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Endomorphins suppress nociception-induced c-Fos and Zif/268 expression in the rat spinal dorsal horn

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Abstract

We evaluated the potency of endomorphin-1 and -2 as endogenous ligands on c-Fos and Zif/268 expression in the spinal dorsal horn by formalin injection to the rat hind paw. Endomorphin-1, -2, or morphine was administered intrathecally or intracerebroventricularly 5 min before formalin injection (5%, 100 μl). All drugs produced marked reductions of formalin-induced c-Fos and Zif/268 immunoreactivity in laminae I and II, and laminae V and VI in the rat lumbar spinal cord. The reductions of Zif/268 expression by endomorphins were greater than those by morphine, while the reductions of c-Fos expression by endomorphins were smaller than those by morphine. These effects of endomorphins were attenuated by pretreatment with naloxone. These results indicate that endomorphin-1 and -2 act as endogenous ligands of μ-opioid receptor in neurons of the spinal dorsal horn and suppress the processing of nociceptive information in the central nervous system.

Keywords: Endomorphin-1; Endomorphin-2; c-Fos; Zif/268; Formalin; Spinal dorsal horn

1. Introduction

Recently, two endogenous peptides, endomorphin-1 (EM-1) and endomorphin-2 (EM-2) were isolated from bovine (Zadina et al., 1997) and human (Hackler et al., 1997) brains. Because these two peptides have a high affinity and selectivity for μ-opioid receptor, they are considered endogenous ligands of this receptor (Zadina et al., 1997). Opioids and their receptors have important roles in the processing of nociceptive information in the central nervous system (CNS) (Basbaum and Fields, 1984; Fields and Basbaum, 1999). Mu opioid receptor is clinically important because of its high affinity for morphine, a potent and widely used alkaloid analgesic. The role of endomorphins as endogenous μ-opioid receptor ligands has been confirmed by examination of their antinociceptive effects on pain-related behaviors; it has been shown that intrathecal (i.t.) or intracerebroventricular (i.c.v.) administration of endomorphins ameliorates acute pain induced by noxious thermal or mechanical stimulation (Stone et al., 1997; Horvath et al., 1999; Przewlocka et al., 1999; Sakurada et al., 1999; Tseng et al., 2000; Ohsawa et al., 2000), inflammatory pain following injection of formalin (Hao et al., 1999; Przewlocka et al., 1999; Soignier et al., 2000) and neuropathic pain (Stone et al., 1997; Przewlocka et al., 1999). These findings suggest that endomorphins may be involved in the processing of nociceptive stimuli. However, their effects on pain-related behaviors differ with i.t. and i.c.v. administration (Zadina et al., 1997; Sakurada et al., 1999).

The involvement of μ-opioid receptor in neuronal processing of nociceptive stimuli has been studied exclusively with the use of morphine. Neurophysiologic studies have demonstrated that discharges of dorsal horn neurons evoked by noxious stimulation are inhibited by the administration of morphine (Yaksh, 1981). In addition, c-Fos expression induced by noxious stimulation is also remarkably reduced by treatment with morphine (Presley et al., 1990; Gogas et al., 1996). c-Fos is a transcription factor encoded by c-*fos* gene, one of the immediate-early genes. The expression of

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c-Fos protein has been used as a general anatomic marker for confirming the activity of nociceptive neurons, since it is induced in the nuclei of dorsal horn neurons in response to noxious stimulation, and since its expression can be evaluated semi-quantitatively by counting the number of positive neurons (Hunt et al., 1987; Harris, 1998). These effects of morphine suggest that μ-opioid receptor is involved in processing neuronal responses in the dorsal horn following noxious stimulation. However, the role of endomorphins in nociceptive processing in these neurons has not been examined extensively.

Recent studies have demonstrated that i.t. administration of EM-1 and EM-2 reduces electrical evoked C-fiber responses in a dose-dependent manner (Chapman et al., 1997). In addition, intraplantar injection of EM-1 produces a dose-dependent reduction of carrageenan-evoked c-Fos expression in the spinal dorsal horn and the effect is attenuated by administration of naloxone (Jin et al., 1999), indicating that EM-1 functions as an endogenous ligand of μ-opioid receptor to reduce c-Fos expression. Hence, it is presumed that EM-2, as well as EM-1, may have a role as an endogenous ligand of μ-opioid receptor in the reduction of c-Fos expression. It is also possible that administration of endomorphins in the CNS as well as peripheral tissue produces considerable effects on noxious stimulus-evoked c-Fos expression, since μ-opioid receptor is distributed densely in both (Basbaum and Fields, 1984). Although c-Fos protein expression has been hitherto used as a representative neuronal marker of gene expression induced by noxious stimulation, it is also evident that such stimulation induces the expression of other members of the immediate-early gene family, including *zif/268* gene (Herdegen et al., 1991; Lanteri-Minet et al., 1993; Buritova et al., 1995). *zif/268* gene encodes Zif/268, also known as NGFI-A, KROX-24, TIS8, and EGR1, and is another transcription factor with zinc fingers (Milbrandt, 1987; Lim et al., 1987; Christy et al., 1988; Sukhatme et al., 1988; Lemaire et al., 1988). To our knowledge, there is little information on the involvement of μ-opioid receptor in the induction of these other immediate-early genes by noxious stimulation (Tölle et al., 1994a,b). Furthermore, the roles of EM-1 and EM-2 as endogenous ligands of μ-opioid receptor have not been examined at the level of the neuron in the CNS.

