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Antianalgesia: stereoselective action of *dextro*-morphine over *levo*-morphine on glia in the mouse spinal cord

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Abstract: We have previously demonstrated that the naturally occurring *levo*-morphine at a sub-analgesic picomolar dose pretreated intrathecally (i.t.) induces antianalgesia against *levo*-morphine-produced antinociception. We now report that the synthetic stereo-enantiomer *dextro*-morphine, even at an extremely low femtomolar dose, induces antianalgesia against *levo*-morphine-produced antinociception using the tail-flick (TF) test in male CD-1 mice. Intrathecal pretreatment with *dextro*-morphine (33 fmol) time-dependently attenuated the i.t. *levo*-morphine-produced TF inhibition for 4 h and returned to the preinjection control level at 24 h. Intrathecal pretreatment with *dextro*-morphine (0.3-33 fmol), which injected alone did not affect the baseline TF latency, dose-dependently attenuated the TF inhibition produced by i.t.-administered *levo*-morphine (3.0 nmol). The ED_{50} value for *dextro*-morphine to induce antianalgesia was estimated to be 1.07 fmol, which is 71,000-fold more potent than the ED_{50} value of *levo*-morphine, indicating the high stereoselective action of *dextro*-morphine over *levo*-morphine for the induction of antianalgesia. Like *levo*-morphine, the *dextro*-morphine-induced antianalgesia against *levo*-morphine-produced TF inhibition was dose-dependently blocked by the non-opioid *dextro*-naloxone and by its stereo-enantiomer *levo*-naloxone, a non-selective μ-opioid receptor antagonist. The antianalgesia induced by both *levo*-morphine and *dextro*-morphine is reversed by the pretreatment with the glial inhibitor propentofylline (3.3-65 nmol), indicating that the antianalgesia is mediated by glial stimulation. The findings strongly indicate that the antianalgesia induced by both *levo*-morphine and *dextro*-morphine is mediated by the stimulation of a novel non-opioid receptor on glial cells.

Introduction:

Naturally occurring *levo*-morphine, which is isolated from the juice of the opium poppy, *papaver somniferum,* is stereochemically identified as a levorotatory isoform of morphine. *levo*-Morphine produces potent analgesic and other major pharmacological effects, which are mainly mediated by the stimulation of μ-opioid receptors. The synthetic *dextro*-enantiomer of *levo*-morphine has minimal activity in the μ-opioid receptor binding assay, the electrically stimulated guinea pig ileum assay and the inhibition of adenylate cyclase activity in the neuroblastoma x glioma hybrid cell homogenates, indicating that it does not interact with μ-opioid receptors (Jacquet et al., 1977). Unlike *levo*-morphine, which produces potent *levo*-naloxone reversible analgesia, *dextro*-morphine microinjected into the periaqueductal gray in rats produces minimal analgesia (Jacquet et al., 1977). In the present study, intrathecal (i.t.) pretreatment with *dextro*-morphine, which injected alone does not affect baseline nociceptive latency, attenuates the antinociception produced by i.t.-administered *levo*-morphine. The phenomenon of the attenuation of *levo*-morphine-produced analgesia by *dextro*-morphine has been defined as antianalgesia (Wu et al., 2004a).

Non-selective μ-opioid receptor antagonist *levo*-naloxone and non-opioid receptor antagonist *dextro*-naloxone were used to identify the opioid or non-opioid nature of the *dextro*-morphine-induced antianalgesia. We have previously reported that i.t. pretreatment with *levo*-morphine at a picomolar dose dose-dependently attenuates the antinociception produced by i.t.-administered *levo*-morphine using the thermal tail-flick (TF) test in mice. This antianalgesia is blocked by pretreatment with *dextro*-naloxone, indicating that the antianalgesia induced by *levo*-morphine is not mediated by the

stimulation of conventional G-protein coupled μ-opioid receptors (Wu et al., 2004a). On the other hand, the antianalgesia induced by endogenous μ-opioid ligands endomorphin-1 and endomorphin-2 is blocked by *levo*-naloxone but not by *dextro*-naloxone, indicating that the antianalgesia induced by endomorphin-1 and endomorphin-2 is mediated by the desensitization of μ-opioid receptors by endomorphin-1 and endomorphin-2 pretreatment (Wu et al., 2003; Terashvili et al., 2004). Thus, *levo*-morphine has biphasic effects: it produces antinociception or analgesia, which is mediated by the stimulation of μ-opioid receptors, and also induces non-opioidergic antianalgesia. However, it is reasonable to believe that *levo*-morphine at high analgesic doses also induces antianalgesia, but the effect is masked by the analgesic effect of *levo*-morphine. This view is supported by the finding that a small dose of *levo*-naloxone or other opioid antagonists enhances *levo*-morphine-produced analgesia in laboratory animals (Crain and Shen 1995, 2000, 2001) and humans (Gan et al., 1997; Joshi et al., 1999).

By releasing neurotransmitters, including glutamate, ATP and other extracellular signaling molecules, glia can affect neuronal excitability and synaptic transmission and coordinate activity across networks of neurons (Fields and Stevens-Graham, 2002). There are indications from the literature that opiate effects are not only mediated by opioid systems such as μ-, δ-, and κ-opioid receptors but that they are also influenced by immune mediators such as cytokines, chemokines, free radicals and nitric oxide that are released through activation of glial cells. Exposure of the microglia to morphine causes changes in microglial morphology and induces apoptosis, which can be blocked by *levo*-naloxone (Dobrenis et al., 1995; Magazine et al., 1996; Hu et al., 2002). Chronic *levo*-morphine treatment induces a marked proliferation and hypertrophy of microglia and

astrocytes in the spinal dorsal horn (Raghavendra et al., 2002; Narita et al., 2004). Inhibition of glial activation by the glial modulator propentofylline spares *levo*-morphine analgesia in neuropathic rats and reverses the development of *levo*-morphine tolerance and withdrawal hyperalgesia (Raghavendra et al., 2002). The findings indicate that *levo*-morphine also acts on glia to modulate the analgesic and other pharmacological activities of *levo*-morphine.

We have previously shown that *levo*-morphine induces antianalgesia, which is mediated by a non-opioidergic mechanism (Wu et al., 2004a). Present experiments were then undertaken to determine if the non-opioid *levo*-morphine enantiomer *dextro*-morphine would act like *levo*-morphine and induce antianalgesia. The glial inhibitor propentofylline and *dextro*-naloxone were used to determine if the antianalgesic effects induced by *dextro*-morphine and *levo*-morphine are mediated by the stimulation of a non-opioid mechanism on glial cells. We now report for the first time that pretreatment with an ultra-low, femtomolar dose of *dextro*-morphine attenuates the analgesia produced by subsequent injection of *levo*-morphine, and that the antianalgesia induced by either *dextro*-morphine or *levo*-morphine is mediated by the stimulation of ~~a~~ novel non-opioid receptors on glia in the mouse spinal cord.

