Differential Effects of Intrathecally Administered Delta and Mu Opioid Receptor Agonists on Formalin-Evoked Nociception and on the Expression of Fos-like Immunoreactivity in the Spinal Cord of the Rat

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ABSTRACT

This study examined the effects of intrathecally (i.t.) administered mu and delta opioid receptor agonists on the flinching behavior and the expression of Fos-like immunoreactivity (Fos-LI) in the spinal cord elicited by s.c. injection of 5% formalin in one hindpaw of the rat. Intrathecal pretreatment with either the delta-1 opioid receptor agonist [delta]-[Pen^2,5]-enkephalin (DPDPE) or the delta-2 opioid receptor agonist [delta]-Ala^2,Glu^3]-deletorphin (DELT) produced a dose-dependent inhibition of flinching behavior in phase 1 and phase 2 but also nearly completely prevented the expression of Fos-LI in all regions of the spinal cord. These effects were antagonized by pretreatment with the mu opioid receptor antagonist [delta]-Phe-Cys-Tyr-Arg-Trp-Phen-NH_2. The efficacy of i.t. administered DAMGO suggests that a direct spinal action contributes to the inhibition of noxious stimulus-evoked Fos-LI in the spinal cord produced by systemically administered mu opioid receptor agonists such as morphine. The relative lack of effect of DPDPE or DELT suggests that delta opioid receptors do not modulate the early-immediate gene c-fos. Alternatively, because delta opioid receptor agonists inhibit synaptic transmission in the spinal cord by predominantly presynaptic mechanisms and do not hyperpolarize dorsal horn neurons, the excitatory inputs that persist in the presence of these agonists may be sufficient to activate the c-fos gene. Taken together, these results provide new evidence, at the level of a "third messenger," that the antinociception produced by i.t. administration of delta and mu opioid receptor agonists is mediated by different mechanisms.

There is considerable evidence that delta opioid receptor agonists act in the spinal cord to produce antinociception. This evidence includes pharmacological investigations of the antinociceptive effects of i.t. administered delta opioid receptor agonists (Porreca et al., 1984; Malmberg and Yaksh, 1992; Mattia et al., 1992; Stewart and Hammond, 1993), electro-physiological characterization of the effects of these agonists on the response properties of dorsal horn neurons (Dickenson et al., 1987; Hope et al., 1990; Kalso et al., 1992; Duggan and Fleetwood-Walker, 1993) and neurochemical determinations of their effects on the release of neurotransmitters from the spinal cord (Go and Yaksh, 1987; Pohl et al., 1989; Collin et al., 1991; Ueda et al., 1995). Other studies have used immunocytochemical visualization of Fos, the protein product of the immediate-early protooncogene c-fos (Curran et al., 1984; Dragunow and Faull, 1989; Hughes and Dragunow, 1995) to identify populations of neurons that are activated by noxious stimuli (Hunt et al., 1987; Bullitt, 1989; Menetrey et al., 1991).
1998; Herdegen et al., 1991) and to concomitantly examine the ability of opioid receptor agonists to suppress the expression of Fos-LI in the spinal cord (Presley et al., 1990; Hammond et al., 1992; Abbadie et al., 1994; Tolle et al., 1994). However, these studies nearly exclusively examined the effects of morphine, a mu opioid receptor agonist (Takemori and Portoghese, 1987; Corbett et al., 1993). Consequently, the contribution of delta opioid receptors to the regulation of immediate-early gene expression in the spinal cord remains unknown.

Previous studies of the effects of morphine on the expression of Fos-LI in the spinal cord routinely used a systemic route of administration. Because systemically administered morphine distributes to spinal, as well as to peripheral and supraspinal sites of action, the neural mechanism by which opioid receptor agonists suppress Fos-LI in the spinal cord remains unclear. For example, there is considerable evidence that opioid receptor agonists activate bulbospinal pain modulatory pathways that originate in the periaqueductal gray and ventromedial medulla ( Gebhart, 1982; Basbaum and Fields, 1984; Gebhart et al., 1984). Consistent with these data, i.c.v. administration of morphine or of the more selective mu opioid receptor agonist DAMGO produces antinociception and a dose-dependent and naloxone-reversible inhibition of the expression of Fos-LI in the spinal cord (Gogas et al., 1991, 1996a, b). There is also evidence for a contribution of peripheral sites, particularly under conditions of inflammatory nociception. Thus, injection of morphine or other opioid receptor agonists in the hindpaw of the rat attenuates the hyperalgesia produced by i.pl. injection of inflammatory irritants (Joris et al., 1987; Stein, 1993, 1995; Hong and Abbott, 1995). Intraplantar administration of morphine also inhibits the expression of Fos-LI in the spinal cord in a dose-dependent and naloxone-reversible manner (Honore et al., 1996). In contrast, despite the well-established ability of i.t. administered opioid receptor agonists to produce antinociception (Yaksh, 1993), it is not yet known whether this antinociception is associated with a suppression of noxious-stimulus-evoked Fos-LI in the spinal cord.

