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**Differential mechanisms of antianalgesia induced by endomorphin-1 and  
endomorphin-2 in the ventral periaqueductal gray of the rat**

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**d) Abbreviations:**

EM-1, endomorphin-1; EM-2, endomorphin-2; NTI, naltrindole; nor-BNI, nor-binaltorphimine; CCK, cholecystokinin; Dyn, Dynorphin A (1-17); A/S Dyn, antiserum against Dynorphin A (1-17); NRS, normal rabbit serum.

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## Abstract

The effects of pretreatment with endomorphin-1 (EM-1) and endomorphin-2 (EM-2) given into the ventral periaqueductal gray (vPAG) to induce antianalgesia against the tail-flick (TF) inhibition produced by morphine given into the vPAG were studied in rats. Pretreatment with EM-1 (3.5-28 nmol) given into vPAG for 45 min dose-dependently attenuated the TF inhibition produced by morphine (9 nmol) given into vPAG. Similarly, pretreatment with EM-2 (1.7-7.0 nmol) for 45 min also attenuated the TF inhibition induced by morphine. However, a high dose of EM-2 (14 nmol) did not attenuate the morphine-produced TF inhibition. The attenuation of morphine-produced TF inhibition induced by EM-2 or EM-1 pretreatment was blocked by pretreatment with  $\mu$  opioid antagonist (-)-naloxone (55 pmol) but not non-opioid (+)-naloxone (55 pmol). However, pretreatment with a morphine-6 $\beta$ -glucuronide sensitive  $\mu$ -opioid receptor antagonist 3-methoxynaltrexone (6.4 pmol) selectively blocked EM-2-, but not EM-1-induced antianalgesia. Pretreatment with dynorphin A(1-17) antiserum reversed only EM-2-, but not EM-1-induced antianalgesia. Pretreatment with antiserum against  $\beta$ -endorphin, [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin, substance P or cholecystokinin, or with  $\delta$ -opioid receptor antagonist naltrindole (2.2 nmol) or  $\kappa$ -opioid receptor antagonist norbinaltorphimine (6.6 nmol) did not affect EM-2-induced antianalgesia. It is concluded that EM-2 selectively releases dynorphin A(1-17) by stimulation of a novel subtype of  $\mu$ -opioid receptor, tentatively designated as  $\mu_3$  in the vPAG to induce antianalgesia, while the antianalgesia induced by EM-1 is mediated by the stimulation of another subtype of  $\mu_1$  or  $\mu_2$ -opioid receptor.

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Endogenous opioid tetrapeptides, endomorphin-1 (EM-1) and endomorphin-2 (EM-2), have been found to be highly selective for  $\mu$ -opioid receptors (Zadina et al., 1997). Both EM-1 and EM-2 potently compete with  $\mu$ -opioid receptor binding with no appreciable affinity with  $\delta$ - and  $\kappa$ -opioid receptors and selectively activate  $\mu$ -opioid receptor mediated G-proteins (Goldberg et al., 1998; Monory et al., 2000; Narita et al., 1998). The increases of the [<sup>35</sup>S]GTP $\gamma$ S binding and antinociceptive effects induced by both EM-1 and EM-2 are selectively blocked by the pretreatment with selective  $\mu$ -opioid receptor antagonist,  $\beta$ -funaltrexamine or D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH<sub>2</sub>, indicating that the effects are mediated by the stimulation of  $\mu$ -opioid receptors.

The  $\mu$ -opioid receptors have been classified into  $\mu_1$ - and  $\mu_2$ -opioid receptors (Pasternak et al., 1980; Pasternak and Wood, 1986). Recent studies indicate that the antinociceptive effect produced by EM-1 and EM-2 is mediated by the stimulation of different subtypes of  $\mu$ -opioid receptors. This view is evidenced by the findings that pretreatment with  $\mu_1$ -receptor antagonist naloxonazine or a novel morphine-6 $\beta$ -glucuronide sensitive  $\mu$ -opioid receptor antagonist 3-methoxynaltrexone (Brown et al., 1997) selectively attenuates EM-2- but not EM-1-produced antinociception, suggesting that the EM-2-produced antinociception is mediated by the stimulation of  $\mu_1$  and a novel  $\mu$ -opioid subtype receptors (Sakurada et al., 1999, 2000, 2001). Pretreatment with D-Pro<sup>2</sup>-endomorphin-1 blocks the antinociception produced by endomorphin-1, but not endomorphin-2 and pretreatment with D-Pro<sup>2</sup>-endomorphin-2 blocks the antinociception produced by endomorphin-2, but to a much less extent, endomorphin-1 (Hung et al., 2002). Pretreatment with antisense deoxynucleotide against G<sub>i2</sub> protein blocks the

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antinociception induced by EM-2, but not EM-1, while pretreatment with antisense deoxynucleotide against  $G_{i1}$  and  $G_{i3}$  proteins blocks both antinociception induced by EM-1 and EM-2, indicating that  $\mu$ -opioid coupled G proteins such as  $G_{i2}$  is regulated by EM-2 but not EM-1 while  $G_{i1}$  and  $G_{i3}$  are regulated by both EM-1 and EM-2 (Sanchez-Blazquez et al., 1999). Blockade of  $\kappa$ -opioid receptors by pretreatment with norbinaltorphimine (nor-BNI) attenuates only EM-2-, but not EM-1-produced antinociception. These findings strongly support the view that EM-1 and EM-2 stimulate different subtypes of  $\mu$ -opioid receptors to produce their pharmacological functions. The stimulation of this EM-2 sensitive  $\mu$ -opioid receptor subsequently induces the release of dynorphin A(1-17) (Dyn) acting on  $\kappa$ -opioid receptors for producing antinociception (Tseng et al., 2000; Ohsawa et al., 2001).

We have previously demonstrated that intrathecal pretreatment of mice with a small dose of EM-2 attenuates the analgesia produced by intrathecal administration of morphine; the phenomenon has been defined as antianalgesia (Wu et al., 2003). The antianalgesia induced by EM-2 against morphine-produced analgesia has been postulated to be specifically mediated by the stimulation of a novel subtype of  $\mu$ -opioid receptor, tentatively designated as  $\mu_3$ . This view is supported by the findings that EM-2 pretreatment induces antianalgesia, which is blocked by the pretreatment with 3-methoxynaltrexone. Pretreatment with EM-2 subsequently causes the release of Dyn to induce antianalgesia.

The ventral periaqueductal gray area (vPAG) of the mesencephalon is a primary site sensitive to opioid agonists for producing analgesia (Smith et al., 1988; Yaksh et al., 1988). Microinjection of morphine into the vPAG consistently produces analgesia, which

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is blocked by the pretreatment with naloxone. The analgesia produced by opioid agonists from the vPAG is mediated by the activation of the spinopetal descending pain control pathways, which are initiated from the vPAG via the rostral ventromedial medulla projecting to the spinal and trigeminal dorsal horns (Basbaum and Fields, 1984). The present study was then designed to examine the antianalgesic effect of EM-2 against the analgesia produced by morphine microinjected into vPAG. The antianalgesic effect of EM-1 was also studied for comparison.

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## Materials and Methods

**Animals.** Male CD rats (Charles River Laboratories Inc., Wilmington, MA) weighing between 300 to 350 g at the time of surgery were housed in pairs before and after surgery. They were maintained in a room at  $22 \pm 0.5^\circ\text{C}$  with an alternating 12 h light/dark cycle. Food and water were available *ad libitum*. All experiments were approved by and conformed to the guidelines of the Animal Care Committee of the Medical College of Wisconsin.

**Surgical procedures.** Rats were pretreated with methylatropine bromide (5 mg/kg, intraperitoneal), anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal) and were mounted in a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA). A 23 gauge stainless steel guide cannula 12 mm in length was then implanted unilaterally 3 mm down from the surface of the skull and anchored to the skull with three stainless screws and dental cement. The coordinates for placement of the cannula for vPAG microinjection were AP 1.20 mm anterior to interaural point and 0.7 mm lateral to the midline (Paxinos and Watson, 1997). After a recovery period of at least 5 days, animals were used for the experiments.