Materials and Methods:

Animals. Male CD-1 mice weighing 25-30 g (Charles River Breeding Laboratory, Wilmington, MA) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light-dark cycle. Food and water were available *ad libitum*. Each animal was used only once. All experiments were approved by and conformed to the guidelines of the Animal Care Committee of the Medical College of Wisconsin.

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, mice were gently held with the tail put 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 low- and high-intensity heat stimuli were set to provide a pre-drug TF response time of 8 to 10 and 3 to 4 s, respectively. The cutoff times for the low- and high-intensity heat stimulus were set at 20 and 10 s, respectively, to avoid tissue damage. The TF response with a low-intensity heat stimulus was only used in Fig. 1 and high heat intensity was used through all the experiments. To calculate the ED_{50} values of the drug tested, the TF response latencies were then converted to the "percent maximum possible effect (% MPE)", which was calculated as $[(T_1-T_0) / (T_2-T_0)] \times 100$. T_0 and T_1 were the TF latencies before and after i.t. injection of morphine, respectively, and T_2 was the cutoff time, which was set at 10 s .

Experimental Protocols. Intrathecal injection was performed according to the procedure of Hylden and Wilcox (1980), using a 25-μl Hamilton syringe with a 30-gauge needle. The injection volume was 5 μl. The following experiments were performed. (1)

The effects of *dextro*-morphine on the TF latency induced by either a low- or high-intensity heat stimulus were determined. Groups of mice were treated with various doses of *dextro*-morphine and TF were measured at different times for 2 h at high or low thermal stimulus. (2) The time course and the dose-response relationship of *dextro*-morphine for the induction of antianalgesia against *levo*-morphine-produced antinociception were determined. Groups of mice were pretreated i.t. with 33 fmol of *dextro*-morphine for different times (0-24 h) before i.t. administration of *levo*-morphine (3.0 nmol). Other mice were used to test different doses (0.3-330 fmol) of *dextro*-morphine 45 minutes before i.t. administration of *levo*-morphine (3.0 nmol). In both cases, TF responses under high heat intensity were measured at different times thereafter. (3) The effects of the pretreatment with non-opioid antagonist *dextro*-naloxone and non-selective opioid antagonist *levo*-naloxone on the attenuation of *levo*-morphine-produced antinociception induced by *dextro*-morphine-induced antianalgesia were studied. Groups of mice were pretreated i.t. with *dextro*-naloxone (0.03-280 pmol) or *levo*-naloxone (0.03-28 pmol) 10 min (Wu et al., 2004a) before i.t. injection of *dextro*-morphine (33 fmol). *levo*-Morphine (3.0 nmol) was injected i.t. 45 min after *dextro*-morphine injection and the TF responses under high heat intensity were measured at different times thereafter. (4) The effect of propentofylline, a glial inhibitor (Schubert et al., 2000; Sweitzer et al., 2001), on the attenuation of *levo*-morphine-produced TF inhibition induced by separate *dextro*-morphine and *levo*-morphine pretreatment was determined. Groups of mice were co-administered i.t. propentofylline with *dextro*-morphine (33 fmol) or *levo*-morphine (0.3 nmol) 45 min prior to i.t. injection of *levo*-morphine (3.0 nmol) and the TF responses under high heat intensity were then measured at 15 min thereafter.

Drugs. *levo*-Morphine sulfate, *dextro*-morphine base and *dextro*-naloxone were obtained from National Institute of Drug Abuse (Baltimore, MD). *levo*-Naloxone and propentofylline were purchased from Sigma (St. Louis, MO). *levo*-Morphine, *levo*-naloxone, *dextro*-naloxone, and propentofylline were dissolved in 0.9% saline. The *dextro*-morphine was dissolved in 10 N hydrochloric acid and then titrated with 1 N sodium hydroxide to pH 7.4, which then diluted to intended dose in 0.9% saline.

Statistical analysis. The analgesic responses (TF latencies) were presented as the mean ± S.E.M. One-way ANOVA followed by Dunnett's post-test or two-way ANOVA followed by Bonferroni's post-tests was used to test the differences between groups. The non-linear regression model was used to fit the dose-response curve and calculates the ED_{50} value and 95% confidence interval (CI). The *F* test was used to test the difference of $LogED_{50}$ between *dextro*-morphine- and morphine-induced antianalgesia. The GraphPad Prism software was used to perform the statistics (version 4.1; GraphPad Software, Inc., San Diego, CA).

Results:

The tail-flick responses to a high- and low-intensity heat stimulus after the i.t. injection of various doses of *dextro*-morphine The experiment was designed to determine if *dextro*-morphine given i.t. caused any change of the TF latency. The intensity of the thermal stimulation was adjusted so that a high and low intensity of heat stimulus induced TF responses in 3-4 s and 8-10 s, respectively. Groups of male CD-1 mice were injected i.t. with various doses of *dextro*-morphine (0.3, 33 fmol or 3.3 pmol) or saline vehicle and the TF responses were measured at different times after injection for 2 h using either a high or low intensity of heat stimulus. Intrathecal injection of *dextro*-morphine at 0.3, 33 fmol or 3.3 pmol did not cause any change of the TF latency either induced by a high-intensity heat stimulus or a low-intensity heat stimulus (Fig 1). Figure 1 also showed that the curves for TF latencies elicited by a high-intensity heat stimulus were more steady and fluctuated less than the TF latencies induced by a low-intensity heat stimulus. The high-intensity heat stimulus was used in the following experiments

Effects of different times and doses of pretreatment of *dextro*-morphine given i.t. on the TF latency produced by i.t.-administered *levo*-morphine. Groups of mice were pretreated i.t. with *dextro*-morphine (33 fmol) at various times before i.t. injection of *levo*-morphine (3.0 nmol) and the TF response was measured 15 min thereafter. Other groups of mice pretreated i.t. with vehicle served as controls. The i.t. administration of *levo*-morphine (3.0 nmol) produced 9.0 to 9.5 s TF latency in mice pretreated i.t. with vehicle for different times. Intrathecal pretreatment with *dextro*-morphine (33 fmol) time-dependently attenuated the TF latencies produced by i.t. *levo*-morphine (3.0 nmol). The attenuation of the *levo*-morphine-produced TF responses developed immediately, reached

a maximal attenuation (5 to 5.5 s TF latencies) between 30 to 60 min, remained attenuated for 2 to 4 h and returned to the saline control level 24 h after injection (9 s TF latency) (Fig. 2). Pretreatment time of 45 min for *dextro*-morphine was then used for the following experiments.

