

Title

Possible Involvement of Dynorphin A (1-17) Release via  $\mu_1$ -Opioid Receptors in Spinal Antinociception by Endomorphin-2

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a) Running title:

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c) Composition:

The number of text page: 30

The number of tables: 1

The number of figures: 8

The number of references: 39

The number of word in the Abstract: 232

The number of words in the Introduction: 390

The number of words in the Discussion: 1499

d) Abbreviations:

ANOVA	analysis of variance
CSF	cerebrospinal fluid
DAMGO	[D-Ala <sup>2</sup> ,MePhe <sup>4</sup> ,Gly(ol) <sup>5</sup> ]enkephalin
D-Pro <sup>2</sup> -Tyr-W-MIF-1	H-Tyr-D-Pro-Trp-Gly-NH <sub>2</sub>
i.c.v.	intracerebroventricular
i.t.	intrathecal
% MPE	percent of the maximum possible effect
s.c.	subcutaneous
TAPA	H-Tyr-D-Arg-Phe-β-Ala-OH

e) Section:

Neuropharmacology

## ABSTRACT

The antinociception induced by intrathecal (i.t.) or intracerebroventricular (i.c.v.) administration of endomorphins is mediated via  $\mu$ -opioid receptors. However, although endomorphins do not have an appreciable affinity for  $\kappa$ -opioid receptors, pretreatment with the  $\kappa$ -opioid receptor antagonist nor-binaltorphimine markedly reduces the antinociceptive response to i.c.v.- or i.t.-administered endomorphin-2 but not endomorphin-1 (Tseng et al., 2000; Sakurada et al., 2001). These results suggest that endomorphin-2 initially stimulates  $\mu$ -opioid receptors, which subsequently induce the release of dynorphins which act on  $\kappa$ -opioid receptors to produce antinociception. The present study was performed in mice to determine whether the release of dynorphins by i.t.-administered endomorphin-2 is mediated through  $\mu$ -opioid receptors to produce antinociception. Intrathecal pretreatment with an antiserum against dynorphin A (1-17), but not against dynorphin B (1-13) or  $\alpha$ -neo-endorphin, dose-dependently prevented the paw-withdrawal inhibition by endomorphin-2. The pretreatments with these antisera did not affect the endomorphin-1- or DAMGO-induced paw-withdrawal inhibition. The attenuation of endomorphin-2-induced antinociception by i.t. pretreatment with an antiserum against dynorphin A (1-17) or subcutaneous (s.c.) pretreatment with nor-binaltorphimine was blocked dose-dependently by s.c. pretreatment with the  $\mu$ -opioid receptor antagonist  $\beta$ -funaltrexamine or the  $\mu_1$ -opioid receptor antagonist naloxonazine at ultra low doses which are ineffective against  $\mu$ -opioid receptor agonists. These results suggest that the spinal antinociception induced by endomorphin-2 is mediated through the stimulation of a distinct subtype of  $\mu_1$ -opioid receptor that induces the release of the endogenous  $\kappa$ -opioid peptide dynorphin A (1-17) in the spinal cord.

## Introduction

Two endogenous opioid peptides, endomorphin-1 and endomorphin-2, have been recently discovered in the mammalian brain. These two peptides activate  $\mu$ -opioid receptors with high affinity and selectivity (Zadina et al., 1997; Goldberg et al., 1998; Gong et al., 1998; Hosohata et al., 1998). Immunoreactivities for these peptides have been localized in many areas of the central nervous system involved in pain processing, including the dorsal horn of the spinal cord, the caudal trigeminal nucleus, and the periaqueductal gray (Martin-Schild et al., 1997, 1998, 1999; Pierce et al., 1998). Some differences have been noted for the distribution of endomorphin-1 and endomorphin-2 in the brain and spinal cord. Endomorphin-1 is more abundant than endomorphin-2 in the brain, whereas endomorphin-2 dominates in the spinal cord (Martin-Schild et al., 1999). Both of these peptides display high affinity and selectivity for the  $\mu$ -opioid receptor; however, neither endomorphin has an appreciable affinity for  $\delta$ - or  $\kappa$ -opioid receptors (Zadina et al., 1997). Distinct pharmacological properties for the endomorphins have been reported in both electrophysiological (Chapman et al., 1997) and behavioral experiments (Stone et al., 1997; Zadina et al., 1997; Goldberg et al., 1998). The antinociception induced by endomorphin-1 and endomorphin-2 given intrathecally (i.t.) or intracerebroventricularly (i.c.v.) is selectively blocked by pretreatment with  $\mu$ -opioid receptor antagonists, naloxone or  $\beta$ -funaltrexamine (Zadina et al., 1997; Goldberg et al., 1998; Sakurada et al., 1999), indicating that their antinociception is mediated by the stimulation of  $\mu$ -opioid receptors. Furthermore, pretreatment with the  $\mu_1$ -opioid receptor antagonist naloxonazine attenuates the antinociception induced by i.t.- or i.c.v.-administered endomorphin-2 but not endomorphin-1, suggesting that endomorphin-2-induced antinociception is mediated by the stimulation of

$\mu_1$ -opioid receptors (Sakurada et al., 1999, 2000a, 2001, 2002).

Although the antinociceptive effects induced by both endomorphin-1 and endomorphin-2 are mediated by the stimulation of  $\mu$ -opioid receptors, some differences in antinociceptive effects between endomorphin-1 and endomorphin-2 have been noted. The antinociception induced by supraspinally-administered endomorphin-2, but not endomorphin-1, is blocked by i.c.v. pretreatment with the  $\kappa$ -opioid receptor antagonist norbinaltorphimine or an antiserum against dynorphin A (1-17), suggesting the possibility that endomorphin-2 stimulates a different subtype of  $\mu$ -opioid receptor, which subsequently induces the release of dynorphins which act on  $\kappa$ -opioid receptors to produce antinociception (Tseng et al., 2000). The present behavioral pharmacological study was conducted to determine whether the release of dynorphin A (1-17) by spinally administered endomorphin-2 is mediated through the stimulation of the  $\mu$ -opioid receptor.

## Materials and Methods

All experiments were approved by the Committee of Animal Experiments at Tohoku Pharmaceutical University and conformed to their guidelines. Every effort was made to minimize the number of animals and any suffering to the animal used in the following experiments.

**Animals.** Male ddY mice weighing 22-25 g (SLC, Hamamatsu, Japan) were housed in a light- and temperature-controlled room (light on at 09:00 and off at 21:00; 23°C). Food and water were available *ad libitum*. Animals were used only once.

**Drugs and Antisera.** Endomorphin-1 (Peptide Institute Inc., Osaka, Japan), endomorphin-2 (Peptide Institute Inc.), [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Gly(ol)<sup>5</sup>]enkephalin (DAMGO; Sigma Chemical Co., St. Louis, MO), naloxonazine (RBI, Natick, MA),  $\beta$ -funaltrexamine (RBI), nor-binaltorphimine (RBI), antiserum against dynorphin A (1-17) (Phoenix Pharmaceutical Inc., St. Joseph, MO), antiserum against dynorphin B (1-13) (Bachem, San Carlos, CA), and antiserum against  $\alpha$ -neo-endorphin (Bachem) were used. Endomorphins, DAMGO and the antisera were dissolved in sterile artificial cerebrospinal fluid (CSF) containing 7.4 g of NaCl, 0.19 g of KCl, 0.19 g of MgCl<sub>2</sub>, and 0.14 g of CaCl<sub>2</sub> in 1 liter. Naloxonazine,  $\beta$ -funaltrexamine and nor-binaltorphimine were dissolved in saline and injected subcutaneously (s.c.) in a volume of 0.1 ml/10 g body weight 24 h prior to testing.