In the present study, the effects of EM-1 and EM-2 on c-Fos and Zif/268 expression induced by formalin injection into the rat hind paw were examined in the spinal dorsal horn after i.t. or i.c.v. administration, and were compared with the effect of morphine.

2. Materials and methods

2.1. Experimental design

The experimental protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Miyazaki Medical College and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Male Sprague–Dawley rats (300–350 g, Kyudo, Japan) were implanted with a catheter after a 1-week acclimation period at the Experimental Animal Center of Miyazaki Medical College and maintained under a 12/12-h light/dark cycle, with food and water freely available. A catheter was implanted into two different sites: intrathecal or intracerebroventricular sites. I.t. catheterization was performed as described previously by Yaksh and Rudy (1976). Briefly, a polyethylene catheter (PE-10, Clay Adams, Sparks, MD) was stretched in a hot water bath (72 °C) to reduce its diameter. Under anesthesia by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg; Sankyo, Tokyo, Japan) and sodium pentobarbital (25 mg/kg; Abbot Laboratories, Abbot Park, IL), an 8-cm length from the elongated part of the catheter was threaded caudally into the subarachnoid space through a horizontal slit in the atlanto-occipital membrane. The rostral part was sutured to the neck muscle to immobilize the catheter, and the wound was closed in two layers with 3-0 silk. At the end of the experiment, the placement of the i.t. catheter was checked in the vicinity of the lumbar enlargement. In contrast, i.c.v. catheterization was performed according to the method of De Balbian Verster et al. (1971). A 22-gauge stainless steel guide tube (spinal needle introducer 1/4, Becton Dickinson, Franklin Lakes, NJ) was cut to a length of 5 mm. The guide tube was stereotaxically implanted into the lateral cerebral ventricle under anesthesia with ketamine hydrochloride (100 mg/kg, i.p.) and sodium pentobarbital (25 mg/kg, i.p.) and attached to the skull with three stainless steel screws and cranioplastic cement. The coordinates were: 1 mm left lateral to the sagittal suture and 1 mm caudal to the coronal suture (Paxinos and Watson, 1986). A 27-gauge needle (Whitacre needle 1/2, Becton Dickinson) was used for drug injection. At the end of the experiment, a solution of cresyl violet dye was injected to verify the injection site. Rats showing neurologic deficits during the 2-week recovery period after catheterization were excluded from the study.

EM-1 ($n = 4 \times 3$ doses $\times 2$ routes), EM-2 ($n = 4 \times 3 \times 2$), morphine hydrochloride ($n = 4 \times 3 \times 2$), or saline ($n = 4 \times 2$ routes) was administered i.t. and i.c.v. through the catheter under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). EM-1 and EM-2 synthesized by Peptide Institute (Osaka, Japan) were dissolved in distilled water and diluted to 10^{-4}, 10^{-3}, or 10^{-2} M with normal saline solution. In preliminary studies, the lowest concentration of endomorphins that had significant effects on formalin injection-evoked c-Fos expression was 10^{-4} M. Morphine hydrochloride (Takeda, Osaka, Japan) and naloxone hydrochloride (Sankyo) were dissolved in saline. A volume of 10 μl was injected slowly (within 2–3 min) through the i.t. catheter, followed by 10 μl of saline to flush, and 1 μl was delivered

through the i.c.v. catheter over a period of 30 s. Rats that received saline were used as controls. Naloxone hydrochloride (10 mg/kg, $n = 4 \times 2$ drugs $\times 2$ routes) was injected intraperitoneally 5 min before administration of either endomorphin (10^{-2} M).

Noxious stimulation was elicited by injecting 100 μl of a 5% formalin solution subcutaneously into the plantar surface of the hind paw using a 30-gauge needle, 5 min after i.t. or i.c.v. administration of each drug.

2.2. Immunohistochemistry of c-Fos and Zif/268

Two hours after formalin injection, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.)