The dose-response relationships for *dextro*-morphine to induce antianalgesia against i.t.-administered *levo*-morphine-produced TF inhibition were then studied. Groups of mice were pretreated i.t. with different doses (0.3 to 330 fmol) of *dextro*-morphine 45 min before i.t. administration of *levo*-morphine (3.0 nmol) and the TF response was measured thereafter. Intrathecal injection of *levo*-morphine 3.0 nmol caused an increase of TF inhibition in mice pretreated with the saline vehicle. The TF inhibition developed in 5 to 10 min, reached a maximum in 15 min and returned slowly to the control level in 60 min. Intrathecal pretreatment with *dextro*-morphine at doses from 1.0 to 33 fmol dose-dependently attenuated the TF responses produced by i.t.-administered *levo*-morphine observed at different times after injection (Fig. 3A). Figure 3B shows that *dextro*-morphine at doses from 1.0 to 33 nmol dose-dependently attenuated the *levo*-morphine-produced TF inhibition observed at 15 min after *levo*-morphine injection. The attenuation reached a maximum at 33 fmol, and a higher dose (330 fmol) of *dextro*-morphine did not further attenuate the *levo*-morphine-produced TF inhibition. The ED_{50} value for *dextro*-morphine to attenuate *levo*-morphine-produced antinociception was estimated to be 1.07 fmol (95% CI, 0.61-1.88 fmol). Figure 3B also shows that *dextro*-morphine at a dose of 33 fmol given alone did not affect the TF latency, which differs from the mice injected with vehicle and *levo*-morphine at 3 nmol that produced marked TF inhibition.

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Effect of i.t. pretreatments with *dextro*-naloxone and *levo*-naloxone on the i.t. *dextro*-morphine-induced antianalgesia against *levo*-morphine-produced TF responses The non-opioid *dextro*-naloxone and its enantiomer, the non-selective μ-opioid receptor antagonist, *levo*-naloxone, were used to determine if the antianalgesia induced by *dextro*-morphine pretreatment is mediated by the stimulation of a non-opioid or μ-opioid receptor mechanism. We have previously demonstrated that 10 or 30 min, but not 45 or 60 min of *levo*-naloxone pretreatment is the most appropriate treatment time for blocking the *levo*-morphine-induced antianalgesia against *levo*-morphine-induced TF inhibition (Wu et al., 2004a). The pretreatment time of 10 min for *dextro*-naloxone or *levo*-naloxone was used in the present study. Intrathecal pretreatment with *dextro*-naloxone (0.03 to 280 pmol) 10 min before i.t. *dextro*-morphine (33 fmol) treatment dose-dependently reversed the attenuation of the i.t. *levo*-morphine (3.0 nmol)-produced TF response. The reversal reached its maximum at 280 fmol (9.1 ± 0.41 s) (Fig. 4A). Intrathecal pretreatment with *dextro*-naloxone (280 pmol) given alone 55 min prior to i.t. *levo*-morphine (3.0 nmol) injection did not affect *levo*-morphine-produced TF response (9.4 ± 0.29 s).

Similarly, i.t. pretreatment with *levo*-naloxone (0.03-28 pmol) 10 min before i.t. pretreatment with *dextro*-morphine (33 fmol) dose-dependently reversed the attenuation of the i.t. *levo*-morphine-produced TF response, and the reversal reached its maximum at a dose of 28 pmol of naloxone pretreatment (9.7 ± 0.22 s) (Fig. 4B). Intrathecal pretreatment with *levo*-naloxone (28 pmol) given alone 55 min prior to i.t. *levo*-morphine (3.0 nmol) injection did not affect the i.t. *levo*-morphine-produced TF response (9.1 ± 0.34 s).

As shown in Table 1, *dextro*-naloxone is as potent as *levo*-naloxone in reversing *dextro*-morphine-induced antianalgesia. *levo*-Naloxone is also equally potent in reversing both *dextro*-morphine- and *levo*-morphine-induced antianalgesia. However, *dextro*-naloxone is comparably less potent in reversing *levo*-morphine-induced antianalgesia against *levo*-morphine-produced TF inhibition.

Effect of i.t. administration of the glial inhibitor propentofylline on i.t. *dextro*-morphine-induced antianalgesia against *levo*-morphine-produced TF response The glial inhibitor propentofylline was used to determine if the *dextro*-morphine-induced antianalgesia is mediated by the stimulation of glial cells. Groups of mice were co-administered i.t. with different doses of propentofylline (3.3-65 nmol) and 33 fmol of *dextro*-morphine or saline vehicle 45 min before i.t. administration of *levo*-morphine (3 nmol). The TF response was then measured 15 min after *levo*-morphine injection. Intrathecal pretreatment with *dextro*-morphine alone for 45 min attenuated the *levo*-morphine-produced TF inhibition. Pretreatment with propentofylline dose-dependently blocked the i.t. *dextro*-morphine-induced antianalgesia and reversed the attenuation of *levo*-morphine-produced TF inhibition (Fig 5A). The ED_{50} value for propentofylline in reversing the *dextro*-morphine-induced antianalgesia was estimated to be 28.08 nmol. Intrathecal injection of propentofylline (98 nmol) given alone did not cause any effect on TF latency in mice treated with the saline vehicle. Intrathecal pretreatment with propentofylline (98 nmol) for 45 min prior to *levo*-morphine (3.0 nmol) injection did not affect the *levo*-morphine-produced TF responses (Fig. 5A).

Effect of i.t. administration of the glial inhibitor propentofylline on i.t. *levo*-morphine-induced antianalgesia against *levo*-morphine-produced TF response The experiment was then undertaken to determine if the *levo*-morphine-induced antianalgesia is also mediated by glial stimulation. Groups of mice were co-administered i.t. with different doses of propentofylline (3.3-65 nmol) and *levo*-morphine (0.3 nmol) or saline vehicle 45 min before i.t. administration of *levo*-morphine (3 nmol). The TF response was then measured 15 min after *levo*-morphine injection. Intrathecal administration of propentofylline (3.3- 65 nmol) dose-dependently reversed the attenuation of the i.t. *levo*-morphine (3.0 nmol)-produced TF inhibition induced by sub-analgesic *levo*-morphine (0.3 nmol) pretreatment. Propentofylline at 65 or 98 nmol was found to completely reverse the morphine-induced attenuation of *levo*-morphine-produced TF inhibition (Fig. 5B). The ED_{50} values for propentofylline for reversing the dextro-morphine- and *levo*-morphine-induced antianalgesia against *levo*-morphine-produced TF inhibition shown in Table 1 indicate that propentofylline is about equally potent in reversing *dextro*-morphine- and *levo*-morphine-induced antianalgesia.