**Assessment of Antinociceptive Response.** The antinociceptive response was assessed with the thermal paw-withdrawal test, using an automated tail-flick unit (BM kiki, Tokyo, Japan). Mice were adapted to the testing environment for at least 1 h before any stimulation. Each animal was restrained with a soft cloth to reduce visual stimuli, and a light beam as a noxious radiant heat stimulation was applied to the hind-paw. The light beam

focused on the plantar surface of the hind-paw, and the latency for the paw-withdrawal response against the noxious radiant heat stimulation was measured. The intensity of the noxious radiant heat stimulation was adjusted so that the predrug latency for the paw-withdrawal response was 2.5-3.5 sec. The antinociceptive effect was expressed as a percent of the maximum possible effect (% MPE), which was calculated with the following equation:  $[(T_1 - T_0) / (10 - T_0)] \times 100$ , where  $T_0$  and  $T_1$  are the predrug and postdrug latencies for the paw-withdrawal response, respectively. To prevent tissue damage to the paw, the noxious radiant heat stimulation was terminated automatically if the mouse did not lift the paw within 10 sec. The measurement of the paw-withdrawal latency was performed by only one individual who was uninformed about the drug treatment for each mouse.

**Intrathecal Administration.** The i.t. administration was performed according to the procedure described by Hylden and Wilcox (1980) using a 10- $\mu$ l Hamilton microsyringe with a 29-gauge needle. The injection volume for opioid peptides and antisera was 2  $\mu$ l and 4  $\mu$ l, respectively.

**Statistical Analysis.** The data are expressed as the mean  $\pm$  S.E.M for 10 mice. The statistical significance of the differences between groups was assessed with a one-way analysis of variance (ANOVA) followed by either Dunnett's test or Bonferroni's test, or a two-way ANOVA followed by Bonferroni's test.



## Results

**Time Courses of the Paw-withdrawal Inhibition Induced by i.t. Administration of Endomorphin-1, Endomorphin-2 and DAMGO.** Groups of mice were injected i.t. with endomorphin-1 (5 nmol), endomorphin-2 (5 nmol), DAMGO (20 pmol), or the vehicle, and the paw-withdrawal responses were measured at various times after the injection. The inhibition of the paw-withdrawal responses after i.t. administration of endomorphin-1 or endomorphin-2 reached their peaks at 5 min and paw-withdrawal responses returned to the preinjection level in 20 min (Fig. 1). On the other hand, the inhibition of the paw-withdrawal response after i.t. administration of DAMGO reached its peak 5-10 min after the injection, and the response returned to the preinjection level 30 min after the injection. CSF given i.t. did not produce any change in the latency of the paw withdrawal response (Fig. 1).

**Effects of s.c. Pretreatment with  $\beta$ -Funtaltrexamine and nor-Binaltorphimine on the Antinociception Induced by i.t.-administered Endomorphin-1, Endomorphin-2 and DAMGO.** Approximately equipotent doses of endomorphin-1, endomorphin-2 and DAMGO were chosen for the studies. Intrathecal injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol) and DAMGO (20 pmol) produced 83.8%, 80.8% and 72.2% of MPE at 5 min, 5 min and 10 min after the injection, respectively (Fig. 1). Groups of mice were pretreated s.c. with the selective  $\mu$ -opioid receptor antagonist  $\beta$ -funtaltrexamine (40 mg/kg) or the selective  $\kappa$ -opioid receptor antagonist nor-binaltorphimine (10 mg/kg) 24 h prior to the i.t. injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol) or DAMGO (20 pmol). The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2 and DAMGO was measured 5 min, 5 min and 10 min after the treatment, respectively. The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2 or DAMGO was

completely inhibited by the pretreatment with  $\beta$ -funaltrexamine (Fig. 2). On the other hand, the inhibition of the paw-withdrawal response induced by endomorphin-2 was markedly reduced by the pretreatment with nor-binaltorphimine, whereas the effects of endomorphin-1 and DAMGO were insensitive to nor-binaltorphimine (Fig. 2).

**Effects of i.t. Pretreatment with Antisera Against Dynorphin A (1-17), Dynorphin B (1-13) and  $\alpha$ -neo-Endorphin on the Antinociception Induced by i.t.-Administered Endomorphin-1, Endomorphin-2 and DAMGO.** Groups of mice were pretreated i.t. with antisera (1:50 dilution) against dynorphin A (1-17), dynorphin B (1-13) or  $\alpha$ -neo-endorphin for 15 min, 15 min and 10 min prior to the i.t. injection of endomorphin-1 (5 nmol), endomorphin-2 (5 nmol) and DAMGO (20 pmol), respectively. The inhibition of the paw-withdrawal response induced by endomorphin-1, endomorphin-2 and DAMGO was measured 5 min, 5 min and 10 min after the treatment, respectively. Intrathecal pretreatment with an antiserum against dynorphin A (1-17) attenuated the paw-withdrawal inhibition induced by endomorphin-2 in a dose-dependent manner (Fig. 3). However, the paw-withdrawal inhibition induced by endomorphin-2 was not affected by antisera against dynorphin B (1-13) or  $\alpha$ -neo-endorphin. The same pretreatment with these antisera did not affect the paw-withdrawal inhibition induced by endomorphin-1 or DAMGO (Fig. 3).

**Effects of  $\beta$ -Funaltrexamine and Naloxonazine Injected s.c. on the Attenuation of Endomorphin-2-induced Paw-withdrawal Inhibition by s.c. Pretreatment with nor-Binaltorphimine.** Subcutaneous pretreatment with ultra low doses of  $\beta$ -funaltrexamine (0.25-3 mg/kg) or naloxonazine (0.031-2 mg/kg) was ineffective against the paw-withdrawal inhibition induced by endomorphin-2 (5 nmol, i.t.) (Table 1). In the following experiments, these ultra low doses of  $\beta$ -funaltrexamine and naloxonazine were used. Groups of mice were pretreated s.c. with nor-binaltorphimine (10 mg/kg) in combination with  $\beta$ -funaltrexamine

(0.25-3 mg/kg) or naloxonazine (0.25-2 mg/kg) 24 h prior to i.t. treatment with endomorphin-2 (5 nmol). The inhibition of paw-withdrawal response induced by endomorphin-2 was measured 5 min after the treatment. Subcutaneous pretreatment with ultra low doses of  $\beta$ -funaltrexamine at 1.0-3.0 mg/kg markedly reduced the attenuation of endomorphin-2-induced paw-withdrawal inhibition by s.c. pretreatment with nor-binaltorphimine (Fig. 4). A similar phenomenon was observed after s.c. pretreatment with ultra low doses of naloxonazine (1-4 mg/kg), which significantly diminished the inhibitory effect of nor-binaltorphimine on the antinociception of endomorphin-2 (Fig. 5).

**Effects of  $\beta$ -Funaltrexamine and Naloxonazine Injected s.c. on the Attenuation of Endomorphin-2-induced Paw-withdrawal Inhibition by i.t. Pretreatment with an Antiserum Against Dynorphin A (1-17).**

Groups of mice, pretreated s.c. with  $\beta$ -funaltrexamine (0.25-2.0 mg/kg, s.c.) or naloxonazine (0.031-0.5 mg/kg) 24 h earlier, were injected i.t. with an antiserum against dynorphin A (1-17) (1:50 dilution) 15 min before i.t. treatment with endomorphin-2 (5 nmol). The inhibition of the paw-withdrawal response induced by endomorphin-2 was measured 5 min after the treatment. Subcutaneous pretreatment with ultra low doses of  $\beta$ -funaltrexamine at 1 or 2 mg/kg markedly reduced the attenuation of endomorphin-2-induced paw-withdrawal inhibition by i.t. treatment with an antiserum against dynorphin A (1-17) (Fig. 6). Similarly, s.c. pretreatment with ultra low doses of naloxonazine from 0.031 to 0.5 mg/kg dose-dependently blocked the attenuation of endomorphin-2-induced paw-withdrawal inhibition by i.t. treatment with an antiserum against dynorphin A (1-17) (Fig. 7).