**Assessment of Analgesia.** Analgesic responses were measured with the tail-flick (TF) test (D'Amour and Smith, 1941). To measure the latency of the TF response, rats were gently held by hand and their tail positioned on the apparatus (Model TF6; EMDIE Instrument Co., Maidens, VA). The TF response was elicited by applying radiant heat to the dorsal surface of the tail. The intensity of the heat stimulus was set to provide a pre-

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drug TF response time of 3 to 4 s. The cutoff time was set at 8 s to minimize tissue damage.

**Experimental Protocol.** Groups of rats were pretreated with EM-1, EM-2 or vehicle before microinjection of morphine (9 nmol) and the TF response was measured at 5, 10, 20, 30, 40, 60, 90 and 120 min thereafter. The following three experiments were performed: 1) Determine the time course and dose response effects of the development of EM-2-induced antianalgesia against morphine-produced antinociception. Groups of rats were pretreated with EM-2 (7.0 nmol) given into the vPAG at different times (30, 45, 90, and 180 min, respectively) before microinjection of morphine (9 nmol) given into vPAG and the antinociception produced by morphine was determined by the TF test. Similar protocol was also used to determine the dose-response of EM-1-induced antianalgesia. 2) Determine the type of receptors involved in the EM-2- and EM-1-induced antianalgesia against morphine-produced analgesia. Rats were pretreated with opioid receptor antagonists, (-)-naloxone (50 min), (+)-naloxone (50 min), 3-methoxynaltrexone (25 min) (Sakurada et al., 2000), nor-binaltorphimine (nor-BNI) (24 h) (Wu et al., 2003) or naltrindole (NTI) (10 min) (Dervisogullari et al., 1996) into the vPAG before vPAG injection of EM-2 followed by vPAG injection of morphine 45 min thereafter. The TF response was then measured 20 min after morphine injection. Other groups of rats were pretreated with (-)-naloxone (50 min), 3-methoxynaltrexone (25 min) or vehicle given into the vPAG before vPAG injection of EM-1 followed by vPAG injection of morphine 45 min thereafter. 3) Determine what endogenous opioid peptides were released by EM-2 for the induction of antianalgesia. Rats were pretreated into the vPAG with antiserum against Dyn, [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin,  $\beta$ -endorphin, cholecystinin (CCK),

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normal rabbit serum (NRS) or substance P given into the vPAG 1 hr prior to EM-2 vPAG injection, followed by vPAG injection of morphine 45 min thereafter. The tail-flick response was then measured 20 min after morphine injection. Similar protocol using antiserum against Dynorphin A(1-17) (A/S Dyn) was also used to determine if Dyn is involved in EM-1-induced antianalgesia against morphine-produced TF inhibition from the vPAG. Previous studies with antisera to inactivate the endogenous neuropeptides have indicated that 1 hour of pretreatment time and the doses used are sufficient for their specific effects (Tseng and Huang, 1992; Xu and Tseng, 1997; Arts et al., 1992; Wu et al., 2004). To test whether Dyn alone induced antianalgesia against morphine-produced analgesia. Groups of rats were pretreated with different doses of Dyn (0.5, 5 and 47 fmol) (Holmes and Fujimoto, 1992) given into the vPAG 5 min prior to vPAG injection of morphine. TF response was then measured 20 min after morphine injection.

**Drugs and Drug Administration.** EM-2 (Tyr-Pro- Phe-Phe-NH<sub>2</sub>) and EM-1 (Tyr-Pro-Trp-Phe-NH<sub>2</sub>) were obtained from Calbiochem Corp. (La Jolla, CA). Morphine sulfate, nor-BNI and NTI were obtained from the National Institution of Drug Abuse (Baltimore, MD). 3-Methoxynaltrexone hydrochloride and naloxone were obtained from Sigma (St. Louis, MO). Dyn was obtained from Bachem (King of Prussia, PA). The EM-2 and EM-1 were dissolved in sterile saline solution (0.9% NaCl solution) containing 10% hydroxypropyl-beta-cyclodextrin, and morphine sulfate, nor-BNI, NTI and 3-methoxynaltrexone hydrochloride were dissolved in sterile 0.9% NaCl solution. Dyn was dissolved in a 0.9% saline solution containing 0.01% Triton X-100. The antiserum against Dyn,  $\beta$ -endorphin, [Leu<sup>5</sup>]enkephalin, [Met<sup>5</sup>]enkephalin, substance P and CCK were produced by immunization of male New Zealand white rabbits according to the

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method described in previous publications. The potencies and cross-immunoreactivities of these antisera have been characterized (Tseng and Huang, 1992; Wu et al., 2004). The cross-immunoreactivity between EM-1/EM-2 and A/S Dyn was found to be less than 0.001%.

Injections into the vPAG were made by hand with a 30-gauge injection needle attached to a microsyringe via polyethylene tubing. The injection needle was inserted directly into the guide cannula. Injection volume for each microinjection was 0.5  $\mu$ l and solutions were administered over a 30 s period. The injection needles were left in place for an additional 60 s to ensure complete distribution. The stereotaxic coordinates of the vPAG injection site were aimed at AP 1.20 mm anterior to interaural point, 0.7 mm lateral to the midline and 5.8 mm down from the surface of the skull.

**Histological Identification of the Injection Site.** At the end of the experiments 0.5  $\mu$ l of methylene blue solution (2%) was injected into the vPAG. The rats were then sacrificed with CO<sub>2</sub> (100 %) 10 to 20 min after injection. The brains were removed, frozen and sectioned sagittally for microscopic identification of the injection sites. The stereotaxic atlas of rats by Paxinos and Watson (1997) was used as a guide for the identification of anatomical injection sites. Only the data obtained from rats, in which the injection sites were accurately identified to be in vPAG were used for further statistical analysis.

**Statistical Analysis.** The analgesic responses, TF latency (s), were presented as the mean  $\pm$  S.E.M. One-way analysis of variance (ANOVA) followed by Dunnett's post-test, two-way ANOVA followed by Bonferroni's post-test or Student's *t*-test was used to test the difference between groups. Nonlinear regression model was used to fit the dose-

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response curve and calculate the ED<sub>50</sub> values and 95% confidence intervals for EM-2 and EM-1 induced antianalgesia. The GraphPad Prism software was used to perform the statistics (version 3.0; GraphPad Software, Inc., San Diego, CA).

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## Results

**Effects of different times of pretreatment with EM-2 given into the vPAG on the TF inhibition produced by vPAG-administered morphine.** Groups of rats were injected with EM-2 (7.0 nmol) into the vPAG at 30, 45, 90 or 180 min before vPAG injection of morphine (9 nmol). Another group of rats pretreated with vehicle given into the vPAG served as control. vPAG pretreatment with EM-2 time-dependently attenuated the TF inhibition produced by vPAG morphine; the attenuation developed in 30 min, reached its maximum effect at 45 min, and returned to control level in 90 to 180 min after pretreatment (Fig. 1). Pretreatment with EM-2 for 45 min was then used for the following experiments.

**Effect of vPAG pretreatment with different doses of EM-2 or EM-1 on the TF inhibition produced by vPAG-administered morphine** Groups of rats were microinjected into the vPAG with saline vehicle or different doses (1.7 to 14 nmol) of EM-2, and morphine (9 nmol) was microinjected into the vPAG 45 min later. The TF response was measured at different times thereafter. Morphine (9 nmol) microinjected into the vPAG produced TF inhibition in rats pretreated with saline vehicle for 45 min; the TF inhibition developed in 5 to 10 min, reached a maximal inhibition at 20 (7.8 +/- 0.2 sec) min and gradually returned to preinjection control level in 120 min. EM-2 at doses 1.7 to 7.0 nmol given into the vPAG did not cause any change in TF latency observed for 45 min. However, such a pretreatment with EM-2 dose-dependently attenuated the TF inhibition produced by morphine (9 nmol). A high dose of EM-2 (14 nmol) given into vPAG produced TF inhibition; the TF inhibition developed in 2.5-10 min, reached a maximal level at 20 min and returned to preinjection level at 45 min after

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injection. Such a treatment with a high dose of EM-2, however, did not attenuate the TF inhibition produced by morphine (9 nmol) given into vPAG (Fig. 2A and 2B).