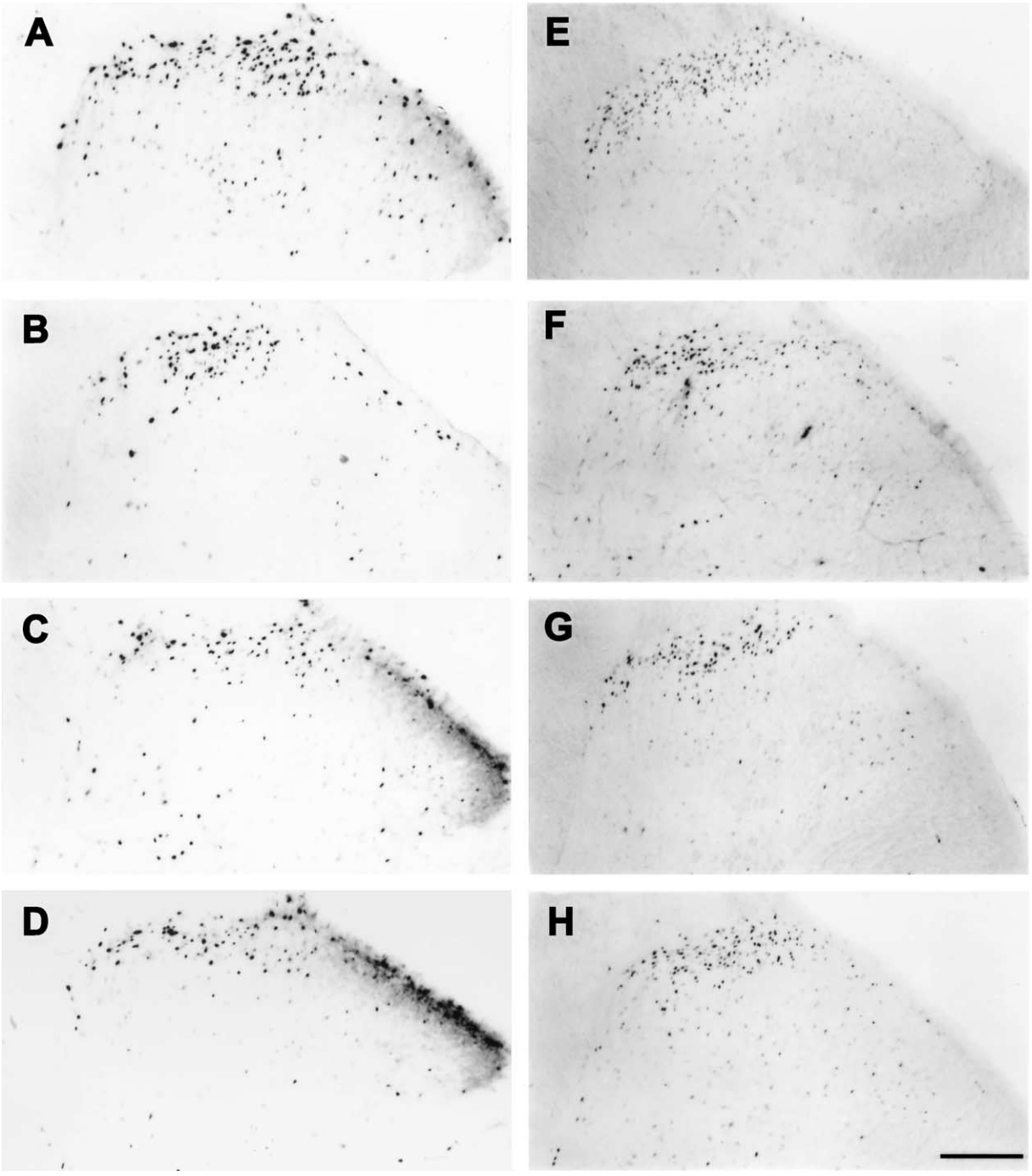

Fig. 1. Photomicrographs showing c-Fos (left side) and Zif/268 (right side) immunoreactivities in the dorsal horn of the lumbar spinal cord in rats that received an injection of 5% formalin solution into the right hind paw 5 min after intrathecal administration of saline (A and E), 10^{-2} M of EM-1 (B and F), 10^{-2} M of EM-2 (C and G), and 10^{-2} M of morphine (D and H). Scale bar = 200 μm.

and perfused intracardially with 200 ml of saline followed by 500 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min. The spinal cord at the level of the lumbar enlargement was removed, postfixed for 1 h in the same fixative, and cryoprotected in 10% sucrose in PB for 1 h and 30% sucrose in PB overnight. Frozen coronal serial sections, 50 μm thick, were cut on a freezing microtome, collected in phosphate-buffered saline (PBS; pH 7.4), and processed as free-floating sections for immunohistochemical staining for c-Fos and Zif/268 proteins. All sections were incubated in 0.1% H_2O_2 for 10 min and 0.5% Triton X-100 for 30 s, and washed with PBS. After incubation in 10% normal goat serum (Nichirei SAB kit, Tokyo, Japan) for 20 min, alternate sections were reacted with a polyclonal rabbit anti-c-Fos antibody (1:7500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Zif/268 antibody (Egr-1, 1:100,000 dilution, Santa Cruz Biotechnology) in PBS with bovine serum albumin for 48 h at 4 °C. The reaction products of biotinylated goat anti-rabbit antiserum and avidin-conjugated horseradish peroxidase (Nichirei SAB kit) were visualized using 0.005% diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.0003% H_2O_2, and intensified by pretreatment with 0.25% cobalt chloride. The sections were mounted onto gelatin-coated glass slides, air-dried, dehydrated with alcohol and xylene, and coverslips were added. Preabsorption of these antibodies with corresponding synthetic peptides (Santa Cruz Biotechnology) or omission of primary antibodies in the protocol abolished staining.