## Discussion

Our previous studies showed that inhibition of the paw-withdrawal response induced by endomorphin-1 or endomorphin-2 given i.t. is blocked by s.c. pretreatment with naloxone and the selective  $\mu$ -opioid receptor antagonist  $\beta$ -funaltrexamine, indicating that the antinociception induced by both endomorphin-1 and endomorphin-2 given spinally is mediated by the stimulation of  $\mu$ -opioid receptors (Sakurada et al., 2000a, 2001). The finding is consistent with evidence that endomorphin-1 and endomorphin-2 have a high affinity for  $\mu$ -opioid receptors but have no appreciable affinity for either  $\delta$ - or  $\kappa$ -opioid receptors (Zadina et al., 1997). Naloxonazine irreversibly binds to  $\mu_1$ -opioid receptors and inhibits supraspinal antinociception (Ling et al., 1986; Paul et al., 1989; Pick et al., 1991). Recent behavioral pharmacological studies suggest the presence of  $\mu_1$ -opioid receptors which are sensitive to naloxonazine in spinal and supraspinal sites as assayed with the formalin test, hot-plate test, tail-pressure test, and tail-flick tests (Sato et al., 1999; Sakurada et al., 1999, 2000a,b). Autoradiographic studies show that  $\mu_1$ - and  $\mu_2$ -opioid receptors are localized in spinal and supraspinal structures involved in the modulation of nociception (Moskowitz and Goodman, 1985). Naloxonazine at a dose of 35 mg/kg (s.c.) has been used to selectively block  $\mu_1$ -opioid receptors in mice (Ling et al., 1986). The antinociceptive activity of endomorphin-2, but not endomorphin-1, given i.t. was completely blocked by pretreatment with this dose of naloxonazine (Sakurada et al., 2001), suggesting that spinal antinociception induced by endomorphin-2 and endomorphin-1 is mediated through the  $\mu_1$ -opioid receptor and the  $\mu_2$ -opioid receptor, respectively.

In the receptor binding assay, endomorphin-2 can bind both  $\mu_1$ - and  $\mu_2$ -opioid receptors, but shows higher affinity for  $\mu_1$ -opioid receptor than for  $\mu_2$ -opioid receptors

(Goldberg et al., 1998). In the mice pretreated s.c. with naloxonazine (35 mg/kg), irreversible antagonist for  $\mu_1$ -opioid receptor, the dose-response curve of endomorphin-2 injected i.t. for antinociception is shifted to the right in a parallel manner (Sakurada et al., 1999). In contrast, endomorphin-2 given i.t. can not produce the antinociception even at higher doses in the mice pretreated s.c. with  $\beta$ -funaltrexamine, irreversible antagonist for both  $\mu_1$ - and  $\mu_2$ -opioid receptors. These evidence suggest that although endomorphin-2 produces the spinal antinociception predominantly mediated through  $\mu_1$ -opioid receptor, endomorphin-2 can produce the antinociception via  $\mu_2$ -opioid receptor in the condition  $\mu_1$ -opioid receptor is occupied.

We found in a recent study that H-Tyr-D-Arg-Phe- $\beta$ -Ala-OH (TAPA), a dermorphin tetrapeptide analog, is highly selective for the  $\mu_1$ -opioid receptor (Mizoguchi et al., 2004). More recently, we have demonstrated that the inhibitory effect induced by i.t.-administered TAPA against i.t. capsaicin-elicited nociceptive responses is more potent than that against i.t. substance P-elicited nociceptive responses and was almost completely attenuated by s.c. pretreatment with naloxonazine, but not by i.t. co-administration of H-Tyr-D-Pro-Trp-Gly-NH<sub>2</sub> (D-Pro<sup>2</sup>-Tyr-W-MIF-1), the  $\mu_2$ -opioid receptor antagonist (Watanabe et al., 2005), suggesting that TAPA may selectively act at  $\mu_1$ -opioid receptors localized presynaptically on excitatory amino acid- and neuropeptide-containing axon terminals in the dorsal horn (Watanabe et al., in press). Similarly, antinociception induced by i.t.-administered endomorphin-2 on capsaicin-induced nociceptive responses, but not substance P-induced nociceptive responses, was completely antagonized by s.c. pretreatment with naloxonazine (unpublished observation). Endomorphin-2-like immunoreactivity is diminished by dorsal rhizotomy or exposure to capsaicin, and is colocalized with calcitonin gene-related peptide or substance P (Martin-Schild et al., 1997; 1998; Pierce et al., 1998). Our above results with

endomorphin-2 on capsaicin-induced nociceptive responses are in agreement with the findings reported by Martin-Schild et al. (1997, 1998) and Pierce et al. (1998). These results suggest that the release of neurotransmitters from nociceptive primary afferents is regulated by endomorphin-2 in primary afferent terminals through the  $\mu_1$ -opioid receptors.

Prodynorphin produces three main peptides:  $\alpha$ -neo-endorphin, dynorphin A (1-17) and dynorphin B (1-13). High levels of dynorphins are found in the brain as well as the dorsal horn of the spinal cord where modulation of nociceptive information occurs (Weber et al., 1982; Slater and Patel, 1983; Tan-No et al., 1997). Since dynorphin family members, such as dynorphin A (1-17), dynorphin B (1-13) and  $\alpha$ -neo-endorphin, have a high affinity for the  $\kappa$ -opioid receptor, dynorphins have been suggested to be the endogenous ligands for  $\kappa$ -opioid receptors (Chavkin and Goldstein, 1981; Chavkin et al., 1982). Dynorphins have been shown to produce antinociception when administered i.t. (Han and Xie, 1982; Piercey et al., 1982).  $\kappa$ -Opioid and  $\mu$ -opioid receptor mRNA is intensely expressed in substance P-containing neurons (Sato and Minami, 1995). Therefore,  $\kappa$ - and  $\mu$ -opioid receptor agonists have been considered to act directly on the primary afferent terminals of substance P-containing neurons to presynaptically modulate the release of substance P (Jessell and Iversen, 1977; Zachariou and Goldstein, 1997). In the present study, we explored the possibility that i.t.-administered endomorphin-2-induced antinociception is mediated by the spinal release of dynorphin A (1-17) via  $\mu$ -opioid receptors. We found that i.t. pretreatment with an antiserum against dynorphin A (1-17) or s.c. pretreatment with the  $\kappa$ -opioid receptor antagonist nor-binaltorphimine attenuated the paw-withdrawal inhibition induced by i.t.-administered endomorphin-2. However, i.t. pretreatment with an antiserum against dynorphin B (1-13) or  $\alpha$ -neo-endorphin did not block the endomorphin-2-induced antinociception, indicating that the effect of endomorphin-2 is selective for the release of

dynorphin A (1-17). This view is supported by the previous findings that the antinociception produced by spinally-administered endomorphin-2 is attenuated by i.t. pretreatment with an antiserum against dynorphin A (1-17) or the  $\kappa$ -opioid receptor antagonist nor-binaltorphimine (Ohsawa et al., 2001; Sakurada et al., 2001).

It should be noted that the pharmacological ability of endomorphin-2 to release dynorphin A (1-17) may be mainly involved in several discrepancies between endomorphin-1 and endomorphin-2 on their pharmacological effects, especially on the rewarding effect and locomotor enhancement. Endomorphin-1 given i.c.v. produces a remarkable rewarding and locomotor-enhancing effect in a dose-dependent manner (Bujdoso et al., 2001a; Narita et al., 2001). In contrast, endomorphin-2 given i.c.v. shows a bell-shaped dose-response curve for the rewarding and locomotor-enhancing effects, and prominently produces an aversive rather than rewarding effect (Bujdoso et al., 2001b; Narita et al., 2001). As is well known, the rewarding and locomotor-enhancing effects of  $\mu$ -opioid receptor agonists are mediated by the disinhibition of mesolimbic and nigrostriatal dopaminergic neurons via the activation of  $\mu$ -opioid receptors located on the GABAergic neurons in the ventral tegmental area and substantia nigra, respectively (Narita et al., 2001). In the terminal of mesolimbic and nigrostriatal dopaminergic neurons on the nucleus accumbens and striatum, respectively, dynorphinergic neurons are localized to inhibit the release of dopamine. As we found on the spinal antinociception in the present study, unlike endomorphin-1, endomorphin-2 has a characteristic pharmacological ability to release dynorphin A (1-17). The release of dynorphin A (1-17) in the nucleus accumbens and striatum may be involved in the lack of the remarkable rewarding effect and locomotor enhancing effect of endomorphin-2. In fact, endomorphin-2 shows a remarkable rewarding effect when dynorphin A (1-17) is blocked by the use of its antiserum (Narita et al., 2001).