The effect of pretreatment with EM-1 given into the vPAG on morphine-produced TF inhibition given into vPAG was also studied. Groups of rats were pretreated with 3.5 to 28 nmol of EM-1 or vehicle given into the vPAG 45 min before vPAG-administered morphine (9 nmol). EM-1 at doses 3.5 and 7.0 nmol did not cause any change in TF latency observed for 45 min, but at a high dose (14 nmol) produced TF inhibition. The TF inhibition developed in 2.5-10 min, reached its peak at 20 min and returned to the preinjection level in 45 min. Such a pretreatment with EM-1 dose-dependently attenuated vPAG morphine-produced TF inhibition. The morphine-produced TF inhibition was almost completely abolished by the pretreatment with 7.0 or 14 nmol of EM-1 (Fig. 3A and 3B). Thus, EM-1 was found to be equally potent as EM-2 in inducing antianalgesia in the vPAG; the ED<sub>50</sub> values (with 95 % confidence intervals) for EM-1 and EM-2 pretreatment into the vPAG were estimated to be 3.28 (1.42 to 7.6) nmol and 3.76 (1.67 to 8.5) nmol, respectively. However, EM-2 was found to be different from that of EM-1 that pretreatment with a high dose (14 nmol) of EM-2 did not attenuate the morphine-produced TF inhibition.

**Effects of vPAG pretreatment with (-)-naloxone, (+)-naloxone, 3-methoxynaltrexone, NTI or nor-BNI on the vPAG EM-2-induced antianalgesia against vPAG morphine-produced TF inhibition** (-)-Naloxone given intracerebrally is a short acting  $\mu$ -opioid receptor antagonist and is expected to block with short duration the antianalgesic effects induced by  $\mu$ -opioid agonists EM-1 or EM-2, but not long enough to block the antinociceptive effect produced by morphine. The first experiment

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was designed to determine the optimal pretreatment time for (-)-naloxone to block EM-1- or EM-2-induced antianalgesia, but not affect the morphine-produced TF inhibition. Groups of rats were injected with (-)-naloxone (55 pmol) or saline vehicle given into the vPAG 55, 65, 75 or 95 min before vPAG injection of morphine (9 nmol) and TF response was performed 20 min after injection. Pretreatment with (-)-naloxone for 55 or 65 min, but not 95 min, significantly attenuated the morphine-produced TF inhibition (Fig 4). Ninety-five min of the pretreatment time for (-)-naloxone (50 min before EM-2 injection) was then chosen for the following studies.

The experiments were undertaken to determine what type of opioid receptors are involved in EM-2-induced antianalgesia against morphine-produced TF inhibition. Pretreatment with  $\mu$ -opioid receptor antagonists (-)-naloxone (55 pmol) or 3-methoxynaltrexone (6.4 pmol) effectively blocked the EM-2-induced antianalgesia and restored the morphine-produced TF inhibition. However, pretreatment with (+)-naloxone (55 pmol), a non-opioid enantiomer of (-)-naloxone, did not affect the attenuation of the morphine-produced TF inhibition induced by EM-2 pretreatment. Pretreatment with selective  $\delta$ -opioid receptor antagonist NTI (2.2 nmol) and  $\kappa$ -opioid receptor antagonist nor-BNI (6.6 nmol) did not affect the antianalgesia induced by EM-2 compared with the vehicle-pretreated group (Fig. 5A).

**Effects of vPAG pretreatment with (-)-naloxone or 3-methoxynaltrexone on the vPAG EM-1-induced antianalgesia against vPAG morphine-produced TF inhibition** The experiments were undertaken to determine whether the antianalgesia induced by EM-1 against morphine-produced TF inhibition is mediated by the same subtype of  $\mu$ -opioid receptor as that of EM-2. Pretreatment with (-)-naloxone (55 pmol)

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effectively blocked the antianalgesia induced by EM-1 and restored the morphine-produced TF inhibition. However, pretreatment with 3-methoxynaltrexone (6.4 pmol) did not affect the attenuation of morphine-produced TF inhibition induced by EM-1 pretreatment (Fig. 5B).

**Effects of vPAG pretreatment with antiserum against Dyn, [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin,  $\beta$ -endorphin, CCK, substance P or normal rabbit serum (NRS) on the vPAG EM-2-induced antianalgesia against vPAG morphine-produced TF inhibition** Groups of rats were pretreated with different doses of A/S Dyn (50, 100, 150 or 200  $\mu$ g) given into the vPAG 1 h before administration of EM-2 (7.0 nmol), and then morphine was administered 45 min afterwards. The TF response was measured 20 min thereafter. Pretreatment with A/S Dyn dose-dependently blocked the EM-2-induced antianalgesia and enhanced the morphine-produced TF inhibition. Rats pretreated with NRS or A/S Dyn (200  $\mu$ g) alone followed by vehicle injection did not affect the morphine-produced TF inhibition (Fig. 6).

Pretreatment with antiserum against [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin,  $\beta$ -endorphin, CCK or substance P given into the vPAG did not affect the attenuation of morphine-produced TF inhibition induced by EM-2 pretreatment (Table 1).

**Effects of vPAG pretreatment with A/S Dyn on the vPAG EM-1-induced antianalgesia against vPAG morphine-produced TF inhibition** Groups of rats were pretreated with 200  $\mu$ g of A/S Dyn given into vPAG 1 h before vPAG administration of EM-1 (14 nmol), and morphine was then administered into the vPAG 45 min thereafter. The TF response was measured 20 min after morphine injection. Pretreatment with A/S

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Dyn did not affect the EM-1-induced antianalgesia against morphine-produced TF inhibition (Fig. 6).

**Effect of vPAG pretreatment with different doses of Dyn on the TF inhibition produced by vPAG-administered-morphine** Since the results of the experiments described above strongly indicate that the antianalgesia induced by EM-2 is mediated by the release of Dyn, the experiment was then taken to determine if pretreatment with Dyn peptide will mimick the effect of EM-2 and produces antianalgesia against morphine-produced TF inhibition. Groups of rats were pretreated with 0.5, 5 or 47 fmol of Dyn or saline given into the vPAG 5 min before administration of morphine (9 nmol) given into the vPAG and the TF response was measured 20 min thereafter. Pretreatment with Dyn dose-dependently attenuated morphine-produced TF inhibition (Fig. 7).