2.3. Data analysis

To evaluate quantitatively the effects of pretreatment with EM-1, EM-2, and morphine on formalin injection-evoked c-Fos and Zif/268 expression, neurons expressing c-Fos or Zif/268 protein in the spinal dorsal horn were counted manually. The dorsal horn was first divided into the superficial layer (laminae I and II), the nucleus proprius (laminae III and IV), and the neck of the dorsal horn (laminae V and VI) by drawing the boundaries between each of these regions according to the criteria described previously (Molander et al., 1984) with a camera lucida attachment under dark-field illumination. Then, their positive neurons were plotted on this drawing under bright-field illumination. In untreated and unstimulated control rats, a small number of c-Fos or Zif/268 positive neurons are found in laminae I and II, laminae III and IV, and laminae V and VI (Herdegen et al., 1991; Tölle et al., 1994a,b). A single investigator, blinded to the type of treatment in each rat,

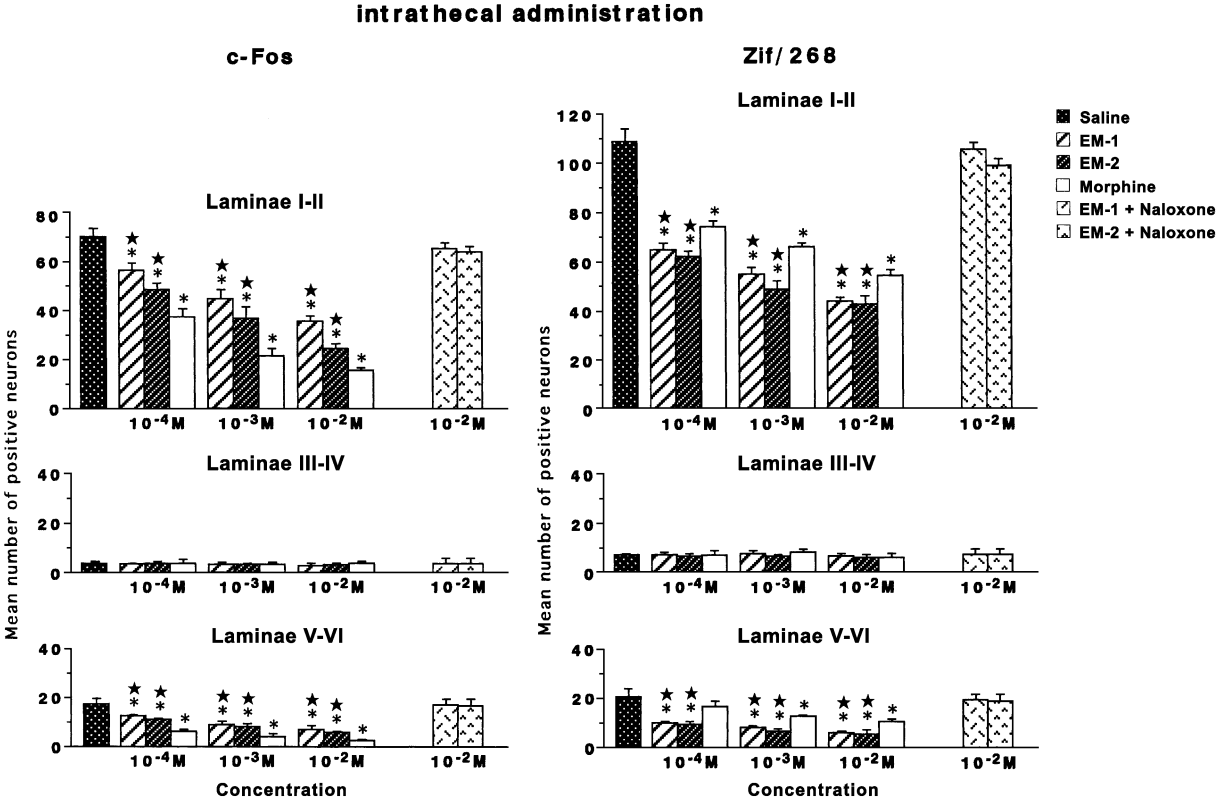

Fig. 2. Histograms showing the number of c-Fos- (left side) and Zif/268- (right side) positive neurons in the dorsal horn induced by formalin injection into the hind paw 5 min after intrathecal administration of different concentrations of EM-1, EM-2, and morphine, and pretreatment with naloxone (i.p.) 5 min before administration of the two endomorphins (10^{-2} M). The data are expressed as mean number of positive neurons ± S.E.M. *: $P<0.05$ compared with saline (control), ★: $P<0.05$ compared with morphine for each concentration.