In the present study, s.c. pretreatment with ultra low doses of  $\beta$ -funaltrexamine or naloxonazine that are ineffective against endomorphin-2, surprisingly prevented the attenuation of i.t.-administered endomorphin-2-induced antinociception by i.t. pretreatment with an antiserum against dynorphin A (1-17) or s.c. pretreatment with nor-binaltorphimine. These findings suggest that endomorphin-2 preferentially stimulates a distinct  $\mu_1$ -opioid receptor ( $\mu_1$ -opioid receptor subtype-1), which is extremely sensitive to naloxonazine and subsequently induces the release of dynorphin A (1-17) which acts on  $\kappa$ -opioid receptors to produce antinociception. However, in the condition that  $\mu_1$ -opioid receptor subtype-1 is occupied by ultra low doses of naloxonazine or  $\beta$ -funaltrexamine, endomorphin-2 should bind to next preferential another  $\mu_1$ -opioid receptor ( $\mu_1$ -opioid receptor subtype-2). Therefore, although the component of antinociception induced by i.t.-administered endomorphin-2 depends on the release of dynorphin A (1-17), endomorphin-2-induced antinociception was not affected by the blockade of released dynorphin A (1-17) after s.c. pretreatment with ultra low doses of naloxonazine or  $\beta$ -funaltrexamine. This finding let us to speculate that when ultra low doses of naloxonazine or  $\beta$ -funaltrexamine interrupt endomorphin-2 in binding to  $\mu_1$ -opioid receptor subtype-1, which regulate the release of dynorphin A (1-17) from dynorphinergic neurons that project to primary afferent terminals, endomorphin-2 at primary afferent terminals may act on  $\mu_1$ -opioid receptor subtype-2 which is not involved in the release of dynorphin A (1-17) (Fig. 8). Judging from the sensitivity to naloxonazine, we firstly propose here that there are two subtypes of  $\mu_1$ -opioid receptors which are involved in endomorphin-2-induced antinociception. One is a  $\mu_1$ -opioid receptor subtype-2 is sensitive to  $\beta$ -funaltrexamine (40 mg/kg, s.c.) and naloxonazine (10-35 mg /kg, s.c.) (Sakurada et al., 2000a), and is not involved in the release of dynorphin A (1-17). The other is a  $\mu_1$ -opioid receptor subtype-1 is involved in the release of dynorphin A (1-17)



acting on  $\kappa$ -opioid receptors as an additional component of endomorphin-2-induced antinociception, and is highly sensitive to both naloxonazine and  $\beta$ -funaltrexamine. The present study provides the important evidence to investigate the spinal neuronal circuit for production of endomorphin-2-induced antinociception.

In conclusion, endomorphin-2 given spinally produces the antinociception via stimulation of multiple  $\mu_1$ -opioid receptors,  $\mu_1$ -opioid receptor subtype-1 and  $\mu_1$ -opioid receptor subtype-2. The  $\mu_1$ -opioid receptor subtype-1, which is extremely sensitive to naloxonazine and  $\beta$ -funaltrexamine, may be involved in the additional component of endomorphin-2-induced antinociception, which is mediated through the release of the endogenous  $\kappa$ -opioid peptide dynorphin A (1-17).

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## Footnotes

a) Financial support

This work was supported by The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private Schools of Japan, a Grant-in-Aid for Scientific Research (C) (KAKENHI 16590058 and 17590065) from the Japan Society for the Promotion of Science, and a Grant-in-Aid for High Technology Research Program from the Ministry of Education, Culture, Sports, Science and Technology Japan.

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## Legends for Figures

**Fig. 1.** Time-course of the antinociceptive effects of endomorphin-1 (EM-1), endomorphin-2 (EM-2) and DAMGO in the mouse paw-withdrawal test. Groups of mice were injected i.t. with EM-1 (5 nmol), EM-2 (5 nmol), DAMGO (20 pmol), or the vehicle, and the paw-withdrawal responses were measured at various times after the injection. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of differences between groups was assessed with a two-way ANOVA followed by the Bonferroni's test. The F-values of the two-way ANOVA for EM-1, EM-2 and DAMGO compared to the vehicle were  $F[1,90] = 79.08$  ( $p < 0.01$ ),  $F[1,90] = 54.94$  ( $p < 0.01$ ) and  $F[1,108] = 127.1$  ( $p < 0.01$ ), respectively.  $*p < 0.05$  and  $**p < 0.01$ , compared to the respective value for the vehicle-treated control group.

**Fig. 2.** Effects of  $\beta$ -funaltrexamine ( $\beta$ -FNA) or nor-binaltorphimine (nor-BNI) on endomorphin-1 (EM-1)-, endomorphin-2 (EM-2)- and DAMGO-induced antinociception in the paw-withdrawal test. Nor-BNI (2.5-10 mg/kg) and  $\beta$ -FNA (40 mg/kg) were administered s.c. 24 h before i.t. administration of EM-1 (5 nmol), EM-2 (5 nmol) and DAMGO (20 pmol). The antinociceptive effects induced by EM-1, EM-2 and DAMGO were measured 5 min, 5 min and 10 min, respectively, after the administration. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The F-values of the one-way ANOVA for DAMGO, EM-1 and EM-2 were  $F[2,27] = 31.37$  ( $p < 0.01$ ),  $F[2,27] = 33.94$  ( $p < 0.01$ ) and  $F[4,45] = 9.763$  ( $p < 0.01$ ), respectively.  $**p < 0.01$ , compared to each agonist alone.



**Fig. 3.** Effects of antisera against dynorphin A (1-17) (Anti-DynA), dynorphin B (1-13) (Anti-DynB) or  $\alpha$ -neo-endorphin (Anti-endo) on endomorphin-1 (EM-1)-, endomorphin-2 (EM-2)- and DAMGO-induced antinociception in the paw-withdrawal test. Anti-DynA, Anti-DynB or Anti-endo was administered i.t. 15 min, 15 min and 10 min before i.t. administration of EM-1 (5 nmol), EM-2 (5 nmol) and DAMGO (20 pmol), respectively. The antinociceptive effects induced by EM-1, EM-2 and DAMGO were measured 5 min, 5 min and 10 min after the administration, respectively. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The F-values of the one-way ANOVA for EM-2 was  $F[5,54] = 10.85$  ( $p < 0.01$ ).  $*p < 0.05$  and  $**p < 0.01$  compared to EM-2 alone.

**Fig. 4.** Effect of  $\beta$ -funaltrexamine ( $\beta$ -FNA) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with nor-binaltorphimine (nor-BNI). Both nor-BNI (10 mg/kg) and  $\beta$ -FNA (0.25-3.0 mg/kg) were administered s.c. 24 h before i.t. administration of EM-2 (5 nmol). The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The F-value of the one-way ANOVA was  $F[6,63] = 6.461$  ( $p < 0.01$ ).  $*p < 0.05$  and  $**p < 0.01$  compared to EM-2 plus nor-BNI.

**Fig. 5.** Effect of naloxonazine (NLZ) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with nor-binaltorphimine (nor-BNI). Both nor-BNI (10

mg/kg) and NLZ (0.25-2.0 mg/kg) were administered s.c. 24 h before i.t. administration of EM-2 (5 nmol). The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The F-value of the one-way ANOVA was  $F[5,54] = 10.57$  ( $p < 0.01$ ).  $**p < 0.01$  compared to EM-2 plus nor-BNI.