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## Discussion

**Pretreatment with EM-2 or EM-1 given into the vPAG dose-dependently induces antianalgesia against vPAG morphine-produced analgesia** We found in the present study that EM-2 (1.7 to 7.0 nmol) or EM-1 (3.5 to 14 nmol) injected into the vPAG did not produce any significant inhibition of TF responses, while the pretreatment with these doses of EM-2 or EM-1 dose-dependently attenuated the TF inhibition produced by morphine. Thus, the production of TF inhibition by EM-1 or EM-2 given into the vPAG is not required for the induction of antianalgesia. The finding clearly indicates dissociation between the production of analgesia by EM-2 or EM-1 and the induction of antianalgesia. We found that a high dose of EM-2 (14 nmol) or EM-1 (28 nmol) given into the vPAG produced TF inhibition. While pretreatment with a high antinociceptive dose of EM-1, but not EM-2, induced antianalgesia, suggesting the involvement of different neural mechanisms for EM-1 and EM-2-induced antianalgesia. Thus both EM-1 and EM-2 given into vPAG produce biphasic antinociception (analgesia) and antianalgesia. The antianalgesia induced by EM-2 and EM-1 is mediated by the stimulation of different subtypes of  $\mu$ -opioid receptors. In early reports, the antinociception induced by EM-1 and EM-2 are thought to be mediated by the stimulation of  $\mu$ -opioid receptors (Stone et al., 1997; Zadina et al., 1997; Goldberg et al., 1998; Narita et al., 1998). However, Sakurada et al. (1999) report that pretreatment with  $\mu_1$ -opioid receptor blocker naloxonazine given systemically blocks the antinociception produced by intracerebroventricular or intrathecal administration of EM-2, but not EM-1, indicating that EM-1 and EM-2 may produce antinociception through the stimulation of the  $\mu_2$  and  $\mu_1$ -opioid receptors, respectively. More recent results intimate that EM-1 and

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EM-2 might act on different subtypes of  $\mu$ -opioid receptors other than classical  $\mu_1$  and  $\mu_2$  receptors. Sakurata et al (2000) report that a novel morphine 6 $\beta$ -glucuronide sensitive  $\mu$ -opioid receptor antagonist, 3-methoxynaltrexone, blocks the antinociception produced by EM-2, but not EM-1, while  $\beta$ -funaltrexamine inhibits both. In the present study, pretreatment with EM-2 or EM-1 into the vPAG dose-dependently attenuated the analgesia produced by morphine given into the vPAG. The antianalgesia induced by EM-2 or EM-1 was blocked by the pretreatment with  $\mu$ -opioid receptor antagonist (-)-naloxone but not by its non-opioid enantiomer (+)-naloxone, indicating that the effects are mediated by the stimulation of  $\mu$ -opioid receptors. The  $\mu$ -opioid receptor antagonist 3-methoxynaltrexone, which selectively blocks EM-2- and morphine-6 $\beta$ -glucuronide-produced antinociception, but not morphine antinociception (Brown et al., 1997; Sakurata et al., 2000; Pasternak, 2004), was used to determine if it blocks the EM-2- and EM-1-induced antianalgesia. We found that 3-methoxynaltrexone pretreatment selectively blocked the EM-2-, but not EM-1-induced antianalgesia. The finding is consistent with previous reports with the studies of EM-2- and EM-1-produced antinociception (Sakurada et al., 2000; Wu et al., 2003), indicating that that the antianalgesia induced by EM-2 is mediated by the stimulation of a novel subtype of  $\mu$ -opioid receptor, tentatively designated as  $\mu_3$ , which is different from  $\mu_1$  and  $\mu_2$  opioid receptors stimulated by EM-1, morphine and other  $\mu$ -opioid agonists. It is likely that both antinociception and antianalgesia produced by EM-2 is mediated by stimulation of this tentative  $\mu_3$  opioid receptor. Whether this newly defined  $\mu_3$ -opioidis belonged to any splice variant of MOR-1 (Pan et al., 2001) is not clear at this point.

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The evidence that EM-1 and EM-2 stimulate different subtypes of  $\mu$ -opioid receptors to induce their pharmacological actions can also be obtained from our previous studies of antinociceptive effects produced by EM-1 and EM-2. Intrathecal pretreatment with antisense oligodeoxynucleotides against exon-1, -4, or -8 of  $\mu$ -opioid receptor clone to knockdown different isoforms of the  $\mu$ -opioid receptor, differentially attenuates the antinociception produced by EM-1 and EM-2 (Wu et al., 2002). These findings strongly indicate that different subtypes of  $\mu$ -opioid receptors are involved in the pharmacological actions produced by EM-1 and EM-2.

**The antianalgesia induced by EM-2, but not EM-1, is mediated by the release of Dyn** Rady and colleagues (2001) have shown that Dyn administered intrathecally attenuates intrathecal morphine-produced analgesia. We found that vPAG pretreatment with A/S Dyn, which neutralizes dynorphin action, blocked the vPAG EM-2-induced antianalgesia. This finding is in line with previous finding that intrathecal pretreatment with A/S Dyn blocks the intrathecal EM-2-induced antianalgesia (Wu et al., 2003). The attenuation of morphine-produced analgesia induced by EM-1 was not affected by the pretreatment with A/S Dyn, indicating that Dyn is not involved in EM-1-induced antianalgesia. These findings indicate that EM-2, but not EM-1, may release Dyn to induce antianalgesia. Furthermore, Dyn given into vPAG was found to mimic the effect of EM-2 and attenuate the morphine-produced analgesia. This is in line with previous studies that intrathecal administration of Dyn induces an antianalgesic action against intrathecally administered morphine (Holmes BB and Fujimoto JM, 1992; Aksu et al., 1993; Rady and Fujimoto, 2001). We have recently found that EM-2, but not EM-1, intrathecally perfused for 3 min dose-dependently increases the release of

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immunoreactive Dyn in the spinal perfusates in urethane-anesthetized rats (Leitermann et al., 2003). The increase of the release of Dyn induced by EM-2 is also blocked by (-)-naloxone and 3-methoxynaltrexone. Thus, our studies with A/S Dyn clearly indicate that Dyn is involved in EM-2- but not EM-1-induced antianalgesia. The exact neural mechanism for EM-1-induced antianalgesia is not clear. It is possible that EM-1 pretreatment may cause endocytosis and internalization of  $\mu$ -opioid receptors and attenuates the morphine-produced analgesia (McConalogue et al., 1999)

**Both analgesia and antianalgesia produced by EM-2 are mediated by the release of Dyn** Thus, Dyn released by EM-2 appears to produce biphasic effects, analgesia (Sakurada et al., 2001; Tseng et al., 2000; Ohsawa et al., 2001) and antianalgesia. This view is supported by the present finding that unlike EM-1, which induced antianalgesia even at a high dose, pretreatment with a high dose of EM-2, which itself produced antinociception, failed to attenuate the morphine-induced antinociception (Figure 2 and 3). It is reasonable to believe that EM-2 even at high doses also induces antianalgesia, but the effect is masked by the analgesic effect of Dyn released by EM-2. The antianalgesia induced by EM-2 from vPAG is not mediated by the stimulation of  $\delta$ - or  $\kappa$ -opioid receptors. This is evidenced by the finding that pretreatment with  $\delta$ -opioid receptor antagonist NTI or  $\kappa$ -opioid receptor antagonist nor-BNI given into the vPAG did not reverse the attenuation of morphine-produced TF inhibition induced by EM-2 pretreatment. The finding is in line with our previous report showing that the blockade of  $\delta$ - and  $\kappa$ -opioid receptors in the spinal cord by i.t. pretreatment with NTI and nor-BNI, respectively, do not block i.t. EM-2-induced antianalgesia (Wu et al., 2003).

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We have previously reported that pretreatment with nor-BNI or A/S Dyn given spinally or supraspinally partially, but significantly blocks the antinociception produced by EM-2 given spinally or supraspinally, indicating that the released Dyn by EM-2 subsequently stimulates  $\kappa$ -opioid receptors to produce antinociception (Tseng et al., 2000; Ohsawa et al., 2001). Thus, Dyn released by EM-2 may produce biphasic responses; an initial release of the Dyn producing analgesia, which is mediated by the stimulation of  $\kappa$ -opioid receptors, while a delay release of Dyn induces antianalgesia, which is not mediated by the stimulation of  $\kappa$ -opioid receptors. Our finding is in line with previous studies that Dyn stimulates neuronal cells via a non-opioid, non-NMDA mechanism (Millan, 1999; Tang et al., 2000; Wang et al., 2001).

**The antianalgesia induced by EM-2 is not mediated by the release of [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin,  $\beta$ -endorphin, CCK or substance P** Intrathecal pretreatment with CCK or [Leu<sup>5</sup>]enkephalin but not [Met<sup>5</sup>]enkephalin attenuates intrathecal morphine-produced analgesia. The antianalgesic effect induced by CCK can be blocked by antisera against CCK (1:1000, i.t., 1 h) and [Leu<sup>5</sup>]enkephalin (200  $\mu$ g, i.t., 1 h), respectively (Rady et al., 2001). We found in the present study that pretreatment with antiserum against [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin or  $\beta$ -endorphin given into the vPAG at the dose sufficient to inactivate endogenous peptides did not block the EM-2-induced antianalgesia. This result indicates that [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin and  $\beta$ -endorphin are not involved in EM-2-induced antianalgesia.