The present study specifically examined the effects of i.t. administration of two prototypic delta opioid receptor agonists, DPDPE and DELT, on the expression of Fos-LI evoked by injection of formalin in one hindpaw of the rat. The ability of these agonists to inhibit formalin-induced flinching behavior in the same animals was also determined. For comparison, the effects of an equianitociceptive dose of the mu opioid receptor agonist DAMGO were also examined. The results indicate that i.t. administration of DAMGO produces a strong reduction in both formalin-induced flinching behavior and in Fos-LI in the spinal cord. In contrast, neither the delta-1 agonist DPDPE nor the delta-2 agonist DELT produce a strong suppression of Fos-LI in the spinal cord despite a significant reduction in formalin-evoked pain behaviors. These data indicate that inhibition of Fos expression can be dissociated from the antinociceptive effects of different opioid receptor agonists. Furthermore, these results provide new evidence, at the level of a “third messenger,” that the antinociception produced by i.t. administration of delta and mu opioid receptor agonists is mediated by different mechanisms. A preliminary report of this work has appeared (Hammond et al., 1995b).

Methods and Materials

Animals. Male Sprague-Dawley rats (Sasco, Madison, WI; 250–350 g) were anesthetized with halothane. One end of a PE-10 catheter was introduced through a slit in the atlantooccipital membrane and threaded caudally for 8 cm in the subarachnoid space, which positioned the tip of the catheter at the L2 segment of the spinal cord. The other end was tunneled subcutaneously and externalized at the top of the head (Yaksh and Rudy, 1976; Hammond, 1998). The rats were housed individually after surgery and allowed 5 to 6 days to recover before testing.

Formalin test and experimental design. Animals were placed individually in Plexiglas testing chambers and allowed to acclimate for at least 15 min. A mirror was situated behind the chamber and another was situated below the floor of the chamber to allow an unobstructed view of the rat’s paws. The first study examined the effect of i.t. pretreatment with DPDPE, DELT or DAMGO on formalin-induced flinching and the expression of Fos-LI in the spinal cord. Either vehicle, 10 or 60 μg of DPDPE, 3.0 or 30 μg of DELT or 0.3 μg of DAMGO was injected i.t. 10 min before the s.c. injection of 100 μl of 5% formalin in the plantar surface of one hindpaw. These doses and the pretreatment time were based on previous studies of the efficacy of these agonists in the tail-flick, hot-plate and carrageenan-inflamed paw-flick tests (Malmberg and Yaksh, 1992; Stewart and Hammond, 1993, 1994). The rats were returned to the testing chamber and the number of flinches that occurred during the next 60 min was recorded in 5-min epochs. Three to five rats were selected from each group for immunocytochemical analysis based on their behavioral scores approximating the mean of the group.

The second study established the pharmacologic specificity of the effects of DPDPE, DELT and DAMGO on formalin-induced flinching and the expression of Fos-LI in the spinal cord. Rats were injected i.t. with a mixture of either 1.0 μg of BNTX and 60 μg of DPDPE, 3.0 μg of NTB and 30 μg of DELT, or 3.0 μg of CTOP and 0.3 μg of DAMGO. These doses of BNTX, NTB and CTOP antagonize the antinociceptive effects of DPDPE, DELT and DAMGO at delta-1, delta-2 and mu opioid receptors, respectively, in the rat (Stewart and Hammond, 1993, 1994; Hammond et al., 1995a; Tseng et al., 1995). Ten minutes after the latter 100 μl of 5% formalin was injected s.c. in the plantar surface of one hindpaw. The rats were returned to the testing chamber and the number of flinches that occurred during the next 60 min was recorded in 5-min epochs. Three to five rats were selected from each group for immunocytochemical analysis based on their behavioral scores approximating the mean of the group. CTOP and NTB were coadministered with their respective agonists so that the effects of the agonist and antagonist would coincide (Malmberg and Yaksh, 1992; Guirmand et al., 1994). Although BNTX is more effective when administered shortly before DPDPE, it was coadministered in this study to ensure its action throughout the observation period (Hammond et al., 1995a).