**Fig. 6.** Effect of  $\beta$ -funaltrexamine ( $\beta$ -FNA) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with an antiserum against dynorphin A (1-17) (Anti-DynA).  $\beta$ -FNA (0.25-2.0 mg/kg, s.c.) and Anti-DynA (1:50 dilution, i.t.) was administered 24 h and 15 min before i.t. administration of EM-2 (5 nmol), respectively. The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The F-value of the one-way ANOVA was  $F[4,45] = 8.527$  ( $p < 0.01$ ).  $*p < 0.05$  and  $**p < 0.01$  compared to EM-2 plus Anti-DynA.

**Fig. 7.** Effect of naloxonazine (NLZ) on the attenuation of endomorphin-2 (EM-2)-induced antinociception by pretreatment with an antiserum against dynorphin A (1-17) (Anti-DynA). NLZ (0.031-0.5 mg/kg, s.c.) and Anti-DynA (1:50 dilution, i.t.) was administered 24 h and 15 min before i.t. administration of EM-2 (5 nmol), respectively. The inhibition of paw-withdrawal response induced by EM-2 was measured 5 min after the treatment. Each value represents the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test. The

F-value of the one-way ANOVA was  $F[4,45] = 13.87$  ( $p < 0.01$ ).  $**p < 0.01$  compared to EM-2 plus Anti-DynA.

**Fig. 8.** The schematic circuit diagram of the spinal dorsal horn with the speculative locations of  $\mu$ -opioid receptor ( $\mu$ -R),  $\kappa$ -opioid receptor ( $\kappa$ -R), N-methyl-D-aspartate receptor (NMDA-R), and neurokinin-1 receptor ( $NK_1$ -R), and their transmitter (endomorphin-2, dynorphin A (1-17), glutamate, and substance P, respectively)-containing neurons.

EM-2, endmorphin-2; Dyn A, dynorphin A (1-17); Glu, glutamate; SP, substance P; NLZ, naloxonazine.

TABLE 1. Effects of s.c. pretreatment with low-doses of the  $\mu$ -opioid receptor antagonists  $\beta$ -funaltrexamine and naloxonazine on the antinociception induced by i.t.-administered endomorphin-2

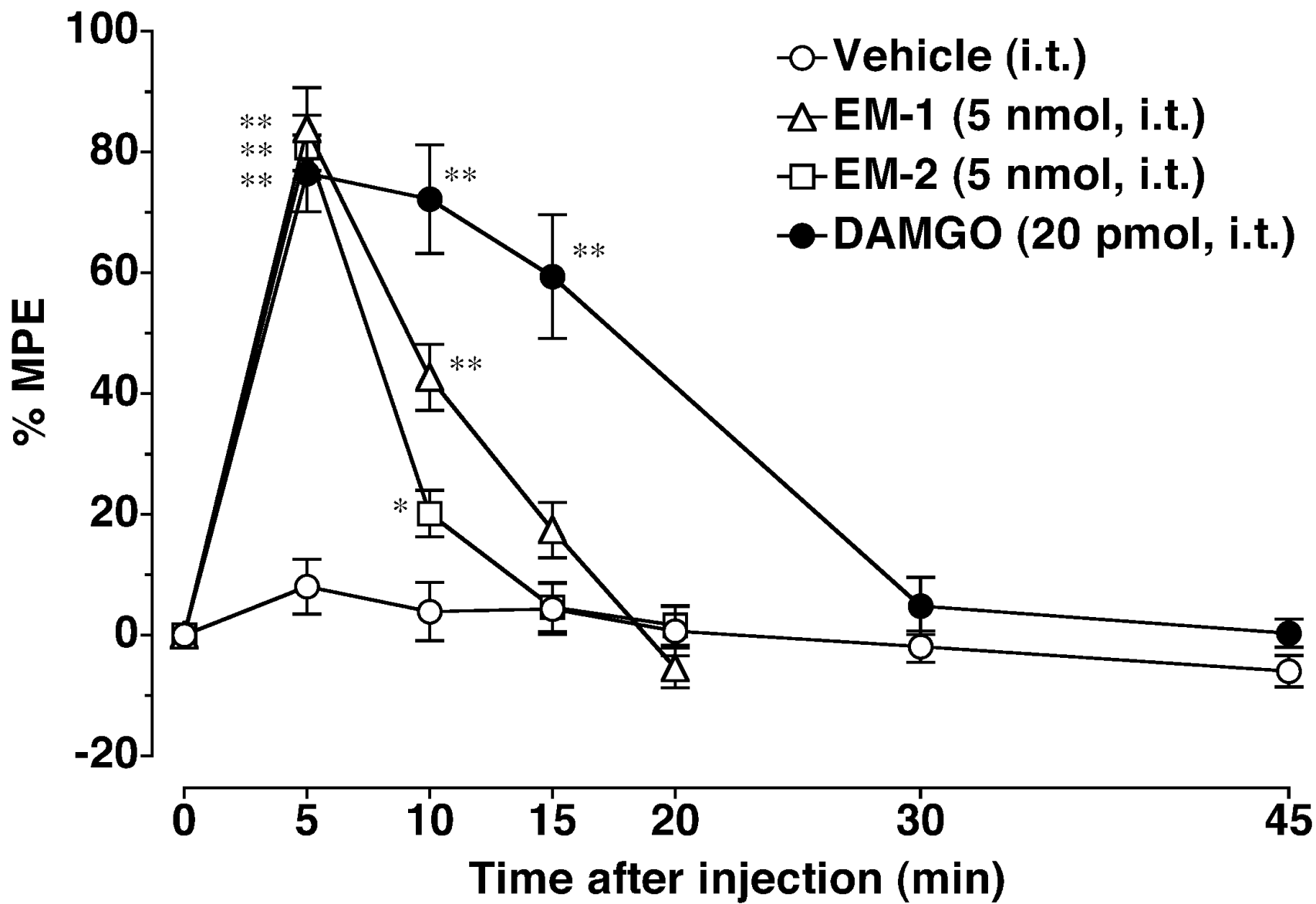
Treatment	Antinociception (% MPE)
Endomorphin-2 (5 nmol, i.t.)	80.81 $\pm$ 5.30
Endomorphin-2 (5 nmol, i.t.) + $\beta$ -funaltrexamine (0.25 mg/kg, s.c.)	86.80 $\pm$ 4.59
Endomorphin-2 (5 nmol, i.t.) + $\beta$ -funaltrexamine (0.5 mg/kg, s.c.)	83.77 $\pm$ 5.32
Endomorphin-2 (5 nmol, i.t.) + $\beta$ -funaltrexamine (1 mg/kg, s.c.)	83.45 $\pm$ 5.29
Endomorphin-2 (5 nmol, i.t.) + $\beta$ -funaltrexamine (2 mg/kg, s.c.)	87.06 $\pm$ 4.95
Endomorphin-2 (5 nmol, i.t.) + $\beta$ -funaltrexamine (3 mg/kg, s.c.)	84.27 $\pm$ 4.16
Endomorphin-2 (5 nmol, i.t.) + naloxonazine (0.031 mg/kg, s.c.)	90.20 $\pm$ 4.64
Endomorphin-2 (5 nmol, i.t.) + naloxonazine (0.125 mg/kg, s.c.)	90.69 $\pm$ 4.48
Endomorphin-2 (5 nmol, i.t.) + naloxonazine (0.25 mg/kg, s.c.)	76.71 $\pm$ 5.70
Endomorphin-2 (5 nmol, i.t.)	