Discussion:

Stereoselective action of *dextro*-morphine over *levo*-morphine in inducing antianalgesia against *levo*-morphine-produced antinociception Unlike naturally occurring *levo*-morphine, which produces analgesia and other μ-opioid receptor mediated pharmacological effects, the synthetic *dextro*-morphine does not have any affinity and efficacy to μ-opioid receptor, and therefore, does not produce analgesia and other *levo*-morphine-like effects (Jacquet et al., 1977). We have previously demonstrated that *levo*-morphine at sub-analgesic doses induce antianalgesia. The antianalgesia induced by *levo*-morphine is blocked by a non-opioid *dextro*-naloxone, indicating the mediation of a non-opioidergic mechanism (Wu et al, 2004a). Like *levo*-morphine, we found in the present studies that *dextro*-morphine, at extremely low femtomolar doses, induces antianalgesia to attenuate *levo*-morphine-produced antinociception. The ED_{50} value for *dextro*-morphine to induce antianalgesia against *levo*-morphine-produced antinociception was estimated to be 1.07 fmol, which is about 71,000-fold more potent than *levo*-morphine for inducing antianalgesia (Wu et al., 2004a). The extremely high stereoselective action of *dextro*-morphine over *levo*-morphine to induce antianalgesia strongly supports the view that the antianalgesia induced by *dextro*-morphine and *levo*-morphine is mediated by the stimulation of a novel receptor and not the traditional G-protein coupled μ-opioid receptors.

The effect of *dextro*-morphine and *levo*-morphine on the baseline nociceptive latency appears to depend on the method of the nociceptive tests used, the route of administration and different strain of animals. We found in the present study using the TF response as the nociceptive test that *dextro*-morphine at the same doses used to induce

antianalgesia given i.t. did not produce any change of the baseline TF latency either using a high or low intensity of heat stimulus for the TF response. However, we recently found that *dextro*-morphine at femtomolar to picomolar doses given i.t. dose-dependently produced thermal hyperalgesia with the thermal paw-withdrawal test and tactile allodynia with the mechanical paw-withdrawal test in mice. We also found that *dextro*-morphine at an extremely low dose (0.1-10 ng/kg) injected subcutaneously dose-dependently produced thermal hyperalgesia using the TF test in mice (unpublished observations). Crain and Shen (2001) reported that *levo*-morphine at a low dose (0.1-1 μg/kg) given subcutaneously produces a decrease of TF latency (hyperalgesia) for a period more than 3 h in Swiss-Webster male mice, but causes an increase of TF latency (analgesia) in 129/SvEvTac mice. The hyperalgesia and allodynia have also been described following various dose of *levo*-morphine in human. Sjogren and his colleagues (1994) report that *levo*-morphine-induced hyperalgesia disappears after discontinuing or substituting *levo*-morphine with other opioid agonists, indicating that *levo*-morphine itself, but not other μ-opioid agonists plays a role in hyperalgesia and allodynia.

Non-stereoselective action of *dextro*-naloxone and *levo*-naloxone in blocking the antianalgesia induced by *dextro*-morphine against *levo*-morphine-produced antinociception *dextro*-Naloxone, an enantiomer of the non-selective μ-opioid receptor antagonist *levo*-naloxone, has been shown not to have any affinity to μ-opioid receptors or block μ-opioid mediated analgesia (Iijima et al., 1978). We found in the present study that pretreatment with *dextro*-naloxone dose-dependently blocked *dextro*-morphine-induced antianalgesia. The finding provides additional evidence that *dextro*-morphine-induced antianalgesia is not mediated by the stimulation of μ-opioid receptors. We have

reported that the *levo*-morphine-induced antianalgesia is blocked by *dextro*-naloxone (Wu et al., 2004a). This neural mechanism of antianalgesia induced by *dextro*-morphine and *levo*-morphine is completely different from that of anti-analgesia induced by selective μ-opioid ligand endomorphin-2. The antianalgesia induced by endomorphin-2 is selectively blocked by *levo*-naloxone, but not by *dextro*-naloxone, indicating that the endomorphin-2-induced antianalgesia is mediated by an opioidergic mechanism (Wu et al., 2004a). However, we also found that the non-selective μ-opioid antagonist *levo*-naloxone was also effective with equal potency to *dextro*-naloxone in blocking *dextro*-morphine-induced antianalgesia. Thus, in addition to blocking the μ-opioid receptors, *levo*-naloxone also shares with *dextro*-naloxone the ability to non-selectively block the novel receptor stimulated by *dextro*-morphine. The finding with *levo*-naloxone clearly indicates that *levo*-naloxone is non-selective as an μ-opioid receptor blocker and should not be used as a reliable marker for identifying the opioidergic mechanism.

Thus, *dextro*-morphine and *levo*-morphine are considered to be agonists to stimulate stereoselectively this novel non-opioid receptor for inducing antianalgesia and *levo*-naloxone and *dextro*-naloxone are antagonists to block non-stereoselectively this receptor and reverse the antianalgesia induced by *dextro*-morphine and *levo*-morphine. Thus, *levo*-morphine has biphasic effects; at high doses, it produces analgesia, which is mediated by μ-opioid receptors, and at low doses, it induces non-opioidergic antianalgesia. However, it is reasonable to believe that *levo*-morphine at analgesic doses also induces antianalgesia, but the effect is masked by the analgesic effect of *levo*-morphine. This view is supported by the findings that a small dose of *levo*-naloxone or other opiate antagonists enhances the *levo*-morphine-produced analgesia in mice (Crain

and Shen, 1995, Shen and Crain, 1997) and in humans (Gan et al., 1997; Joshi et al., 1999).

The fundamental principles of enantiometric selectivity were delineated long before investigators contemplated to isolation of receptors (Taylor and Insel, 1990). Easson and Stedman (1933) suggest that if selectivity of enantiomeric pairs could be seen in a biologic system, then a three-point attachment must occur between the enantiomer and a dissymmetric surface. We found that *dextro*-morphine exhibits extremely high stereoselective action over *levo*-morphine in a more than four orders of magnitude for stimulating this receptor to induce antianalgesia, while *dextro*-naloxone and *levo*-naloxone blocks non-stereoselectively the *dextro*-morphine- and *levo*-morphine-induced antianalgesia. This finding suggests that at least three-point attachment on the receptor for the agonists and only a single or two binding sites on the receptor for the antagonists.