The third study examined the effect of post-treatment with the highest effective dose of DPDPE or DELT. These rats received a s.c. injection of 100 μl of 5% formalin in the plantar surface of the left hindpaw. After measurement of the first phase response to formalin (i.e., 0–5 min), the rats were injected i.t. with either vehicle, 60 μg of DPDPE or 30 μg of DELT, and returned to the testing chamber. The number of flinches that occurred during the subsequent 50 min was recorded in 5-min epochs. Immunocytochemical analysis was not conducted on these animals.

A final study evaluated whether injection of the agonists, by themselves, induced an expression of Fos-LI in the absence of formalin. This study controlled for possible nonspecific stimulatory effects of the agonists that could mask a suppression of Fos-LI. For this study, the rats were injected i.t. with either 60 μg of DPDPE (n = 2) or 30 μg DELT (n = 3) and returned to the testing chamber for 60 min. Tissue from these rats was processed for immunocytochemical visualization of Fos-LI.
**Immunocytochemistry.** Sixty minutes after the injection of formalin, the rats were deeply anesthetized with 60 mg/kg i.p. pentobarbital and perfused intracardially with 50 ml of 0.05 M PBS, pH 7.4 at 37°C followed by 500 ml of 4% formalin in 0.1 M phosphate buffer pH 7.4 at 4°C. The spinal cord was postfixed in *situ* for 90 min, removed from the vertebral canal and placed in fresh fixative at 4°C for an additional 90 min. The tissue was then cryoprotected in phosphate-buffered 30% sucrose buffer for at least 48 hr.

Fos-LI was visualized by ABC/glucose oxidase immunocytochemistry with use of commercially available kits (*Elite* Vectastain; Vector Laboratories, Burlingame, CA). The tissue was coded so that the person performing the immunocytochemistry had no knowledge of the treatment condition. Fifty-micron frozen serial sections were cut through the lumbar enlargement of the spinal cord and collected in 0.05 M PBS. After immersion in PBS containing 3% normal goat serum and 0.3% Triton X-100 for 1 hr, the sections were incubated for 48 hr at 4°C in a rabbit polyclonal antiserum directed against an *in vitro* translated protein product of the c-fos gene (courtesy of Dr. Dennis Slamon, Department of Hematology and Oncology, UCLA) at a dilution of 1:20,000 in PBS containing 1% normal goat serum and 0.3% Triton X-100. This antiserum does not recognize the Fos-related antigens and had been preabsorbed with acetone-dried rat liver powder for 1 hr at 37°C and 2 hr at 4°C to reduce background staining. After incubation in the primary antibody, the tissue was transferred to a goat anti-rabbit biotinylated secondary IgG complex for 1 hr at room temperature and then exposed to the ABC *Elite* complex for 1 hr at room temperature. Tissue sections were thoroughly rinsed with Tris buffer, mounted from tap water onto gelatin-coated slides, air dried, dehydrated in alcohol in a graded manner, cleared in xylenes and coverslipped.

**Quantitation of Fos-LI.** Four sections from the L4 or L5 segments of the spinal cord of each rat were randomly selected for quantification of Fos-LI. The sections were photographed at low power (4×) using Kodak technical pan film and a Nikon Microphot-FXA microscope. The film was developed with HC110 Dilution E developer, stopped with Kodak stop bath and fixed with Kodak rapid fixative. The individual sections were printed at 60× enlargement and overlaid with an acetate sheet on which the distribution of Fos-LI neurons was then plotted by a person with no knowledge of the treatment condition. For quantitation, we divided the spinal cord into four regions of interest: (1) the superficial laminae (laminae I, IIo and III); (2) the nucleus proprius (laminae III and IV); (3) the neck of the dorsal horn (laminae V and VI); and (4) the ventral horn (laminae VII–X). The number of Fos-LI neurons in each region was determined by averaging the counts made in the four sections for each rat. The number of Fos-LI neurons in a treatment group was then expressed as the mean ± S.E.M. of these values.

**Statistical analysis.** The number of flinches was expressed as the mean ± S.E.M. Phase 1 was defined as the 5-min period immediately after the injection of formalin. Phase 2 was defined as the period 20 to 60 min after the injection of formalin.