+ naloxonazine (0.5 mg/kg, s.c.)	87.05 ± 4.65
Endomorphin-2 (5 nmol, i.t.)	
+ naloxonazine (1 mg/kg, s.c.)	76.65 ± 6.81
Endomorphin-2 (5 nmol, i.t.)	
+ naloxonazine (2 mg/kg, s.c.)	76.38 ± 8.15

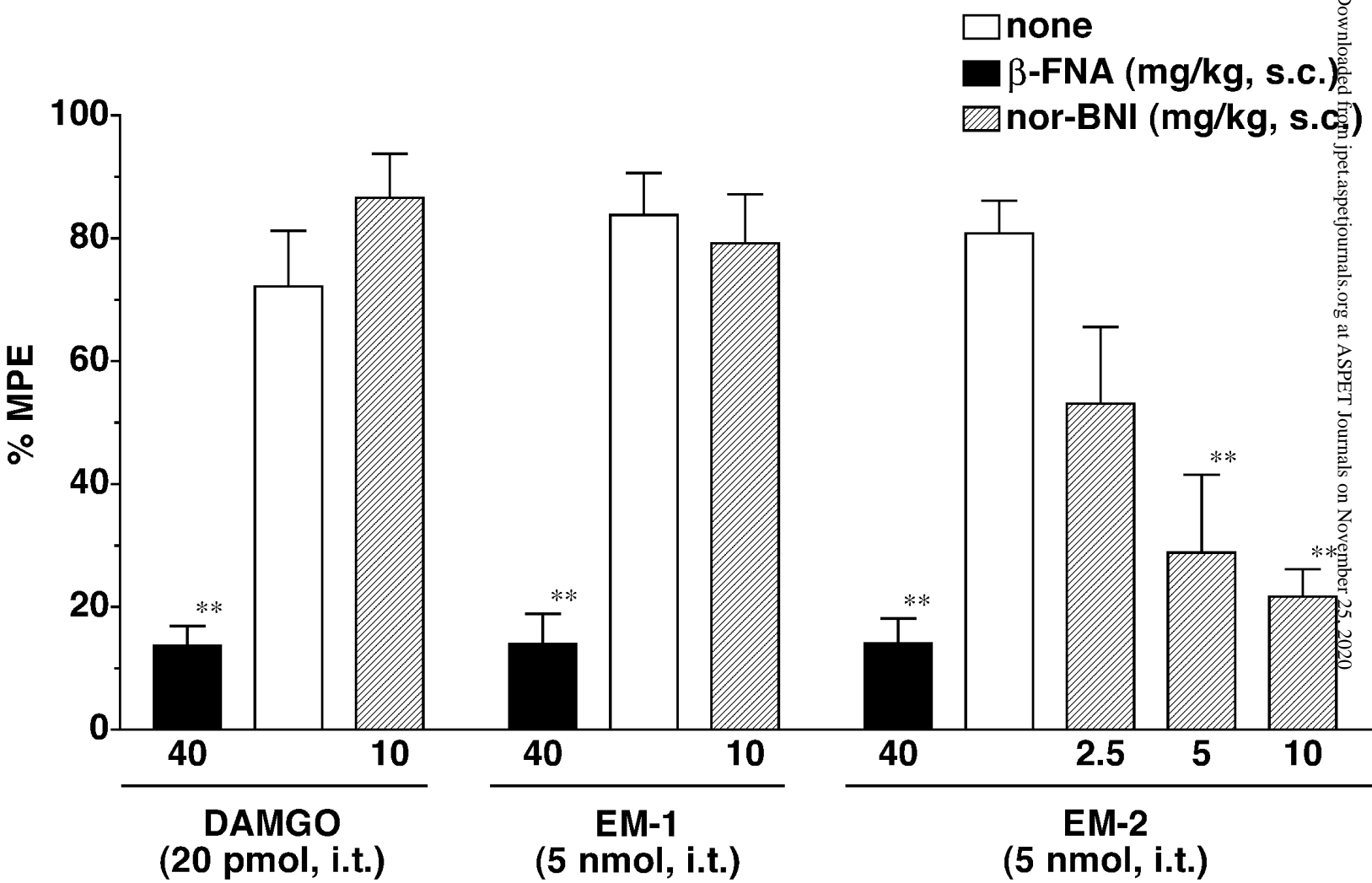
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Groups of mice were pretreated s.c. with various doses of  $\beta$ -funaltrexamine (0.25-3 mg/kg) or naloxonazine (0.031-2 mg/kg) 24 h prior to the i.t. administration of endomorphin-2 (5 nmol). The inhibition of the paw-withdrawal response induced by endomorphin-2 was measured 5 min after the treatment. The data represent the mean  $\pm$  S.E.M. for 10 mice. The statistical significance of the differences between groups was assessed with a one-way ANOVA followed by the Bonferroni's test.