Antianalgesia induced by *dextro*-morphine and *levo*-morphine is mediated by the glial stimulation Propentofylline**,** a methylxanthine derivative, exhibits neuroprotective effects through multiple mechanisms, which include an inhibition of glutamate release (Miyashita et al., 1992), an increase in nerve growth factor secretion (Shinoda et al., 1990), and an attenuation of glial activation (Schubert et al., 2000). The specific mechanism by which propentofylline exhibits such diverse effects is not understood due to its multiple mechanisms of actions, which include nonspecific inhibition of phosphodiestease enzyme (Meskini et al., 1994; Schuber et al., 1997) and its ability to inhibit adenosine re-uptake (Parkinson et al., 1993). Both molecular mechanisms exert neuroprotective effects. The glial inhibitory property of

propentofylline (Raghavendra and DeLeo, 2004) was then used to determine if *dextro*-morphine- and *levo*-morphine-induced antianalgesia is mediated by glial stimulation.

It has been documented that *levo*-morphine stimulates glial cells to induced the release of cytokines, chemokines and free radicals. Exposure of microglia to *levo*-morphine caused marked changed in cellular morphology, including assumption of a rounded shape and retraction of cytoplasmic process. These morphological changes can be blocked by the opioid antagonist *levo*-naloxone. In contrast, several opioid peptides do not produce effects (Dobrenis et al., 1995; Magazine et al., 1996). *Levo*-morphin causes the release of pro-inflammatory cytokines such as tumor necrosis factor (Chao et al., 1994). Chronic administration of *levo*-morphine also activates spinal microglia and astrocytes and up-regulates pro-inflammatory cytokines. Chronic systemic treatment with *levo*-morphine given in rats causes a significant increase in complement receptor type 3 alpha subunit (OX-42) and glial fibrillary acidic protein immunoreactivity and increases the mRNA for interleukin-1β, interleukin-6 and tumor necrosis factor α in the lumbar spinal cord of rats (Raghavendra et al., 2002). The increase of spinal glial fibrillary acidic protein as well as antinociceptive tolerance (antianalgesia) to i.t. *levo*-morphine induced by i.t. sub-analgesic *levo*-morphine treatment is blocked by co-treatments with fluorocitrate, a specific and reversible inhibitor of glial cells (Song and Zhao, 2001). Inhibition of glia activation by chronic treatment with the glial inhibitor propentofylline spares *levo*-morphine analgesia in neuropathic rats and reverses the development of *levo*-morphine-induced antinociceptive tolerance and withdrawal-induced hyperalgesia. Attenuation of pain behaviors by propentofylline is associated with the inhibition of glial activation and the subsequent pro-inflammatory immune activation in the lumbar spinal

cord (Sweitzer et al., 2001; Raghavendra et al., 2002). In present studies, we found that glial inhibition with propentofylline (Schubert et al., 1997; Sweitzer et al., 2001) inhibited *dextro*-morphine- and *levo*-morphine-induced antianalgesia with similar potency. The results of the studies provide the evidence that antianalgesia induced by *dextro*-morphine and *levo*-morphine is mediated by the stimulation of glial cells. The stimulation of glial cells by *dextro*-morphine or *levo*-morphine may subsequently cause the releases of cytokines, chemokines and other free radicals for the induction of antianalgesia.

Damage to the peripheral nerves or nerve roots produces intense microglial and astrocyte activation in the central nervous systems (Gehrmann et al., 1991; Colburn et al., 1999; Hashizume et al., 2000). The glial activation leads to the development of hyperalgesia, allodynia and the ineffectiveness or attenuation of morphine-produced analgesia (antianalgesia) (Watkins et al., 2001; Raghavendra and DeLeo, 2004; Arner and Meyerson, 1988; Rowbotham et al., 2003). Similarly, activation of glia by spinal injection of lipopolysaccharide induces hyperalgesia and antianalgesia, which is blocked by glial inhibitor (Wu et al., 2004b; Johnston and Westbrook, 2005). These observations are consistent with the present finding that activation of glia by *dextro*-morphine causes the induction of antianalgesia against *levo*-morphine-produced analgesia.

It is concluded that *dextro*-morphine at femtomolar doses or *levo*-morphine at picomolar doses given spinally induces antianalgesia against spinal *levo*-morphine-produced analgesia. The antianalgesia induced by *dextro*-morphine or *levo*-morphine is mediated by the stimulation of a novel and non-opioid receptor on glial cells. Blockade of the receptor by *dextro*-naloxone or inhibition of the glia by propentofylline reverses the

attenuation of *levo*-morphine-produced analgesia induced by *dextro*-morphine or *levo*-morphine.

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b) Leon F. Tseng, Ph.D.

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Legends for figures:

Fig. 1 The TF responses to i.t. administration of *dextro*-morphine or vehicle. Groups of mice were injected i.t. with *dextro*-morphine (33 fmol), or vehicle (5 μl) and the TF responses were then measured at different times thereafter. The baseline TF latencies induced by high- and low-heat intensities were 3-4 s and 8-10 s, respectively. Each point represents the mean and vertical bar represents the S.E.M. TF latency with 7-8 mice in each group. The two-way ANOVA followed by Bonferroni's post-test was used to test the difference between groups. The $F_{\text{interaction, treatment, time}} = 0.84, 3.37, 0.93$ for high intensity and 0.46, 2.20, 0.27 for low intensity.

Fig. 2 Effects of different pretreatment times with *dextro*-morphine given i.t. on the TF inhibition produced by i.t.-administered *levo*-morphine. Groups of mice were pretreated i.t. with *dextro*-morphine (33 fmol) or vehicle 0, 0.5, 0.75, 1, 2, 4, 8, 24 h before i.t. administration of *levo*-morphine (3.0 nmol) and the TF latency was measured 15 min thereafter. Each column represents the mean and the vertical bar represents the S.E.M. with 8 to 10 mice in each group. The two-way ANOVA followed by Bonferroni's post-test was used to test the difference between groups. The $F_{\text{interaction, treatment, time}} = 11.24, 46.65, 9.08$; * $p < 0.01$, ** $p < 0.001$.

Fig. 3 Effect of different doses of *dextro*-morphine given i.t. on the TF response produced by i.t.-administered *levo*-morphine. (A) Groups of mice were pretreated i.t. with *dextro*-morphine (0.3-330 fmol) or vehicle 45 min prior to i.t. injection of *levo*-morphine (3.0 nmol) and TF latencies were measured at different times thereafter. (B)

The TF latency was measured 15 min after *levo*-morphine (3.0 nmol) administration based on the data from figure 3 A. Each column represents the mean and the vertical bar represents the S.E.M. with 8 to 10 mice in each group. The two-way ANOVA followed by Bonferroni's post-test (A) or one-way ANOVA followed by Dunnett's post-test (B) was used to test the difference between groups. The $F_{\text{interaction, treatment, time}} = 5.52, 57.52, 153$ (A); $F = 27.68$ (B); * $p < 0.01$, ** $p < 0.001$.