**Drugs and injections.** DPDPE (lot no. 13HS58451), DELT (lot no. 63H06631) and DAMGO (lot no. 121HS58152) were purchased from Sigma Chemical Co. (St. Louis, MO). BNTX hydrochloride (lot no. WY-III-69B) was obtained courtesy of Research Biochemicals (Natick, MA) and the NIDA Technology Branch. CTOP (lot no. FRY-297A) was purchased from Research Biochemicals (Natick, MA). NTB was a gift from G.D. Searle (lot no. XXI-146.3; Skokie, IL). DPDPE, DAMGO and BNTX were dissolved in saline, which served as their respective vehicle control. DELT and NTB were dissolved in 5% Molecusol (hydroxypropyl-β-cyclodextrin; Pharmatec; Alachua, FL), which served as their respective vehicle control. Drug solutions were made fresh and injected i.t. in a volume of 10 μl followed by a 10-μl volume of saline. The location of the catheter was verified by direct visualization of the tip of the catheter after laminectomy.

**Results**

**Effects of intrathecally administered DPDPE on formalin-induced flinching and expression of Fos-LI in the spinal cord.** Intrathecal pretreatment with DPDPE dose-dependently decreased the number of flinches in both phase 1 and phase 2 (fig. 1A). Intrathecal administration of 60 μg of DPDPE inhibited formalin-induced flinching behavior to a greater extent and for a longer duration than did 10 μg of DPDPE. The inhibition of flinching produced by 60 μg of DPDPE was attenuated by coadministration of 1.0 μg of BNTX (fig. 1A). In these rats, the number of flinches did not differ from the number of flinches observed in rats pretreated with 10 μg of DPDPE. The antinociceptive effect of 60 μg of DPDPE was completely antagonized by coadministration of 10 μg of BNTX (data not shown). However, because this dose of BNTX has additional effects at delta-2 and mu receptors (Hammond et al., 1995a) it was not suitable for further study.

Intrathecal post-treatment with 60 μg of DPDPE, administered 7 min after the injection of formalin, also significantly inhibited flinching behavior in phase 2 (fig. 1B). The magnitude of the inhibition was similar to the magnitude of inhibition observed in rats in which this same dose of DPDPE was administered 10 min before the injection of formalin (P > .6 for the 15- to 60-min period).

Figure 2, A to C, illustrates the distribution and number of formalin-evoked Fos-LI neurons in the spinal cords of rats pretreated with saline, 60 μg of DPDPE or a mixture of 60 μg of DPDPE and 1.0 μg of BNTX. As reported previously (Presley et al., 1990; Gogas et al., 1991), s.c. injection of formalin induced the expression of large numbers of Fos-LI neurons in the medial aspects of ipsilateral laminae I, IIo and III and laminae V–VI, with fewer numbers present in laminae III–IV and the ventral horn of saline-pretreated rats (figs. 2A and 3A). This concentration of formalin also induced an expression of Fos-LI in the contralateral spinal cord, but the numbers of Fos-LI neurons were significantly less than in the ipsilateral spinal cord in each region (data not shown).

In rats pretreated with 10 μg of DPDPE, the distribution and number of Fos-LI neurons did not differ from that of saline-pretreated rats (data not shown). Intrathecal administration of 60 μg of DPDPE produced a modest decrease in the number of Fos-LI neurons in the ipsilateral laminae I, IIo and III, as well as in laminae V–VI and the ventral horn, but did not decrease the number of Fos-LI neurons in laminae III–IV (figs. 2B and 3A). The number of Fos-LI neurons was reduced to 80.1 ± 7.6% of control in laminae I, IIo and III, to 70.4 ± 12.3% of control in laminae V–VI and to 62.6 ± 9.0% of control in the ventral horn. Thus, 60 μg of DPDPE suppressed Fos-LI to a similar extent in each of these three regions (P > .4). This dose of DPDPE also significantly decreased the number of Fos-LI neurons in the contralateral laminae V–VI and ventral horn (data not shown). Coadministration of 1.0 μg of BNTX completely prevented the decrease in numbers of Fos-LI neurons produced by 60 μg of DPDPE (figs. 2C and 3A).

**Effects of intrathecally administered DELT on formalin-induced flinching and expression of Fos-LI in
Intrathecal post-treatment with 30 μg of DELT, administered 7 min after formalin, also inhibited formalin-induced flinching behavior in phase 2 (fig. 4B). However, the inhibition was modest and was much less than that observed when this dose was administered 10 min before formalin (compare fig. 4, A and B; P < .01 for the 15- to 60-min period). The magnitude of this inhibition was equivalent to the magnitude of inhibition produced by pretreatment with 3.0 μg of DELT, a 10-fold lower dose (P = .6).