plotted the positive neurons. Four rats in each experimental group were analyzed, and 10 sections in the L4 and L5 segments were selected from each rat, according to the largest number of positive neurons per section. The mean number of positive neurons was used to evaluate quantitatively the effect of administration of these drugs into the two regions of the brain.

Data were expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were examined for statistical significance using one-way analysis of variance, followed by Fisher's protected least significant difference for multiple comparisons. A P value less than 0.05 denoted the presence of a statistically significant difference.

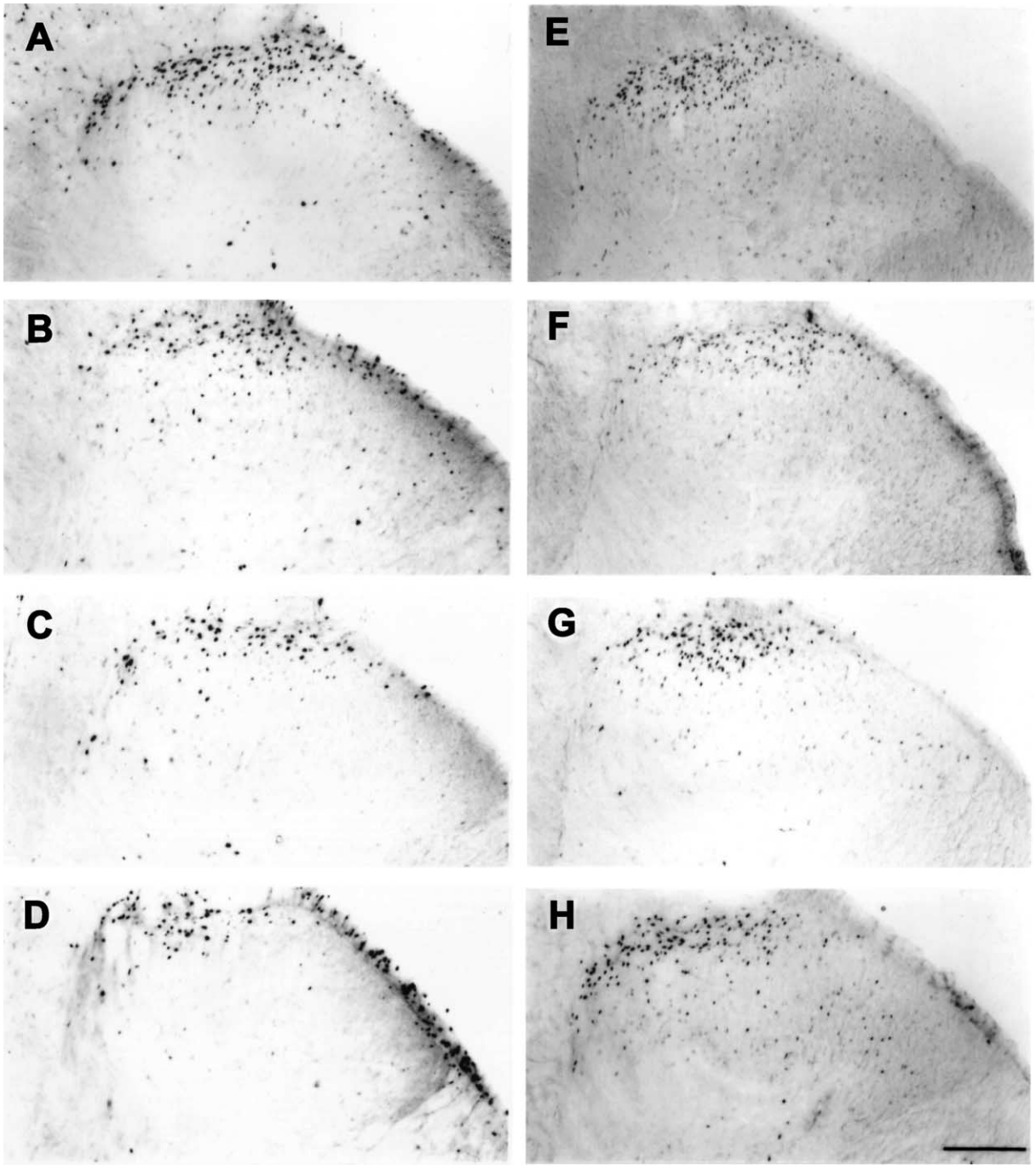

Fig. 3. Photomicrographs showing c-Fos (left side) and Zif/268 (right side) immunoreactivities in the dorsal horn of the lumbar spinal cord in rats that received an injection of 5% formalin solution into the right hind paw 5 min after intracerebroventricular administration of saline (A and E), 10^{-2} M of EM-1 (B and F), 10^{-2} M of EM-2 (C and G), and 10^{-2} M of morphine (D and H). Scale bar = 200 μm.