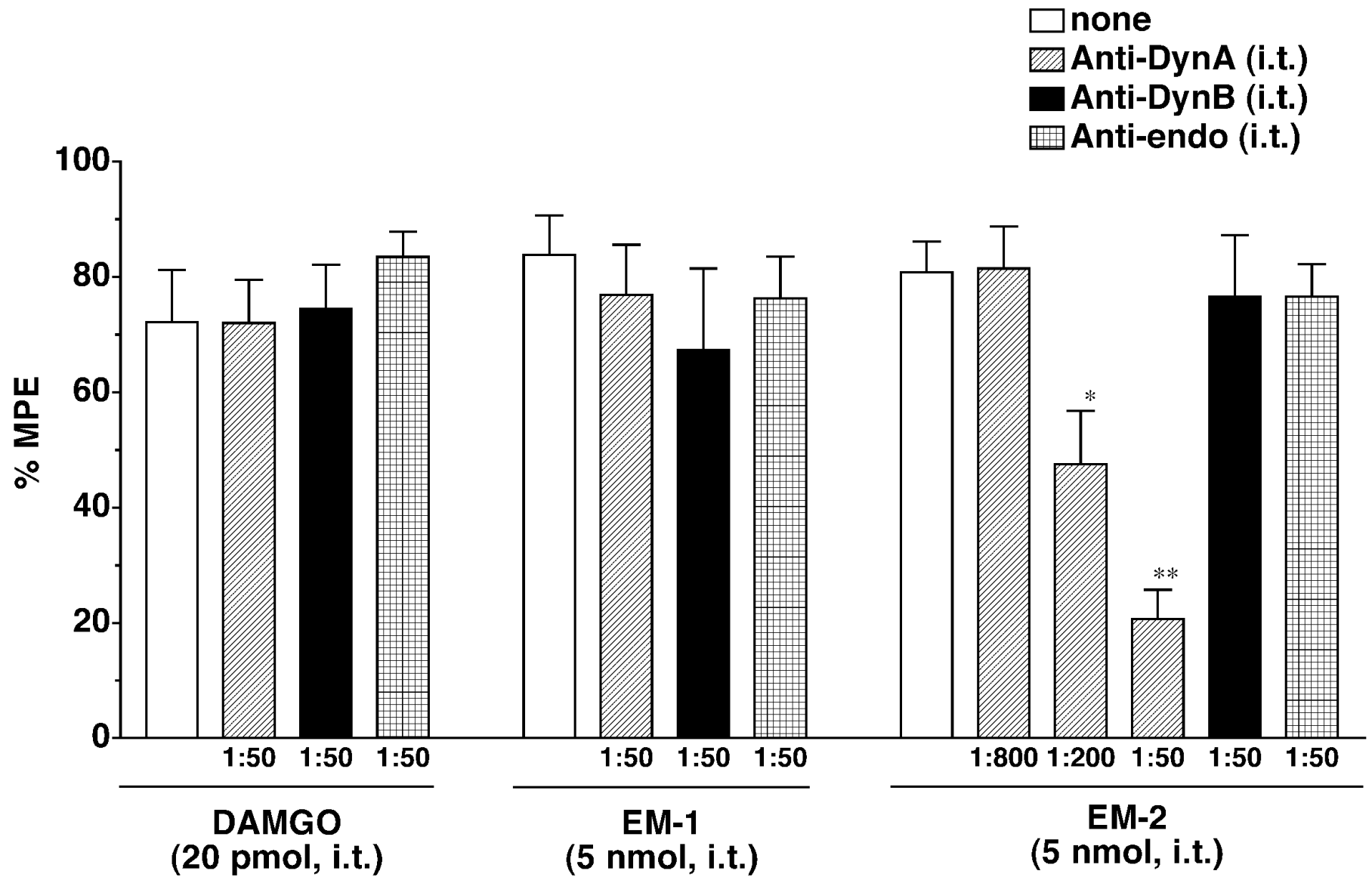
**Figure 1**



**Figure 2**

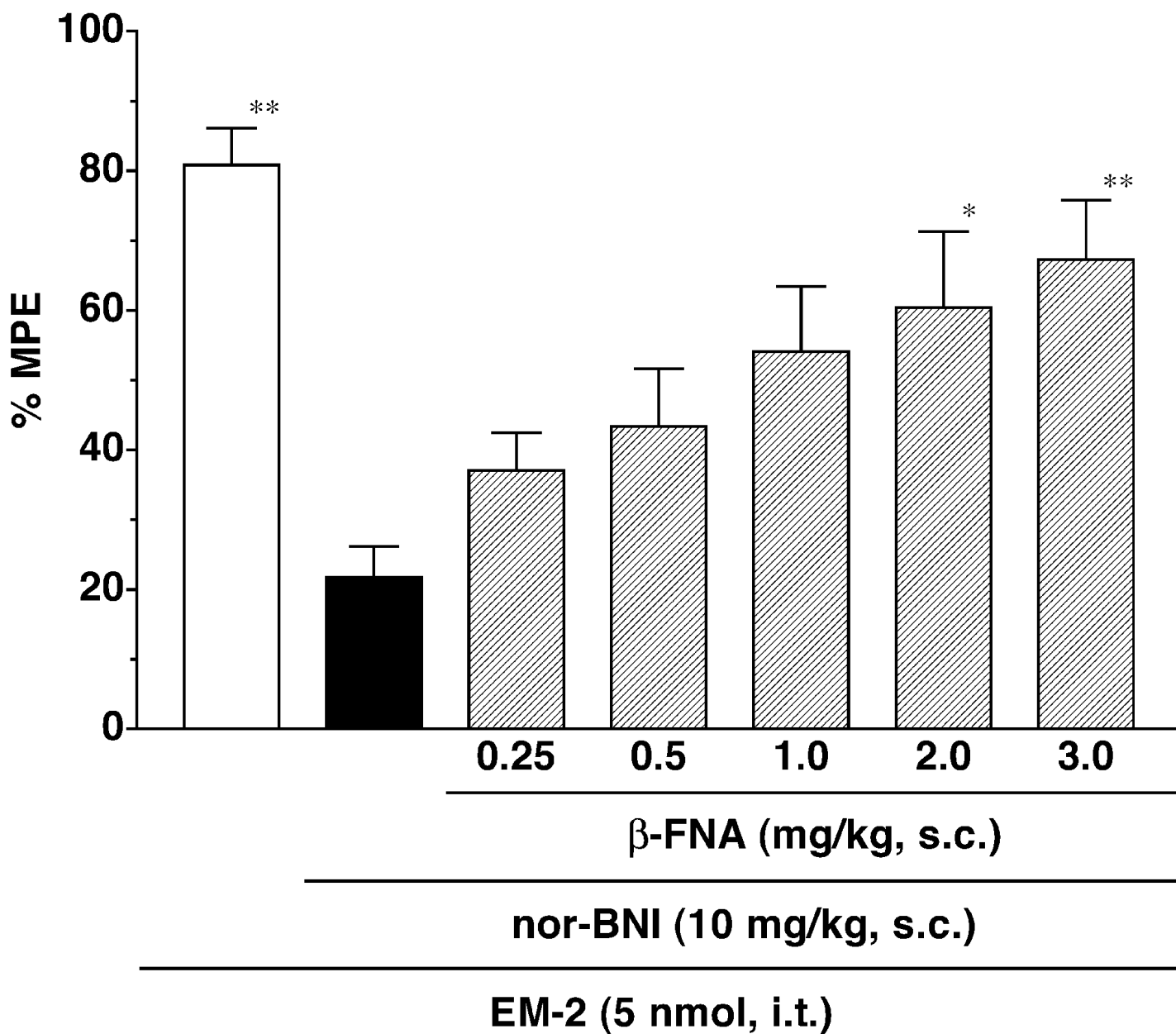


**Figure 3**

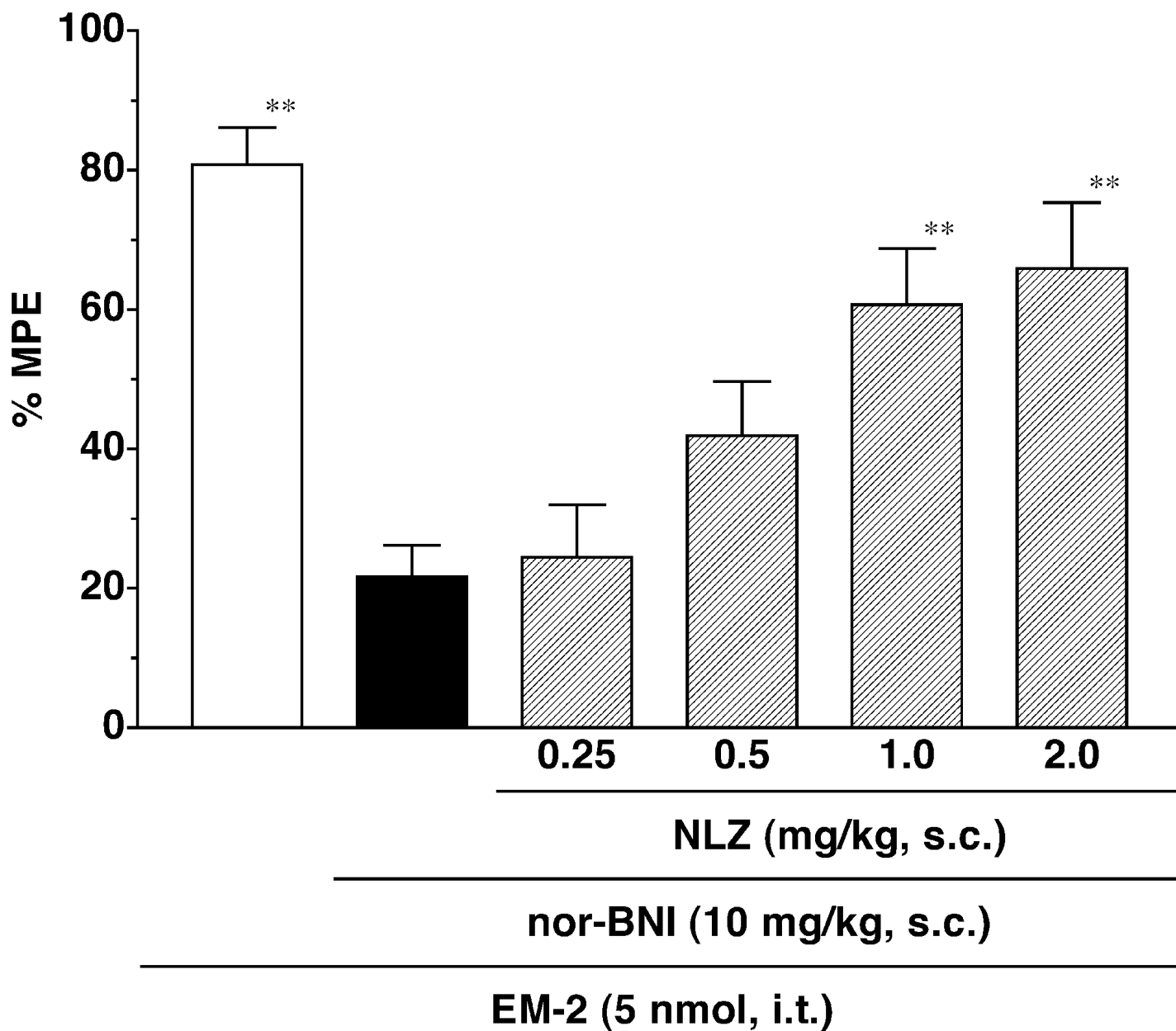