Figure 5, A to C, illustrates the distribution of Fos-LI neurons in the spinal cord of rats pretreated with either Molecusol, 30 μg of DELT or a mixture of 30 μg of DELT and 3.0 μg of NTB. The distribution of Fos-LI immunoreactive neurons in the spinal cord of Molecusol-pretreated rats was similar to that observed in saline-pretreated rats, although the numbers of neurons in laminae I, Ilo and III and in laminae III–IV tended to be greater in Molecusol-pretreated rats (fig. 3, A and B). Neither 3.0 μg nor 30 μg of i.t. administered DELT significantly decreased the number of Fos-LI neurons in any of the four regions of the spinal cord as compared with values in Molecusol-pretreated rats (figs. 3B and 5B). Coadministration of 3.0 μg of NTB with 30 μg of DELT was without effect.

Effects of intrathecally administered DAMGO on formalin-induced flinching and expression of Fos-LI in the spinal cord. Intrathecal pretreatment with 0.3 μg of DAMGO significantly decreased the number of flinches in both phase 1 and phase 2 (fig. 6). This dose of DAMGO inhibited flinching to the same extent as did either 30 μg of DELT or 60 μg of DPDPE in the 45 min after injection of formalin (P > .3). The inhibition of flinching produced by 0.3 μg of DAMGO was completely antagonized by coadministration of 3.0 μg of CTOP (fig. 6).

Unlike either DELT or DPDPE, i.t. pretreatment with DAMGO produced a very profound suppression of Fos-LI in each of the four regions of the spinal cord (figs. 3C and 7B). The numbers of Fos-LI neurons were reduced to 32.7 ± 7.2% of control in laminae I, Ilo and II, to 44.2 ± 9.3% of control in laminae III and IV, to 22.0 ± 7.0% of control in laminae V–VI and to 19.6 ± 5.3% of control in laminae VII–X ipsilateral to the injection site. These values did not differ from one another (P > .1), which indicates that this dose of DAMGO produced an equivalent suppression of Fos-LI in each region of the spinal cord (P > .1). DAMGO also reduced the numbers of Fos-LI neurons in each region of the contralateral spinal cord to a similar extent (data not shown). Coadministration of 3.0 μg of CTOP completely prevented the decrease in number of Fos-LI neurons produced by 3.0 μg of DAMGO in all laminae, with the exception of laminae VII–X in which the antagonism was only partial (figs. 3C and 7).

Effects of intrathecally administered DELT or DPDPE on the expression of Fos-LI in the spinal cord in the absence of formalin. Low numbers of Fos-LI neurons were observed in the spinal cord of rats treated with 60 μg of DPDPE or 30 μg of DELT in the absence of formalin. Numbers of Fos-LI neurons ranged between 13 and 30/region and were uniformly distributed among the four regions of interest on both sides of the spinal cord (figs. 2D and 5D). Also, the intensity of staining of Fos-LI neurons was much lighter than in rats in which formalin had been injected. The pial surface of the spinal cord often exhibited large numbers of densely stained Fos-LI cells.

**Fig. 1.** Panel A depicts the effects of i.t. pretreatment with saline (○; n = 6), 10 μg DPDPE (●; n = 7), 60 μg DPDPE (■; n = 7) or a combination of 60 μg DPDPE and 1.0 μg BNTX (□; n = 5) 10 min before the s.c. injection of 5% formalin into the plantar surface of one hindpaw of the rat. Panel B depicts the effects of i.t. post-treatment with saline (○; n = 5) or 60 μg of DPDPE (■; n = 5) 7 min after the injection of formalin. The arrowhead in panel B indicates the time at which the DPDPE or saline was administered. Each symbol represents the mean ± S.E.M. The arrows indicate the time at which formalin was injected. Values that differ from one another (P < .05) or daggers (P < .01).

**Fig. 4A.** Moreover, beginning 40 min after the injection of formalin, the number of flinches in these rats significantly exceeded those in Molecusol-pretreated rats.
Discussion