It has been suggested that CCK and substance P may be involved in antianalgesia against morphine-produced analgesia. Tortorici et al. (2002) showed that CCK has anti-opioid activity in the PAG and that tolerance to morphine in the PAG can be prevented or

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restored if pretreatment with the non-selective CCK receptor antagonist proglumide. Substance P can be released not only from the spinal cord but also from brain regions that are involved in endogenous pain suppression (Basbaum and Fields, 1984). The findings that substance P microinjection into the PAG produces a naloxone-reversible analgesia (Mohrland and Gebhart, 1979) suggest that substance P within the brain may play a neuromodulatory role in transmitting nociceptive information. Art et al (1992) demonstrated that substance P given intrathecally attenuates morphine-produced analgesia indicating that substance P is also antianalgesic. We found in the present study that the vPAG pretreatment with antiserum against CCK or substance P did not affect the EM-2-induced antianalgesia, indicating that CCK and substance P are not involved in the EM-2-induced antianalgesia from vPAG.

It is concluded that pretreatment with EM-2 or EM-1 given into the vPAG dose- and time-dependently induces antianalgesia against morphine-produced analgesia. The antianalgesic effects induced by EM-2 and EM-1 from vPAG is mediated by the stimulation of different subtypes of  $\mu$ -opioid receptors. The antianalgesia induced by EM-2 from vPAG is mediated by the release of Dyn acting on non-opioid receptors. [Met<sup>5</sup>]-enkephalin, [Leu<sup>5</sup>]-enkephalin,  $\beta$ -endorphin, CCK and substance P are not involved in EM-2-induced antianalgesia.

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## Footnotes

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### Legends for figures

Fig 1. Effects of different pretreatment times with EM-2 given into the vPAG on the TF inhibition produced by vPAG-administered morphine. Groups of rats were pretreated into the vPAG with 7.0 nmol of EM-2 or vehicle (0.5 ml) 30, 45, 90 or 180 min before vPAG injection of morphine (9 nmol). The tail-flick latency for vehicle or EM-2 pretreated groups for 30, 45, 90 or 180 min before morphine injection are  $3.3 \pm 0.13$ ,  $3.49 \pm 0.24$ ,  $3.18 \pm 0.15$  and  $3.13 \pm 0.09$  sec, respectively. The tail-flick latency for EM-2 pretreated groups for 30, 45, 90 or 180 min before morphine injection are  $2.81 \pm 0.12$ ,  $2.98 \pm 0.23$ ,  $2.91 \pm 0.22$  and  $3.1 \pm 0.15$  sec, respectively. The tail-flick response was measured 20 min after injection of morphine. Each column represents the mean and vertical bar represents the S.E.M. with number of animal studies in each group indicated in the column. Two-way ANOVA followed by Bonferroni's post-test was used to test the difference between groups. For group of rats injected with EM-2 versus vehicle, interaction  $F_{(3, 48)} = 1.79$ , treatment  $F_{(1, 48)} = 68.00$ , time  $F_{(3, 48)} = 2.64$ , \*  $p < 0.001$ .

Figure 2. Effects of different doses of EM-2 pretreatment given into the vPAG on the TF inhibition produced by vPAG-administered morphine. Pretreatment with EM-2 dose-dependently inhibits the TF response induced by morphine observed at different times after morphine injection (A) and 20 min after morphine injection (B). Groups of rats were injected with 1.7, 3.5, 7.0 or 14.0 nmol of EM-2 or saline vehicle given into the vPAG 45 min before vPAG injection of morphine or vehicle. TF responses were measured at different times after EM-2 and morphine injections. The number of animals studied in each group is indicated in parenthesis in (A) or in the column in (B). Two-way ANOVA

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followed by Bonferroni's post-test (A) and One-way ANOVA followed by Dunnett's post-test (B) were used to test the difference between groups. For the group of rats injected with EM-2 versus vehicle, interaction  $F_{(40, 288)} = 3.26$ , treatment  $F_{(5, 288)} = 65.64$ , time  $F_{(8, 288)} = 34.26$  (A) and  $F_{(4, 27)} = 9.32$  (B), \*  $p < 0.01$ , \*\*  $p < 0.001$ .

Figure 3. Effects of different doses of EM-1 pretreatment given into the vPAG on the TF inhibition produced by vPAG-administered morphine. Pretreatment with EM-1 dose-dependently inhibits the TF response induced by vPAG-administered morphine observed at different times after morphine injection (A) and 20 min after morphine injection (B). Groups of rats were injected with 3.5, 7, 14 or 28 nmol of EM-1 or saline vehicle given into the vPAG 45 min before vPAG injection of morphine (9 nmol) or vehicle. TF responses were measured at different times after EM-1 and morphine injections. Two-way ANOVA followed by Bonferroni's post-test (A) and One-way ANOVA followed by Dunnett's post-test (B) were used to test the difference between groups. The number of animals studied in each group is indicated in parenthesis in (A) or in the column in (B). For the group of rats injected with EM-1 versus vehicle, interaction  $F_{(40, 324)} = 3.31$ , treatment  $F_{(5, 324)} = 38.51$ , time  $F_{(8, 324)} = 13.29$  (A) and  $F_{(4, 31)} = 7.84$  (B); \*  $p < 0.01$ , \*\*  $p < 0.001$ .

Figure 4. Effects of different pretreatment times with (-)-naloxone given into the vPAG on the TF inhibition produced by vPAG-administered morphine. Groups of rats were pretreated into the vPAG with 55 pmol of (-)-naloxone or vehicle (0.5  $\mu$ l) 55, 65, 75 or 95 min before vPAG injection of morphine (9 nmol). The tail-flick response was

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measured 20 min after injection of morphine. Each column represents the mean and vertical bar represents the S.E.M. with number of animal studies in each group indicated in the column. Two-way ANOVA followed by Bonferroni's post-test was used to test the difference between groups. For the group of rats injected with EM-2 versus vehicle, interaction  $F_{(3, 42)} = 3.66$ , treatment  $F_{(1, 42)} = 40.11$ , time  $F_{(3, 42)} = 3.40$ , \*  $p < 0.001$ .

Figure 5. (A) Effects of vPAG pretreatment with (-)-naloxone, (+)-naloxone, 3-methoxynaltrexone (3-MN), NTI or nor-BNI given into the vPAG on the reversal of vPAG morphine-produced TF inhibition induced by vPAG EM-2 pretreatment, and (B) effects of vPAG pretreatment with (-)-naloxone or 3-methoxynaltrexone (3-MN) on the reversal of vPAG morphine-produced TF inhibition induced by vPAG EM-1 pretreatment. Groups of rats were pretreated into the vPAG with (-)-naloxone (55 pmol) 50 min, (+)-naloxone (55 pmol) 50 min, 3-methoxynaltrexone (6.4 pmol) 25 min, NTI (2.2 nmol) 10 min or nor-BNI (6.6 nmol) 24 h before vPAG injection of EM-2 (7.0 nmol) or EM-1 (14 nmol) and were then injected into the vPAG with morphine (9 nmol) 45 min after EM-2 or EM-1 injection. Other groups were injected into the vPAG with saline vehicle and served as control. The TF response was measured 20 min after morphine injection. Each column represents the mean and the vertical bar represents the S.E.M. with 6 to 7 rats in each group. Student t-test was used to test difference between groups. For group of rats pretreated with (-)-Naloxone or 3-methoxynaltrexone versus vehicle followed by EM-2 challenge,  $t=4.95$ ,  $7.99$  and  $df=11$ ,  $10$ , respectively (A). For each group of rats pretreated with (-)-Naloxone versus vehicle followed by EM-1 challenge,  $t=8.88$ ,  $df=12$  (B); \*  $p < 0.001$ .