3. Results

3.1. Control experiment

In saline-treated control rats, formalin injection into the plantar surface of the hind paw produced marked increases in c-Fos and Zif/268 immunoreactivities in the dorsal horn of the ipsilateral L4 and L5 segments 2 h after injection. Neurons positive for c-Fos or Zif/268 protein were identified by dark blue-stained nuclei and scant staining of the cytoplasm. Most c-Fos- and Zif/268-positive neurons were localized within the medial half of the spinal dorsal horn, and they were numerous in the superficial layer, especially in lamina I and the outer part of lamina II. The numbers of positive neurons in laminae III and IV, and laminae V and VI were few. The number of neurons expressing c-Fos protein was always lower than that of Zif/268 in the spinal dorsal horn, especially in laminae I and II, and laminae III and IV (Fig. 1A and E, control in Fig. 2 and Fig. 3A and E, and control in Fig. 4). Quantitative analysis in the i.t. administration group revealed that the numbers of neurons expressing c-Fos and Zif/268 in laminae I and II were 77.0% and 79.7% of the total positive neurons in the spinal dorsal horn, respectively. The route of administration of saline did not have any significant influence on the number of positive neurons in each region.

3.2. Effects of intrathecal administration of EM-1 and EM-2 on c-Fos and Zif/268 expression

I.t. administration of EM-1, EM-2, and morphine produced remarkable reductions in formalin injection-evoked c-Fos immunoreactivity in the spinal dorsal horn (Fig. 1B, C, and D). Similarly, the immunoreactivity of Zif/268 was markedly reduced in the spinal dorsal horn by administration of these drugs (Fig. 1F, G, and H). The effects of all three drugs on c-Fos and Zif/268 immunoreactivities were conspicuous in laminae I and II, and laminae V and VI. Quantitative analysis revealed that the numbers of c-Fos- and Zif/268-positive neurons in laminae I and II, and laminae V and VI were significantly lower in rats given 10^{-4} M of each drug, and further decreases were noted with higher concentrations of these drugs (Fig. 2). No effect of the three drugs was found in laminae III and IV.

There was a slight difference between the effects of these drugs on the reduction of c-Fos and Zif/268 expression in the dorsal horn. Administration of morphine produced a consistently larger decrease in the number of c-Fos-positive neurons in laminae I and II, and laminae V and VI than did the endomorphins, and the difference in the numbers of positive neurons between morphine-treated and endomorphin-treated groups was statistically significant for each