Antinociceptive effects of delta opioid receptor agonists in the formalin test. One of the principal findings of this study was that i.t. administered delta opioid receptor agonists produce antinociception in the formalin test in the rat. Previous investigations of the effects of i.t. administered opioid receptor agonists in this model of persistent, inflammatory nociception focused on mu or kappa opioid receptor agonists (Pelissier et al., 1990; Yamamoto and Yaksh, 1992; Malmberg and Yaksh, 1993; Fujibayashi and Iizuka, 1995). The only study that examined delta opioid receptor agonists concluded that they were without effect in the mouse at doses that did not produce adverse motor effects (Murray and Cowan, 1991). The present study is therefore the first to identify a contribution of spinal delta opioid receptors in the modulation of nociceptive behaviors in the formalin test. Specifically, it determined that i.t. pretreatment with the delta-1 opioid receptor agonist DPDPE or the delta-2 opioid receptor agonist DELT dose-dependently suppressed flinching behavior in both phase 1 and phase 2 of the formalin test at doses that did not adversely affect motor function. Moreover, this antinociception was attenuated by the delta-1 opioid receptor antagonist BNTX and the delta-2 opioid receptor antagonist NTB, respectively. Finally, each delta opioid receptor agonist was also effective when administered after the injection of formalin. These results extend earlier reports of the antinociceptive efficacy of i.t. administered delta opioid receptor agonists in the carrageenan-inflamed paw-flinch test (Hylden et al., 1991; Stanfa et al., 1992; Stewart and Hammond, 1994) to a second model of persistent, inflammatory nociception. They also complement previous investigations of the antinociceptive effects of these two agonists in models of acute nociception, such as the tail-flinch and hot-plate tests, in the rat (Malmberg and Yaksh, 1992; Stewart and Hammond, 1993; Hammond et al., 1995a).

Additional evidence of a modulation of formalin-induced pain behaviors by delta opioid receptors is provided by the report that s.c. administration of the nonselective delta opio-
oid receptor antagonist naltrindole increased the number of flinches elicited in phase 2 by i.pl. injection of a submaximal concentration of formalin (Ossipov et al., 1996). These data suggested that formalin evokes a release of enkephalins that act at delta opioid receptors in the spinal cord. Our observation that the number of flinches in rats that received NTB and DELT exceeded those in the vehicle control group in the last 20 min may reflect additional antagonism of the effects of endogenously released enkephalins.

**Lack of effect of delta opioid receptor agonists on the expression of Fos-LI in the spinal cord.** Another principal finding of this study was that i.t. pretreatment with the delta-1 opioid receptor agonist DPDPE only marginally decreased formalin-induced expression of Fos-LI in the spinal cord and that the delta-2 opioid receptor agonist DELT was without significant effect. Yet, each agonist produced a robust decrease in flinching behavior in the formalin test that was dose-dependent and was attenuated by coadministration of the appropriate antagonist. The relative lack of effect of the delta opioid receptor agonists is in stark contrast to the profound reduction in the number of Fos-LI neurons in the spinal cord of rats treated with an equianalgesic dose of the mu opioid receptor agonist DAMGO. The lack of inhibition cannot be attributed to a nonspecific stimulatory effect. 

![Figure 3](image-url) **Fig. 3.** Effects of i.t. pretreatment with (A) DPDPE, (B) DELT or (C) DAMGO alone or in the presence of its respective antagonist on the number of Fos-LI neurons evoked by the s.c. injection of 5% formalin in one hindpaw of the rat. Data are the mean ± S.E.M. number of Fos-LI neurons in the different laminar regions on the side of the spinal cord ipsilateral to the site of formalin injection. The number of animals in each treatment group ranges from 3 to 5. Values that are significantly different from the vehicle control are indicated by * (P < .05) and ** (P < .01).

![Figure 4](image-url) **Fig. 4.** Panel A depicts the effects of i.t. pretreatment with 5% Molecusol (○; n = 5), 3.0 μg of DELT (●; n = 8), 30 μg of DELT (■; n = 6), or a combination of 30 μg DELT and 3.0 μg NTB (▲; n = 6) 10 min before the s.c. injection of 5% formalin into the plantar surface of one hindpaw of the rat. Panel B depicts the effects of i.t. post-treatment with 5% Molecusol (○; n = 5) or 30 μg of DELT (■; n = 6) 7 min after the injection of formalin. The arrowhead in panel B depicts the time at which DELT or Molecusol was administered. Each symbol represents the mean ± S.E.M. The arrows indicate the time at which formalin was injected. Values that differ from the corresponding vehicle control are indicated by asterisks (P < .05) or daggers (P < .01).
effect of these agonists by themselves because neither DP-DPE nor DELT increased the expression of Fos-LI in the spinal cord in the absence of formalin.