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Figure 6 Effect of vPAG pretreatment with various doses of antiserum against dynorphin A(1-17) (A/S Dyn) on the reversal of vPAG morphine-produced TF inhibition induced by EM-2 or EM-1 vPAG pretreatment. Groups of rats were administered into the vPAG with various doses of A/S Dyn or normal rabbit serum (NRS) 60 min before vPAG injection of EM-2 (7.0 nmol) or EM-1 (14 nmol) and morphine (9 nmol) was injected into the vPAG 45min after EM-2 or EM-1 injection. The tail-flick response was measured 20 min after morphine injection. Each column represents the mean and the vertical bar represents the S.E.M. with 6 to 7 rats in each group. Two-way ANOVA followed by Bonferroni's post-test was used to test the difference between groups. For each group of rats pretreated with A/S Dyn versus vehicle followed by EM-2 or EM-1 challenge, interaction  $F_{(6, 75)} = 6.40$ , treatment  $F_{(1, 75)} = 23.59$ , time  $F_{(6, 75)} = 38.14$ , \*  $p < 0.001$ .

Figure 7 Effect of vPAG pretreatment with various doses of dynorphin A(1-17) Dyn on the TF inhibition produced by vPAG-administered morphine. Groups of rats were administered into the vPAG with various doses (0.5, 5 or 47 fmol) of Dyn or vehicle 5 min before vPAG injection of morphine (9 nmol). The TF response was measured 20 min after morphine injection. Each column represents the mean and the vertical bar represents the S.E.M. with 6 to 7 rats in each group. One-way ANOVA followed by Dunnett's post-test was used to test the difference between groups. For each group of rats pretreated with Dyn versus vehicle,  $F_{(3, 22)} = 15.09$ ; \*  $p < 0.001$ .

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**Table 1. Effects of pretreatment with antiserum against Dynorphin A (1-17), [Met<sup>5</sup>]enkephalin, [Leu<sup>5</sup>]enkephalin,  $\beta$ -endorphin, cholecystokinin or substance P on the attenuation of morphine-produced analgesia induced by EM-2 pretreatment.**

Groups of rats were administered antiserum against dynorphin A (1-17) (200  $\mu$ g), [Met<sup>5</sup>]enkephalin (200  $\mu$ g), [Leu<sup>5</sup>]enkephalin (200  $\mu$ g),  $\beta$ -endorphin (200  $\mu$ g), cholecystokinin (200  $\mu$ g), substance P (10  $\mu$ g) or normal rabbit serum (200  $\mu$ g) given into the vPAG 1 h before vPAG injection of EM-2 (7.0 nmol) and were injected given with morphine (9 nmol) given into vPAG 45 min thereafter. The tail-flick response was measured 20 min after morphine injection.

Antiserum (N) <sup>b</sup>	EM-2 (7.0 nmol)	TF latency (s)
	vPAG pretreatment	by vPAG morphine (9 nmol)
Normal rabbit serum (6)	No	7.23 $\pm$ 0.71
A/S Dynorphin A(1-17) (7)	No	7.31 $\pm$ 0.29
Normal rabbit serum (7)	Yes	4.26 $\pm$ 0.39
A/S <sup>c</sup> Dynorphin A(1-17) (6)	Yes	7.48 <sup>a</sup> $\pm$ 0.52
A/S [Met <sup>5</sup> ]enkephalin (6)	Yes	3.6 $\pm$ 0.14
A/S [Leu <sup>5</sup> ]enkephalin (6)	Yes	3.78 $\pm$ 0.25
A/S $\beta$ -endorphin (6)	Yes	3.7 $\pm$ 0.17
A/S cholecystokinin (7)	Yes	3.64 $\pm$ 0.18
A/S substance P (6)	Yes	3.45 $\pm$ 0.16

<sup>a</sup> Data were expressed as mean  $\pm$  S.E.M. One-way ANOVA followed by Dunnett's post-test was used to test the difference between groups. \*  $p < 0.001$ .

<sup>b</sup> N represented the number of rats in each group.