Fig. 4 Effect of different doses of *dextro*-naloxone (A) and *levo*-naloxone (B) on i.t. morphine-produced TF response in mice pretreated i.t. with *dextro*-morphine. Groups of mice were pretreated i.t. with *dextro*-naloxone (0.03-280 pmol) (A) or *levo*-naloxone (0.03-28 pmol) (B) or vehicle 10 min before i.t. injection of *dextro*-morphine (33 fmol) followed by i.t. injection of *levo*-morphine (3.0 nmol) 45 min thereafter. The TF latency was measured 15 min after *levo*-morphine administration. Each column represents the mean and the vertical bar represents the S.E.M. with 7 to 10 mice in each group. The four columns in the right hand side of the figure represent different control data, which were not used for statistic purpose. The one-way ANOVA followed by Dunnett's post-test was used to test the difference between groups. The $F = 12.30$ (A) and 10.57 (B); * $p < 0.05$, ** $p < 0.01$.

Fig. 5 Effect of i.t. co-administration of propentofylline and *dextro*-morphine on i.t. *levo*-morphine-produced TF inhibition in mice. Groups of mice were co-administered i.t. with (A) propentofylline (3.3-65 nmol) and *dextro*-morphine (33 fmol) or (B) propentofylline (0.3-98 nmol) and *levo*-morphine (0.3 nmol) 45 min before i.t. injection of *levo*-morphine

(3.0 nmol) 45 min and the TF response was measured 15 min thereafter. Groups of mice injected i.t. with *dextro*-morphine or *levo*-morphine alone served as control. Each column represents the mean and the vertical bar represents the S.E.M. with 7 to 9 mice in each group. The four columns in the right-hand side of the figure represent different control data, which were not used for statistic purpose. The one-way ANOVA followed by Dunnett's post-test was used to test the difference between groups. $F = 14.90$ (A) and 8.38 (B); * $p < 0.05$, ** $p < 0.01$.

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Table 1. The ED_{50} values of propentofylline, *dextro*-naloxone, and *levo*-naloxone for blocking *dextro*-morphine- and *levo*-morphine-induced antianalgesia against *levo*-morphine-produced TF inhibition. Groups of mice were co-administered i.t. with propentofylline (0.3-98 nmol) and *dextro*-morphine (33 fmol) or *levo*-morphine (0.3 nmol) 45 min prior to i.t. injection of *levo*-morphine (3.0 nmol). Other groups of mice were pretreated i.t. with *dextro*-naloxone (0.03-280 pmol) or *levo*-naloxne (0.03-28 pmol) for 10 min before i.t. injection of *dextro*-morphine (33 fmol) or *levo*-morphine (0.3 nmol) injection and were then injected i.t. with *levo*-morphine (3.0 nmol) 45 min after *dextro*-morphine injection. The tail-flick response was measured 15 min after *levo*-morphine injection.

	ED_{50} (95% CI) [c]		
	Propentofylline	*dextro*-naloxone	*levo*-naloxone
Pretreatment			
dextro-morphine	28.7 nmol[a] (9.28-88.47)	0.1 pmol[a] (0.05-0.21)	0.09 pmol[a] (0.02-0.49)
levo-morphine	16.9 nmol[a] (0.08-3507)	1.79 pmol[b] (0.68-4.71)	0.21 pmol[b] (0.04-1.17)

[a] The ED_{50} was calculated based on the data in Fig. 4 and 5.

[b] The data were obtained from Wu et al. (2004).

[c] The *F* test was used to test the difference of $LogED_{50}$ between *dextro*-morphine and *levo*-morphine pretreatment, which showed no significant difference in present study.

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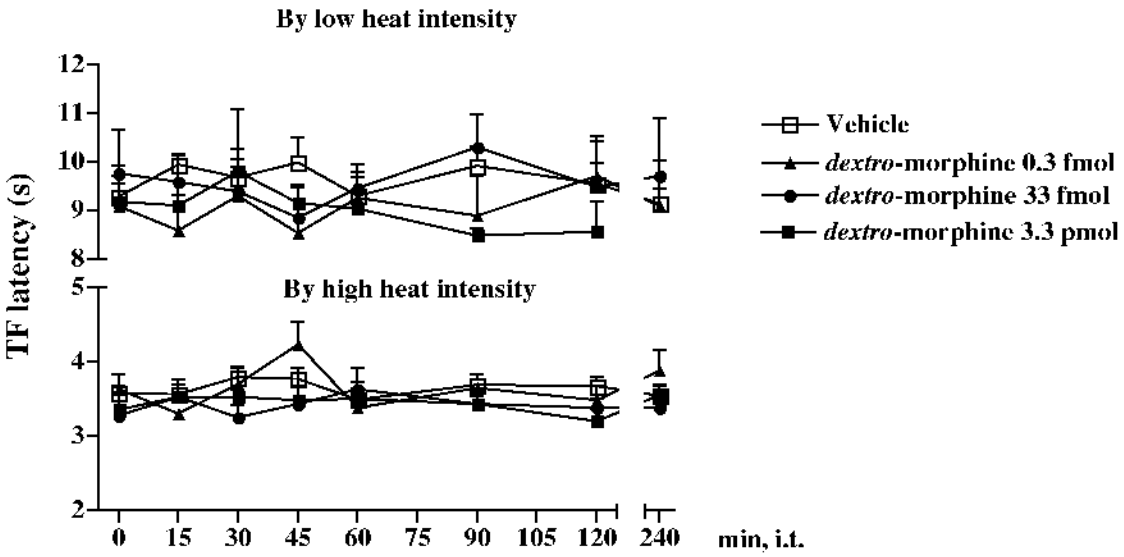