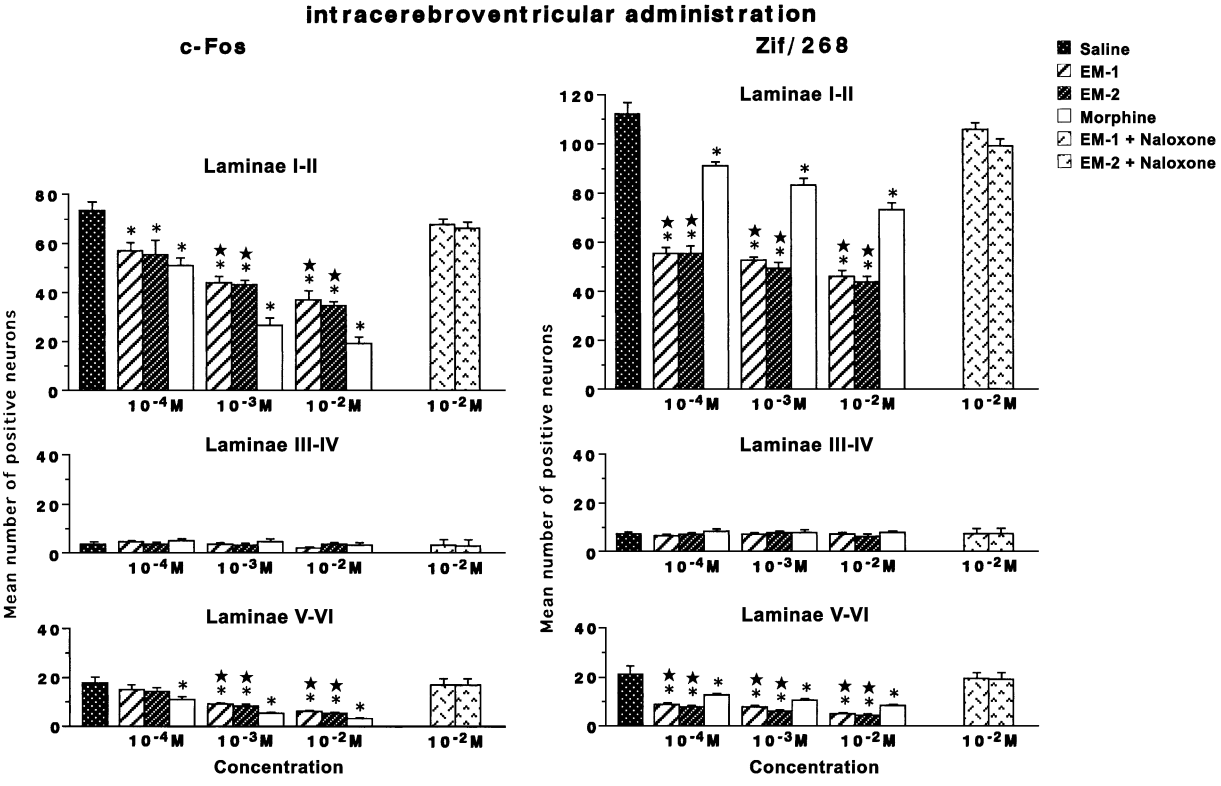

Fig. 4. Histograms showing the number of c-Fos- (left side) and Zif/268- (right side) positive neurons in the dorsal horn induced by formalin injection into the hind paw 5 min after intracerebroventricular administration of different concentrations of EM-1, EM-2, and morphine, and pretreatment with naloxone (i.p.) 5 min before administration of the two endomorphins (10^{-2} M). The data are expressed as mean number of positive neurons ± S.E.M. *: $P<0.05$ compared with saline (control), ★: $P<0.05$ compared with morphine for each concentration.

concentration ($P<0.05$) (Fig. 2). In contrast, the effect on Zif/268 expression was the opposite of that on c-Fos expression ($P<0.05$) (Fig. 2). The effects of i.t. administration of EM-1 and EM-2 on c-Fos and Zif/268 expression were clearly attenuated by pretreatment with naloxone (Fig. 2).

3.3. Effects of intracerebroventricular administration of EM-1 and EM-2 on c-Fos and Zif/268 expression

I.c.v. administration of EM-1, EM-2, and morphine also produced remarkable reductions in c-Fos immunoreactivity in the spinal dorsal horn (Fig. 3B, C, and D). In common with i.t. administration, the immunoreactivity of Zif/268 was also markedly reduced (Fig. 3F, G, and H). The pattern of the effects of all three drugs on formalin injection-evoked c-Fos and Zif/268 immunoreactivities was similar to that seen after i.t. administration. Quantitative analysis revealed that the number of c-Fos-positive neurons in laminae I and II and the numbers of Zif/268-positive neurons in laminae I and II, and laminae V and VI were significantly lower in rats given 10^{-4} M of each drug, and further decreases were noted with higher concentrations of these drugs (Fig. 4). No effect of the three drugs was seen in laminae III and IV.

Similar to what was seen with i.t. administration, there was a slight difference between the effects of the three drugs on the reduction of c-Fos and Zif/268 expression in the dorsal horn. Administration of morphine produced a consistently larger decrease in the number of c-Fos-positive neurons in laminae I and II, and laminae V and VI than did the endomorphins, and the difference in the numbers of positive neurons between morphine-treated and endomorphin-treated groups after administration of 10^{-3} and 10^{-2} M of these drugs was statistically significant ($P<0.05$) (Fig. 4). In contrast, the effect on Zif/268 expression was the opposite of that on c-Fos expression ($P<0.05$) (Fig. 4). The effect of i.c.v. administration of EM-1 and EM-2 on c-Fos and Zif/268 expression was clearly attenuated by pretreatment with naloxone (Fig. 4).

4. Discussion

The present results demonstrate that i.t. and i.c.v. administration of EM-1, EM-2, and morphine dose-dependently reduced the expression of c-Fos and Zif/268 proteins in laminae I and II, and laminae V and VI induced by formalin injection into the rat hind paw. The reductions by endomorphins were greater for Zif/268 expression and smaller for c-Fos expression compared with morphine. The effect of the endomorphins was attenuated by administration of naloxone. Overall, EM-1 and EM-2 act as endogenous ligands of μ-opioid receptor on nociceptive neurons.