<sup>c</sup> A/S: antiserum

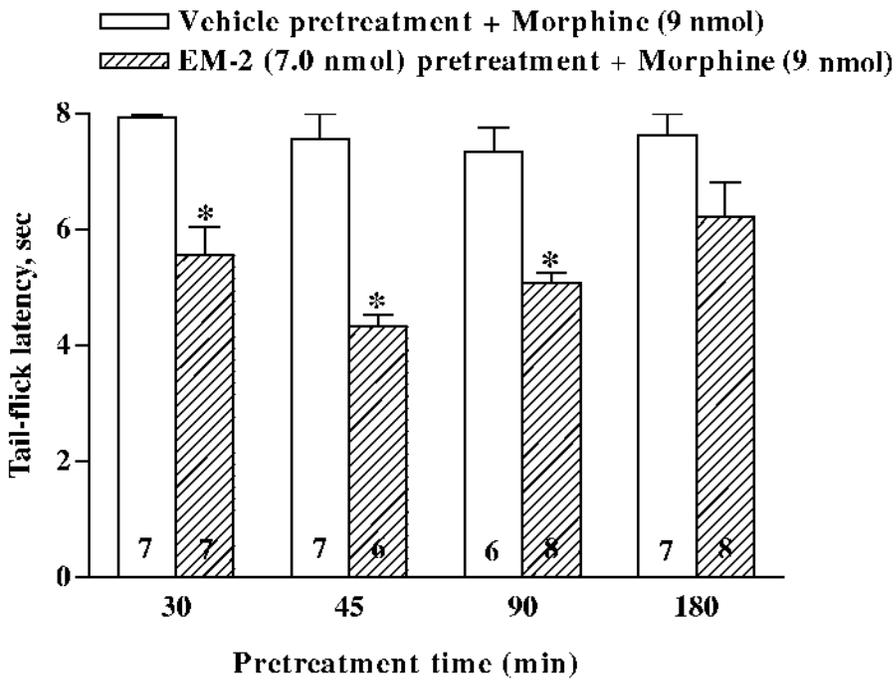


Fig 1

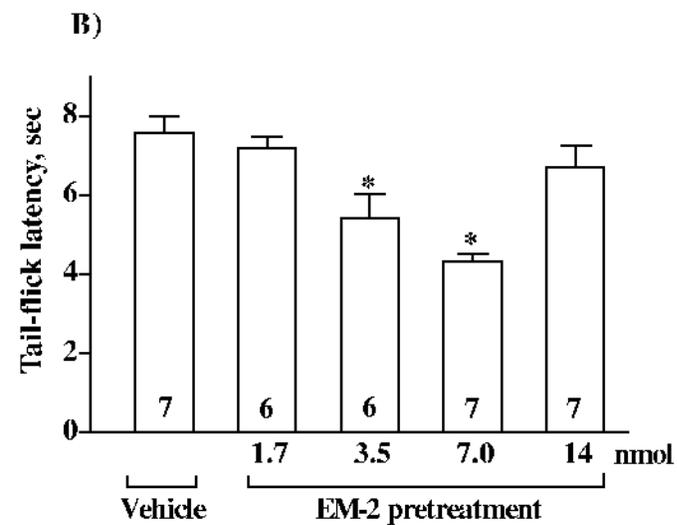
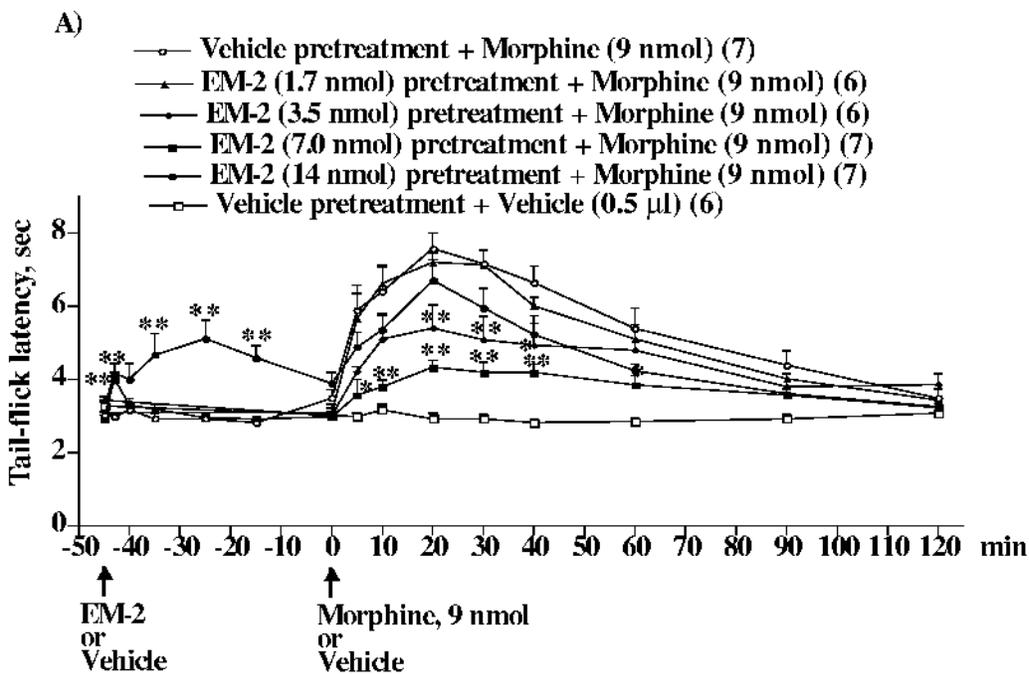


