McKenna, J.E., Sharma, S.K., Henry, J.L., Coderre, T.J., 1998. Comparison of the effects of treatment with intrathecal lidocaine given before and after formalin on both nociception and Fos expression in the spinal cord dorsal horn. Anesthesiology 88, 157–164.

Zhang, R.-X., Wang, R., Chen, J.-Y., Qiao, J.-T., 1994. Effects of descending inhibitory systems on the c-Fos expression in the rat spinal cord during formalin-induced noxious stimulation. Neuroscience 58, 299–304.