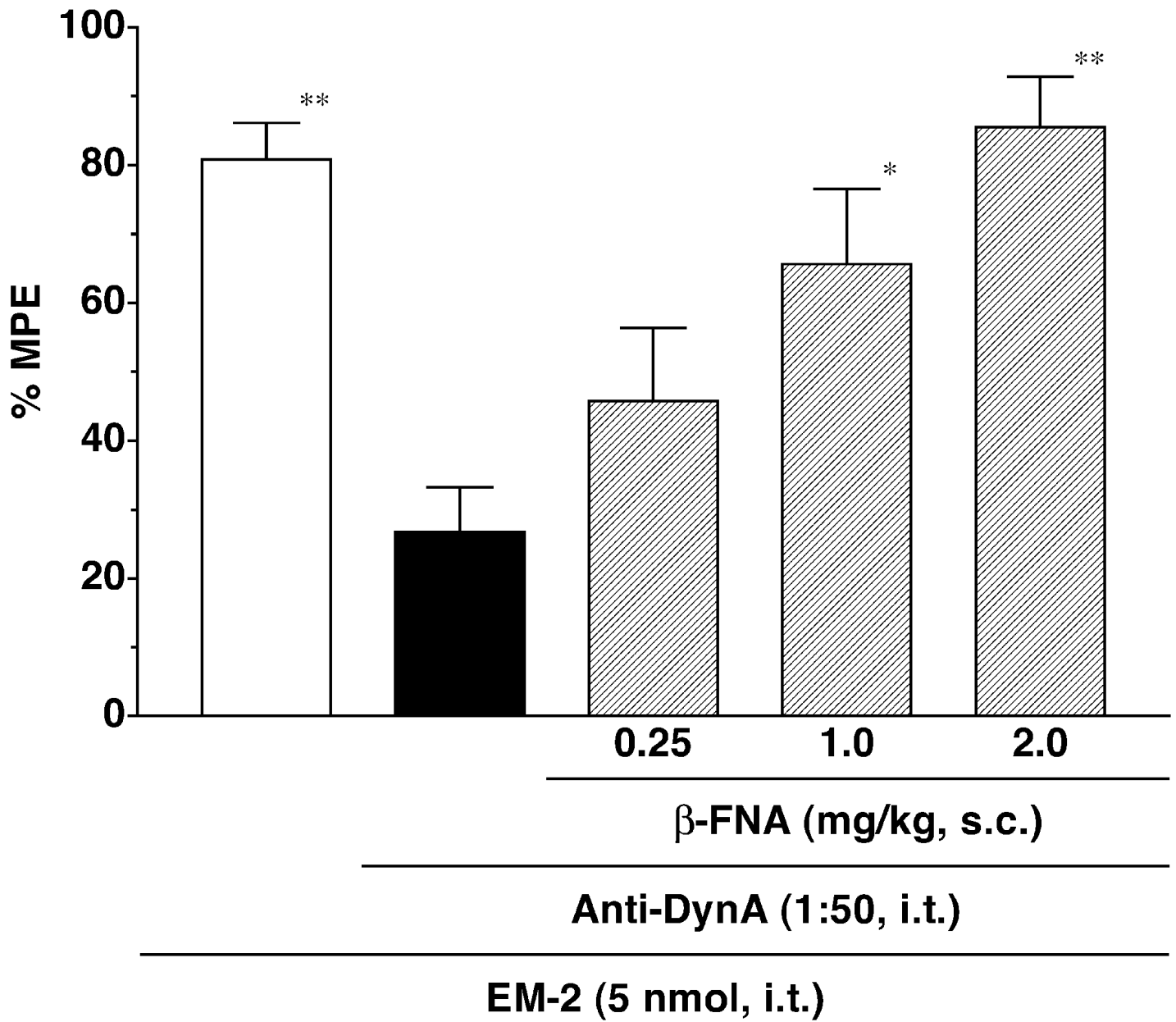
**Figure 4**



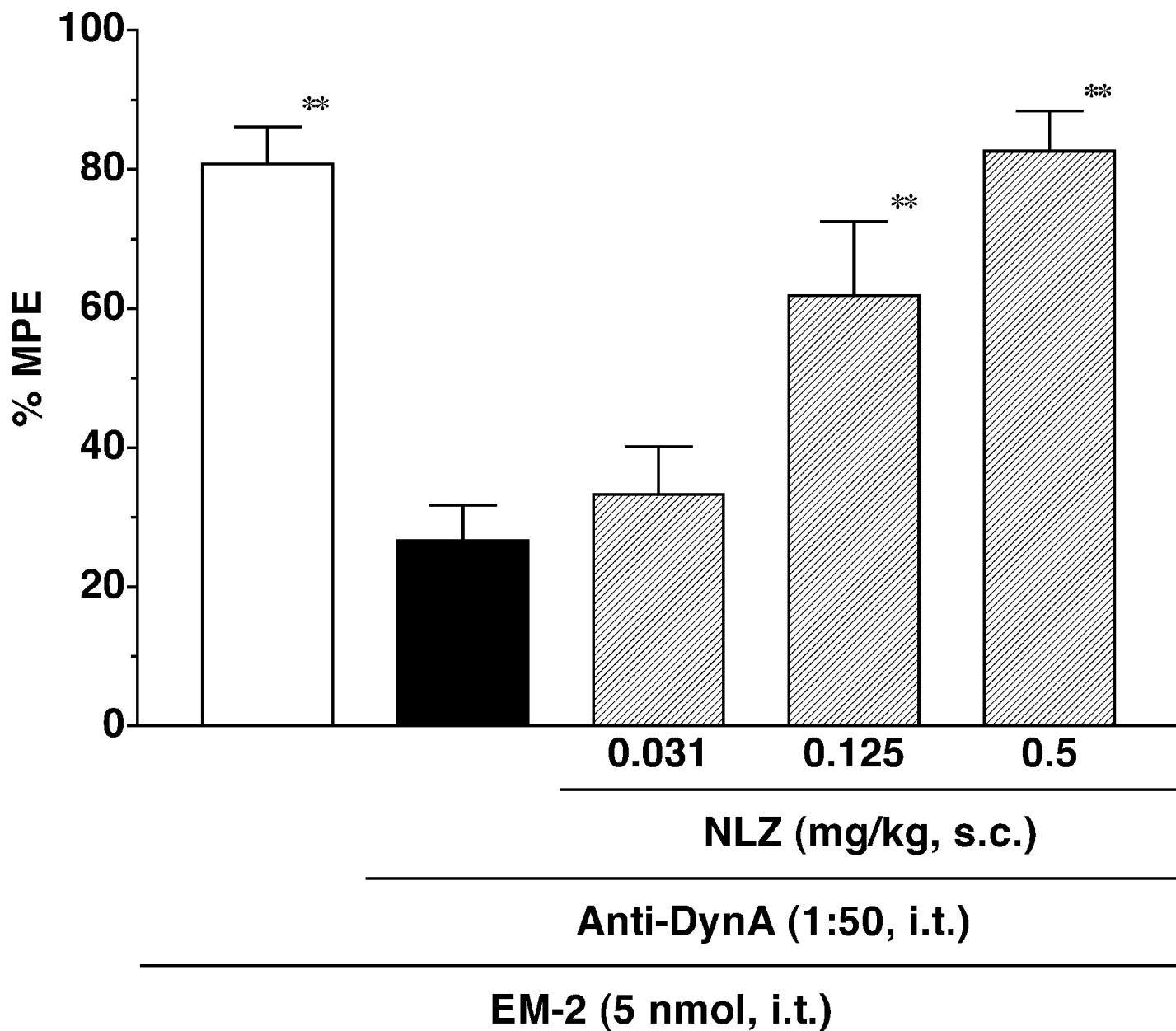
**Figure 5**



**Figure 6**



**Figure 7**



# Figure 8