The present study examined the effects of the opioid receptor agonists on Fos-LI 1 hr after the injection of formalin. This time was selected because it was desirable to assess the effects of the opioid agonists during their time of peak effect. Also, the sensitivity of the method for immunocytochemical detection of Fos-LI has increased compared with previous studies that required a 2-hr interval for optimal detection of Fos-LI (Presley et al., 1990). Although the relative timing of drug administration, application of the noxious stimulus and perfusion of the animal can be important variables in studies of the effects of drugs on Fos-LI (Tolle et al., 1994; Honoré et al., 1995), the 1-hr interval used in this study is unlikely to significantly influence its findings for several reasons. First, the inhibitory effect of i.t. administered DAMGO on spinal Fos-LI was readily observed in this study (see below). Second, the suppression of formalin-evoked Fos-LI in the spinal cord by i.c.v. administration of DAMGO, morphine or the kappa opioid receptor agonist CI-977 was also evident at this interval (Gogas et al., 1991, 1996a, b). Finally, Fos protein detected immunocytochemically at 1 hr would reflect events of the first 30 min after formalin injection that influence the transcription and translation of c-fos. Because DP-DPE and DELT significantly decreased flinching behavior to an extent similar to DAMGO throughout the first 30 min, a decrease in Fos-LI should have been readily detectable under these conditions.

**Effect of a mu opioid receptor agonist on the expression of Fos-LI in the spinal cord.** The finding that i.t. administered DAMGO reduced both formalin-induced flinching behavior and Fos-LI in the spinal cord establishes the sensitivity of this study and complements previous reports that the inhibition of formalin-induced pain behaviors by systemic or i.c.v. administration of morphine or DAMGO is accompanied by a decrease in formalin-evoked Fos-LI in the spinal cord (Presley et al., 1990; Gogas et al., 1991, 1996a). It also suggests that the suppression of noxious-stimulus-evoked Fos-LI in the spinal cord by systemically administered mu opioid receptor agonists is likely to result from a coincident activation of inhibitory bulbospinal pathways, as well as a direct action of the opioids on local circuits in the spinal cord. Intrathecal administration of DAMGO suppressed formalin-induced Fos-LI in laminae I, IIo and III to the same extent as in laminae V–VI and VII–X. By comparison, systemic (Presley et al., 1990; Tolle et al., 1994) or i.c.v. (Gogas et al., 1991, 1996a) administration of mu opioid re-
ceptor agonists consistently suppressed Fos-LI in laminae V–VI and VII–X to a greater extent than in the superficial laminae. The ability of i.t. administered DAMGO (and of DPDPE) to produce an equivalent suppression of Fos-LI in the superficial as in the deeper laminae is likely to reflect the “topical” nature of its application in the spinal cord.

Possible bases for the discrepant effects of delta and mu opioid receptor agonists on formalin-induced Fos-LI. Despite producing an equivalent antinociception in the formalin test, DPDPE and DELT differed markedly from DAMGO in their effects on the expression of Fos-LI in the spinal cord. The most parsimonious explanation for the inability of antinociceptive doses of either DPDPE or DELT to significantly decrease formalin-induced Fos-LI in the spinal cord is that c-fos is not regulated by delta opioid receptors. Whether delta opioid receptor agonists regulate the transcription and translation of other immediate early genes such as jun or krox, as for morphine (Tölle et al., 1994), remains to be determined. Alternatively, differences in the effects of DPDPE, DELT and DAMGO on Fos-LI may reflect the different mechanisms by which these agonists modulate synaptic transmission in the spinal cord. For example, it is well established that mu receptors comprise the largest percentage (70–80%) of opioid receptors in the spinal cord; the percentage of delta receptors is much smaller (10–15%) (Besse et al., 1991; Stevens et al., 1991). Autoradiographic studies (Besse et al., 1990; Stevens and Seybold, 1995) and immunocytochemical visualization of the mu (Arvidsson et al., 1995; Ding et al., 1996) and delta (Dado et al., 1993; Arvidsson et al., 1995; Cheng et al., 1995) opioid receptors indicate that both receptors are located postsynaptically on dorsal horn neurons, as well as presynaptically on the terminals of primary afferent neurons. However, the results of intracellular or whole-cell recordings from dorsal horn neurons in the superficial laminae of slices of rat spinal cord suggest that mu and delta opioid receptor agonists modulate synaptic transmission by different mechanisms. In these latter studies, mu opioid receptor agonists inhibited both spontaneous and evoked EPSP/Cs at low concentrations and, at slightly higher concentrations, also hyperpolarized a portion of the dorsal horn neurons (Murase et al., 1982; Jeftinija, 1988; Glaum et al., 1994; Grudt and Williams, 1994). Bath application of DPDPE or DELT similarly inhibited evoked EPSP/Cs (Glaum et al., 1994). However, even high concentrations of these agonists did not appreciably alter resting membrane potential (Jeftinija, 1988; Glaum et al., 1994). Taken together, these observations suggest that delta opioid receptor agonists produce antinociception by a predominantly presynaptic mechanism, i.e., inhibition of neurotransmitter release, and that they are unlikely to effectively hyperpolarize and reduce the excitability of dorsal horn neurons. Conceivably, any excitatory synaptic input that persists in the presence of the delta opioid receptor agonists is sufficient to alter the disposition of intracellular calcium and activate c-fos. In contrast, DAMGO is likely to not only presynaptically inhibit neurotransmitter release, but to also hyperpolarize and reduce the excitability of dorsal horn neurons. In the added presence of this hyperpolarization, the synaptic input that persists may be unable to depolarize the neuron to a sufficient extent to increase intracellular calcium and activate c-fos. Finally, the polysynaptic nature of the pathways that transmit nociceptive information provides several loci for postsynaptic inhibition by mu opioid receptors.