Morphine produces antinociception by acting at spinal opiate receptors to block the transmission of nociceptive messages to the brain. The existence of μ-opioid receptor in the CNS was initially proposed by autoradiography with radioisotope-labeled ligand (Pert et al., 1976; Atweh and Kuhar, 1977), and the location of this receptor has been reconfirmed in the spinal dorsal horn by using immunohistochemical method (Arvidsson et al., 1995; Mansour et al., 1995; Ding et al., 1996; Moriwaki et al., 1996). Accordingly, i.t. or i.c.v. administration of morphine inhibits spinal nociceptors via its action at the spinal cord level and produces analgesia. On the other hand, immunohistochemical studies have revealed that both EM-1 and EM-2 are localized in the dorsal horn, especially in laminae I and II (Martin-Schild et al., 1999; Wu et al., 1999), and a release of EM-2 by electrical stimulation has been demonstrated in the dorsal horn by the antibody microprobe technique (Williams et al., 1999; Dun et al., 2000). Thus, the presence of endomorphins was consistent with the region of a dense population of μ-opioid receptor. Both the coincidence of the distributions of endomorphins and μ-opioid receptors in the dorsal horn and the marked reductions of formalin injection-evoked c-Fos and Zif/268 expression in laminae I and II, and laminae V and VI by endomorphin administration strengthened further the idea that EM-1 and EM-2 may serve as endogenous ligands of μ-opioid receptor that plays a role in the modulation of the expression of these proteins. Furthermore, electron microscopy studies have revealed that this receptor is localized on both primary afferents and neurons located in laminae I and II (Cheng et al., 1996), indicating that the endomorphins are able to bind to both. The present study showed only the effect of EM-1, EM-2, and morphine on formalin injection-evoked c-Fos and Zif/268 expression in the spinal dorsal horn; therefore, it remains unclear whether the effect of these drugs is due to the contribution of μ-opioid receptors located on primary afferents or to μ-opioid receptors located on neurons in laminae I and II. Further clarification may be important for evaluating an exact role of μ-opioid receptor in the transmission of nociceptive information into the dorsal horn.

There was little difference in the reduction of c-Fos and Zif/268 expression between i.t. and i.c.v. administration of EM-1 or EM-2. In the previous study on pain-related behaviors (Zadina et al., 1997; Sakurada et al., 1999), endomorphin was more effective via i.t. administration than via i.c.v. administration. In contrast, another study demonstrated that EM-1 displayed significantly higher potency spinally, while EM-2 did so supraspinally (Goldberg et al., 1998). Although these results are inconsistent, the effects of endomorphins may be responsible for different μ-opioid receptor subtypes (Goldberg et al., 1998; Sakurada et al., 1999). Mu1-receptors mediate supraspinal analgesia although μ2-receptors are responsible for spinal analgesia. The endomorphins may produce antinociception via the distinct μ1- and μ2-subtypes of μ-opioid receptors at both supraspinal and spinal levels.

There was a slight difference between the effects of the endomorphins and morphine on formalin injection-induced c-Fos and Zif/268 expression in our study, although both are potent agonists of μ-opioid receptor. Indeed, the reductions of Zif/268 expression by endomorphins were greater than

those by morphine, while the reductions of c-Fos expression by endomorphins were smaller than those by morphine. However, it is not likely that the differential effect between the endomorphins and morphine is derived from the differences in penetration of these drugs into the CNS or the half-life of these drugs in the CNS. It has been shown that the mechanism of action of the endomorphins was some different from morphine in the previous study on pain-related behaviors (Przewlocka et al., 1999). It is conceivable that the difference may be due to the different patterns of G-protein activation, the different μ-opioid receptor subtypes, the mediation of nitric oxide, or the release of dynorphinA (Przewlocki et al., 1999; Sanchez-Blazquez et al., 1999; Tseng et al., 2000). Although the relations of these factors remain unknown in our experiment, the different reductions between c-Fos and Zif/268 expression indicate that there are somewhat substantial differences in responses of nociceptive neurons to endomorphins and morphine. Furthermore, it is possible that some non-opioid receptors may mediate the effects of endomorphins and morphine, and the effects of endomorphins and morphine may be different between pre- and postsynaptic sites of action.

The time course and distribution of c-Fos and Zif/268 expression in dorsal horn neurons by peripheral noxious stimulation are similar in the acute phase (Herdegen et al., 1991). In this experiment, Zif/268 expression, as well as c-Fos expression, was markedly induced by formalin injection. The different inductions between c-Fos and Zif/268 expression would reveal the difference of the effects of endomorphins and morphine. Thus, Zif/268 expression, in addition to c-Fos expression, is useful as an anatomic marker for confirming the activity of nociceptive neurons.

In summary, the administration of EM-1 and EM-2 reduced c-Fos and Zif/268 expression in the spinal dorsal horn induced by formalin injection, and these effects were attenuated by pretreatment with naloxone. To the best of our knowledge, this is the first report that the roles of EM-1 and EM-2 as endogenous ligands of μ-opioid receptor were examined at the level of the neuron in the CNS using the marker of c-Fos and Zif/268 proteins. The effect of the two endomorphins on c-Fos and Zif/268 expression was very potent, suggesting that it may be possible to substitute EM-1 and EM-2 for morphine in the treatment of pain.

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