Fig. 1

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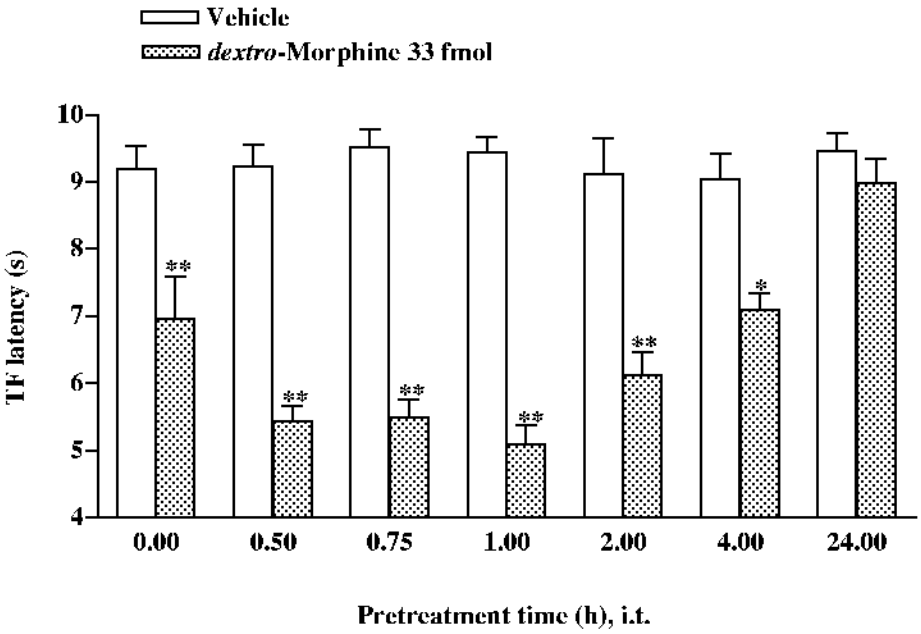

Fig. 2

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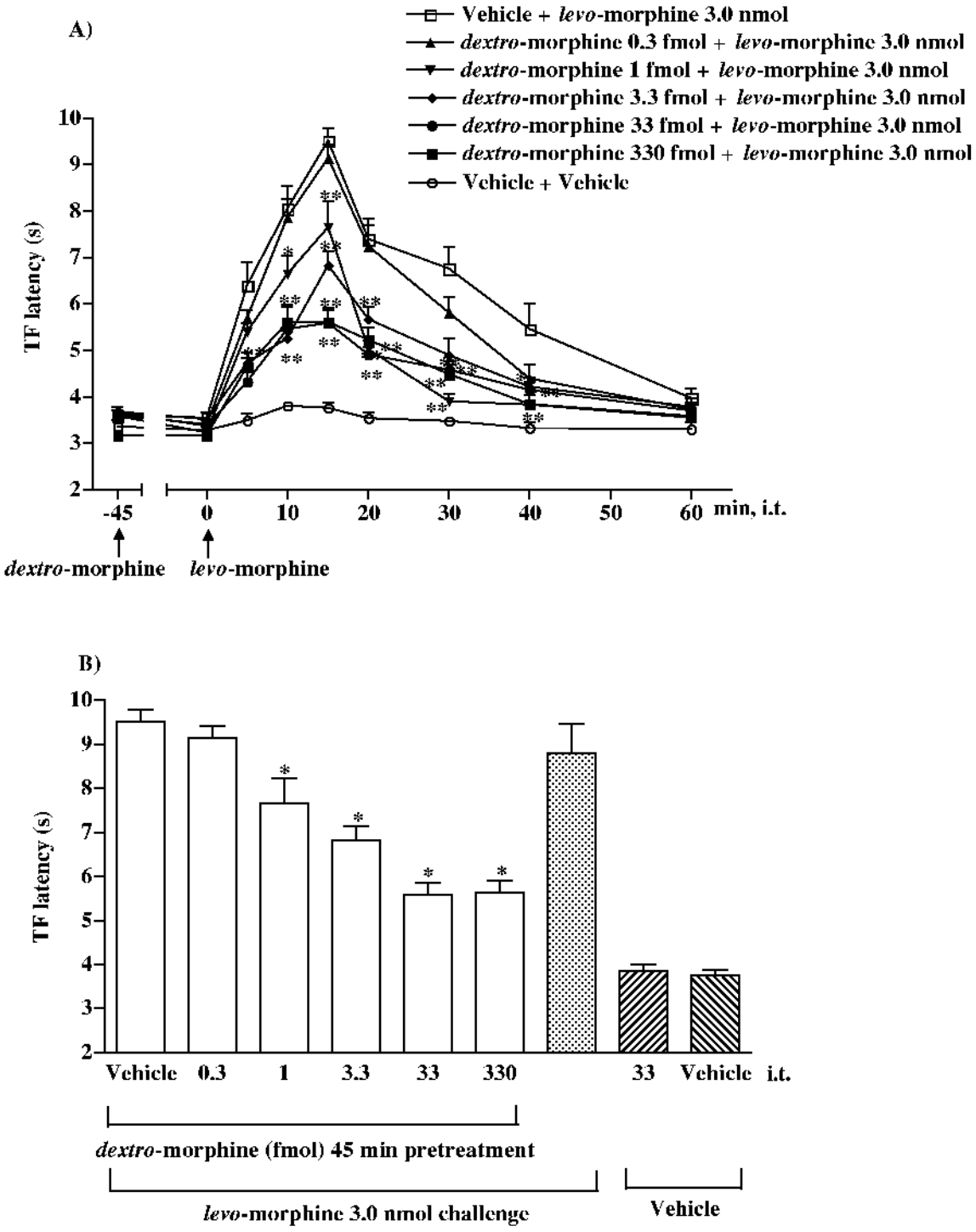

Fig. 3

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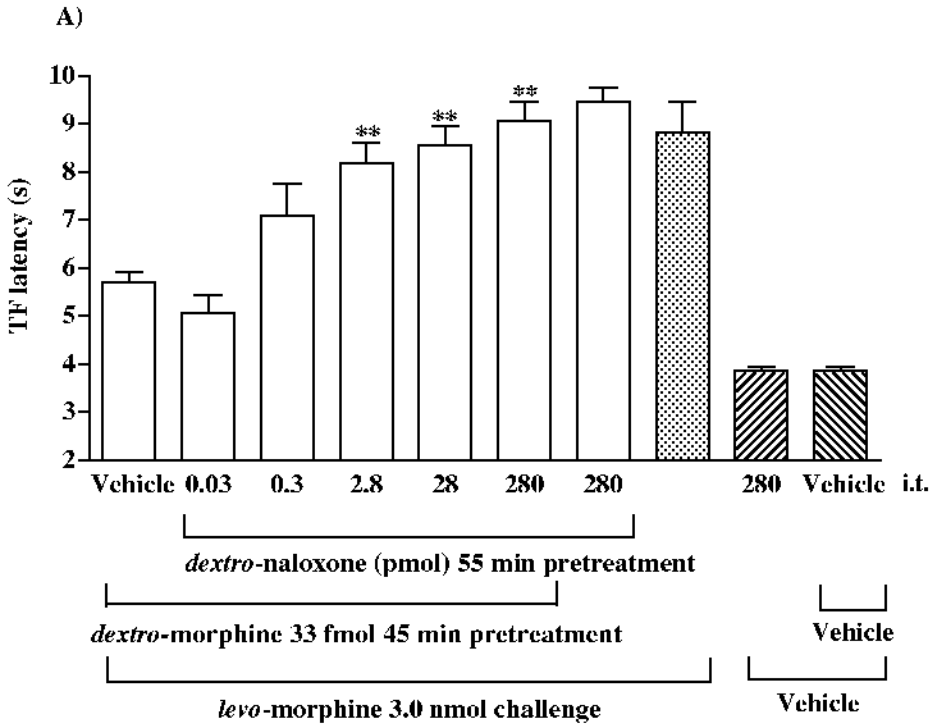