Fig 2 A,B

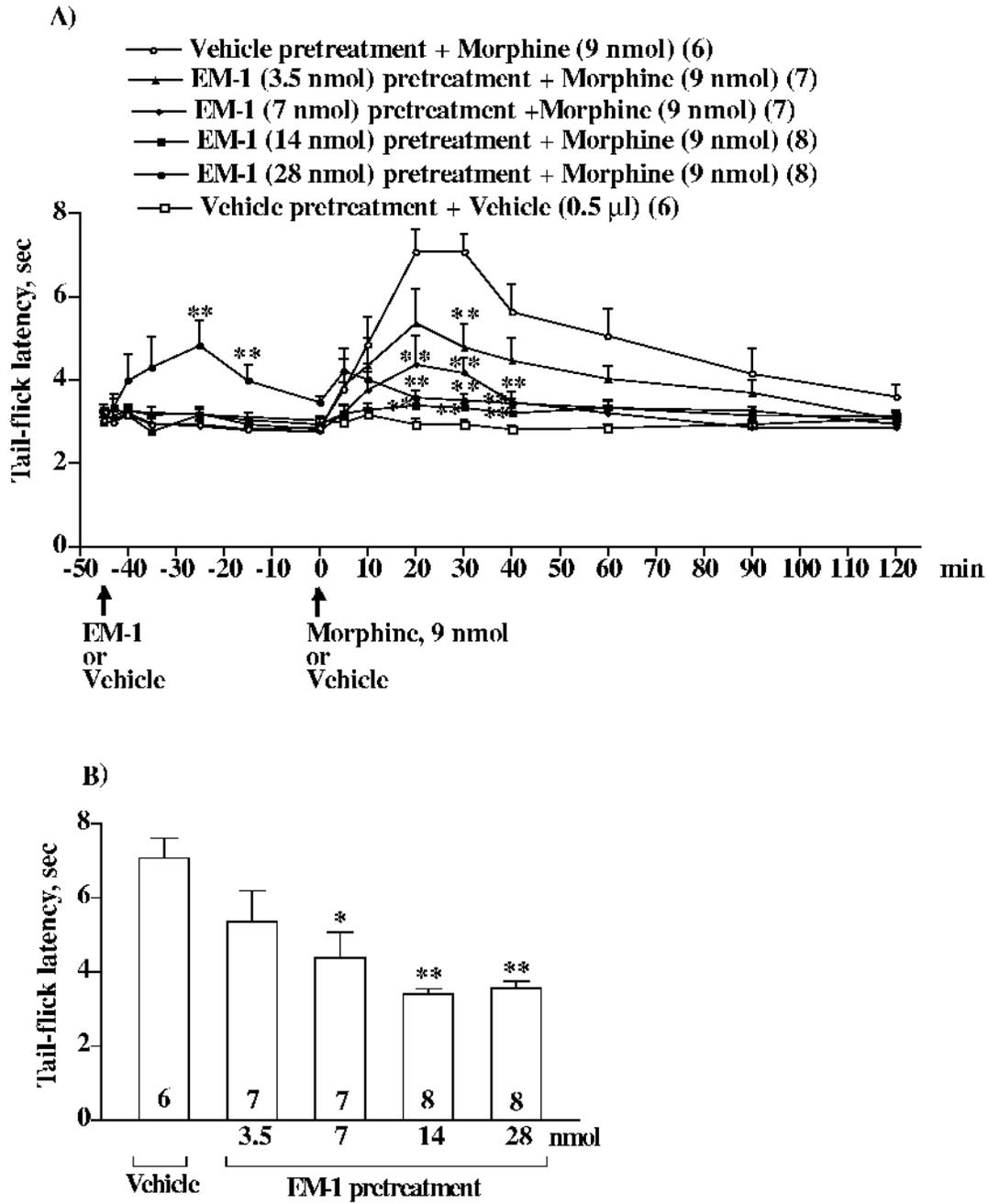


Fig 3 A,B

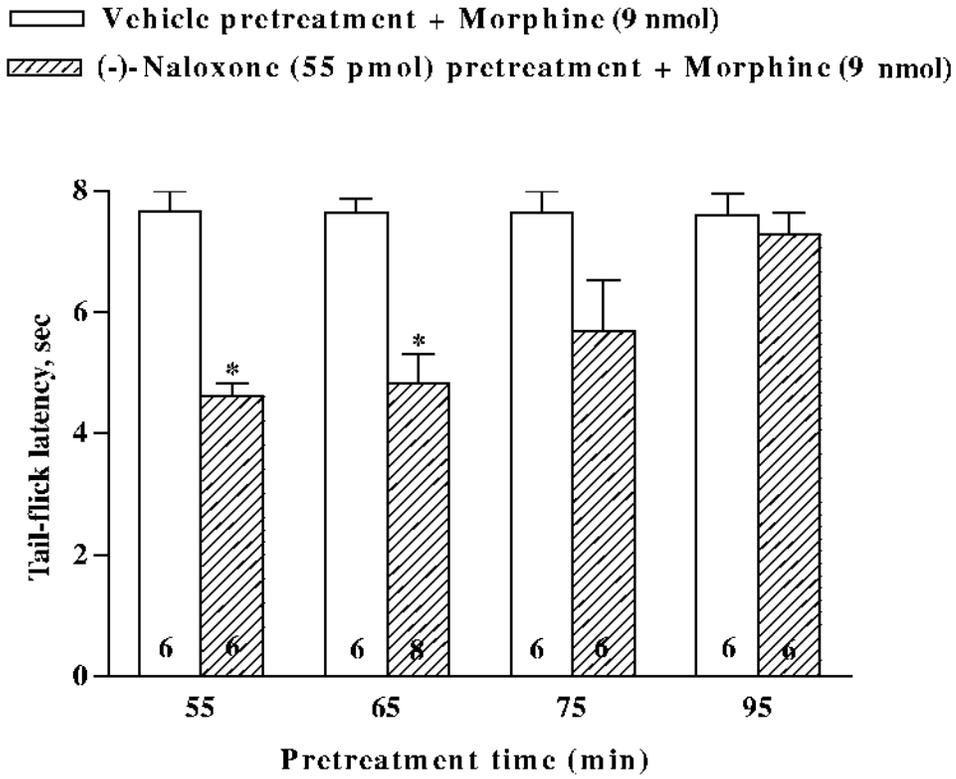


Fig 4

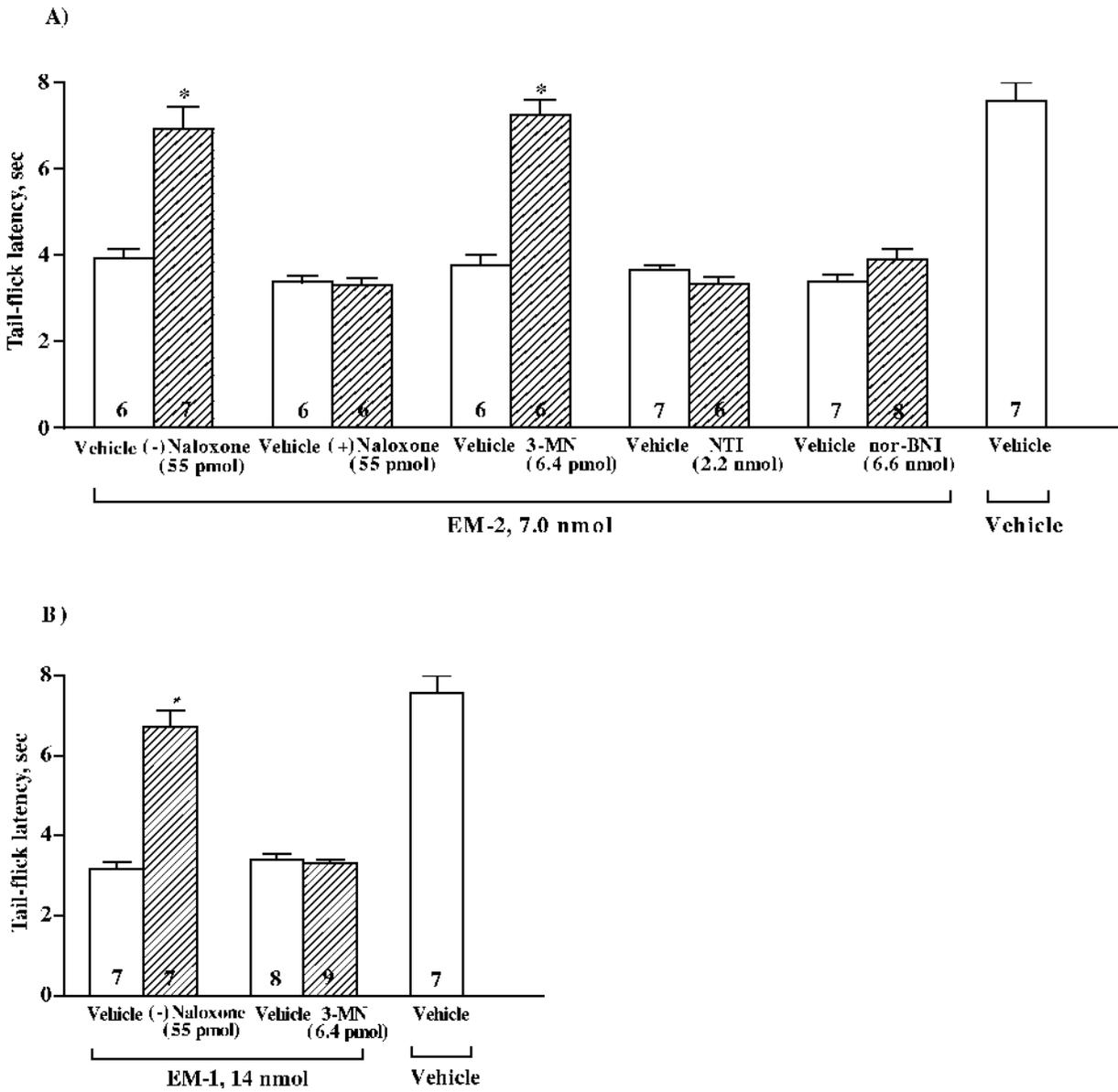


Fig 5 A,B

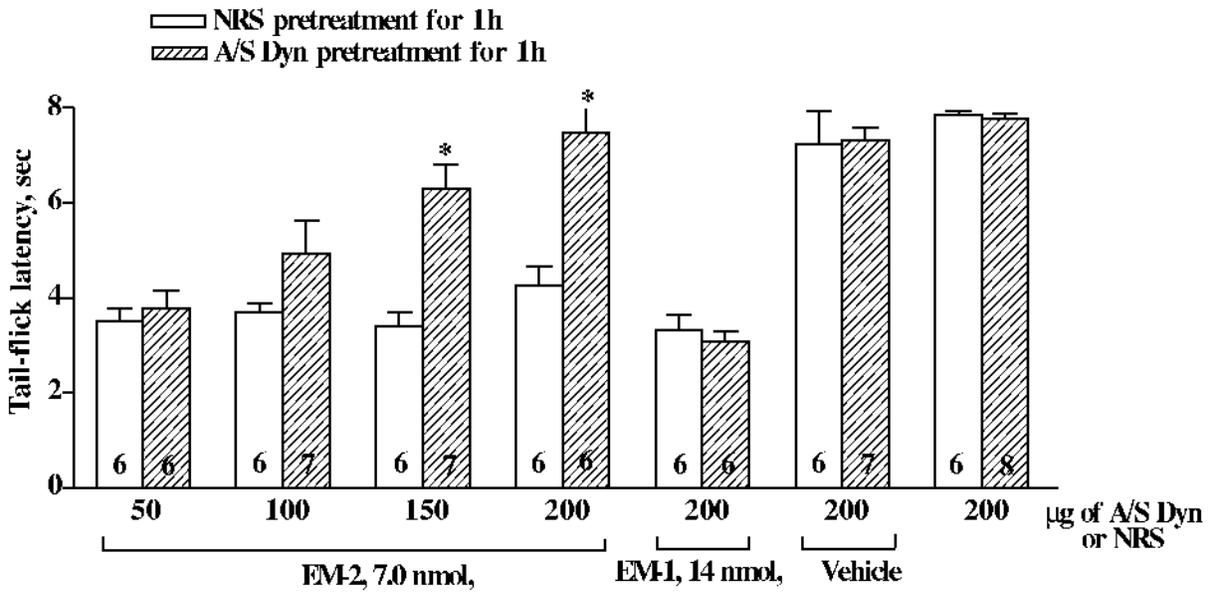


Fig 6

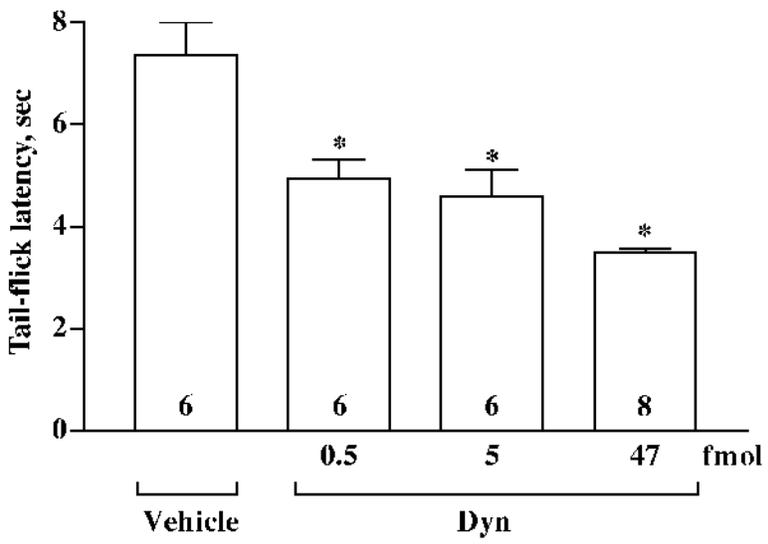


Fig 7