Fig. 6. Effects of i.t. pretreatment with saline (○; n = 6), 0.3 μg of DAMGO (●; n = 6) or the combination of 0.3 μg of DAMGO and 3.0 μg of CTOP (■; n = 6) 10 min before the s.c. injection of 5% formalin into the plantar surface of one hindpaw of the rat. Each symbol represents the mean ± S.E.M. Values that differ from the corresponding vehicle control are indicated by asterisks (P < .05) or daggers (P < .01).

Fig. 7. Photomicrographs of transverse sections of the ipsilateral lumbar spinal cord illustrating the distribution of formalin-evoked Fos-LI neurons in rats pretreated i.t. with either (A) saline, (B) 0.3 μg of DAMGO or (C) a mixture 0.3 μg of DAMGO and 3.0 μg of CTOP 10 min before the s.c. injection of 5% formalin in one hindpaw. Scale bar, 400 μm.
agonists and could serve to “amplify” the inhibitory effects of drugs such as DAMGO and morphine on the expression of Fos-LI.

**Fos-LI as a measure of antinociception.** There is strong evidence that the expression of Fos-LI in the spinal cord is a function of the intensity and duration of the noxious stimulus (Hunt et al., 1987; Williams et al., 1988; Abbade et al., 1994). Furthermore, many studies have determined that opioid receptor agonists suppress Fos-LI in the spinal cord in a dose-dependent and naloxone-reversible manner (Presley et al., 1990; Hammond et al., 1992; Abbade et al., 1994) and that there can be an excellent correlation between the magnitude of antinociception and the extent to which Fos-LI is suppressed (Gogas et al., 1991; Hammond et al., 1992). However, closer examination of the literature reveals important disparities. For example, complete behavioral antinociception can be produced without complete suppression of Fos-LI in the spinal cord. Similarly, moderate behavioral antinociception can be produced in the absence of significant decreases in Fos-LI in the superficial laminae (Presley et al., 1990; Gogas et al., 1991, 1996a; Jasmin et al., 1994). Conversely, a decrease in the number of Fos-LI neurons in the superficial laminae has been observed in the absence of behavioral antinociception (Kehl et al., 1991; Gogas et al., 1996a, b). Thus, although there is strong evidence that the expression of Fos-LI in the spinal cord is an appropriate measure of nociception, it is not as clear that the suppression of Fos-LI in the spinal cord, and particularly in the superficial laminae, is an equally good measure of antinociception. The present finding with the delta opioid receptor agonist is perhaps the most extreme example to date of the disparity that can exist between behavioral antinociception and the expression of Fos-LI in the spinal cord.

In conclusion, i. pretreatment with agonists of the delta-1 and delta-2 opioid receptor produced a dose-dependent and reversible antinociception in phase 1 and phase 2 of the formalin test. However, neither DPDP nor DELT produced a robust decrease in formalin-induced Fos-LI in the spinal cord. This finding contrasts with the ability of an equianitrogenic dose of DAMGO, a mu opioid receptor agonist, to inhibit Fos-LI in the spinal cord. The efficacy of i. administered DAMGO suggests that a direct spinal action contributes to the inhibition of noxious-stimulus-evoked Fos-LI in the spinal cord produced by systemic administration of mu opioid receptor agonists, such as morphine. The relative lack of effect of DPDP or DELT suggests that delta opioid receptor agonists do not regulate the immediate early gene c-fos. Alternatively, differences in the mechanisms by which delta and mu opioid receptor agonists modulate synaptic transmission of nociceptive information in the spinal cord may underlie the disparate effects of equally antinociceptive doses of DPDP, DELT, and DAMGO on the expression of formalin-evoked Fos-LI in the spinal cord. Collectively, these data provide new evidence, at the “third messenger” level, that the mechanisms by which i. administered delta and mu opioid receptor agonists modulate nociception differ.

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**References**


Honore P, Buritova J and Besson J-M (1996) Intraplantar morphine depresses spinal...