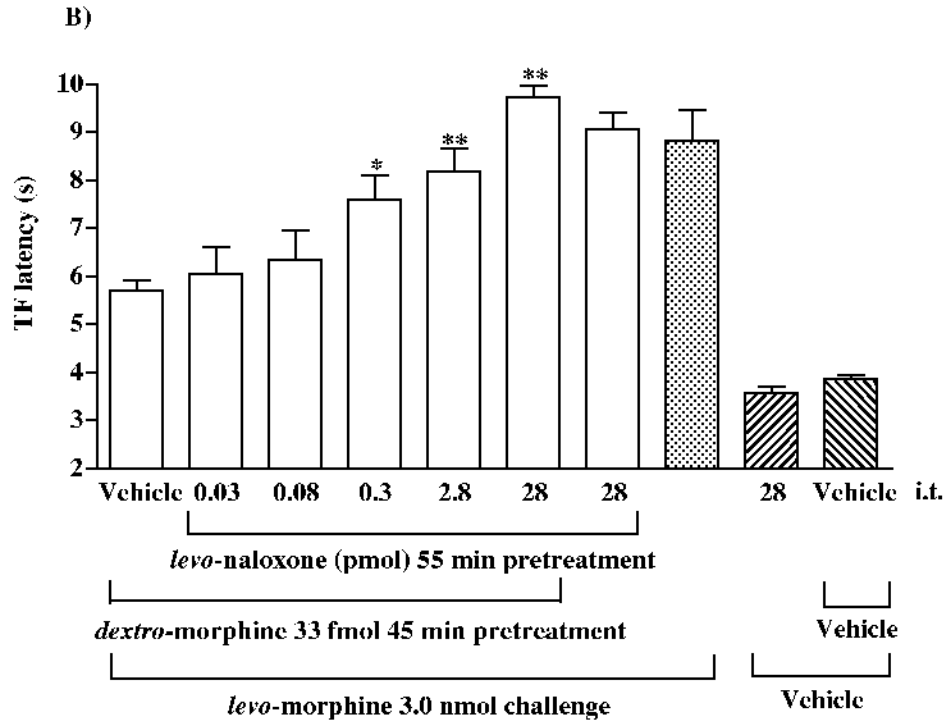

Fig. 4

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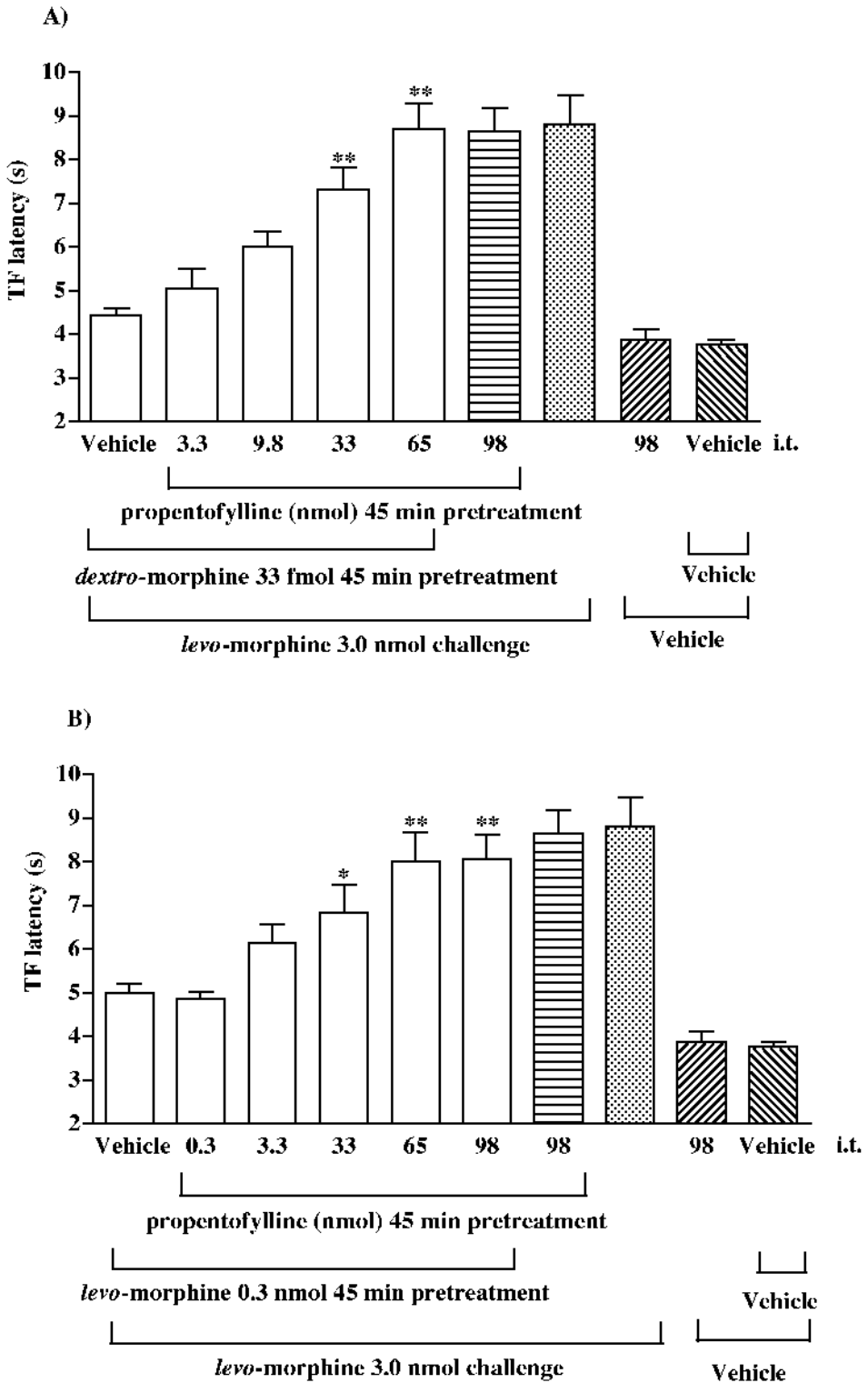

Fig. 5