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ENDOGENOUS OPIOID PEPTIDES CONTRIBUTE TO ANTINOCICEPTIVE POTENCY OF INTRATHECAL [DMT^{1}]DALDA

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Running Title: Endogenous opioid peptides and intrathecal [Dmt[1]]DALDA

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Abbreviations:

[Dmt[1]]DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2',6'-dimethyltyrosine; hMOR, cloned human μ opioid receptor; hDOR, cloned human δ opioid receptor; hKOR, cloned human κ opioid receptor; i.t., intrathecal; i.c.v., intracerebroventricular; DAMGO, H-Tyr-D-Ala-Gly-NMePhe-Gly-ol; DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen; U69,593, (5α,7α,8β)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl)-benzeneacetamide; s.c. subcutaneous.

Abstract

[Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2',6'-dimethyltyrosine) is a dermorphin analog that shows high affinity and selectivity for the μ opioid receptor. The intrathecal potency of [Dmt^1]DALDA far exceeded its affinity at μ receptors, and suggests that other mechanisms must be involved in its action in the spinal cord. The affinity and selectivity of [Dmt^1]DALDA was determined using cell membranes expressing hMOR, hDOR and hKOR. Competitive displacement binding with [^{3}H][Dmt^1]DALDA, [^{3}H]DPDPE (H-Tyr-D-Pen-Gly-Phe-D-Pen) and [^{3}H]U69,593 ((5α,7α,8β)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl)-benzeneacetamide) revealed Ki of 156 ± 26 pM for MOR, 1.67 ± 0.04 μM for DOR and Ki of 4.4 ± 1.7 nM for KOR, respectively. [Dmt^1]DALDA increased [^{35}S]GTPγS binding in MOR, DOR and KOR membranes, with EC_{50} being 17 (8.8 – 33) nM, 2 (1.2 – 3.2) μM and 124 (15 – 1000) nM, respectively. Intrathecal [Dmt^1]DALDA inhibited the tail-flick response in mice with ED_{50} = 1.22 (0.59 – 2.34) pmol. Intrathecal administration of an antiserum against dynorphin A(1-17) or [Met^5]enkephalin significantly attenuated the response to i.t. [Dmt^1]DALDA, resulting in ED_{50} of 6.2 (3.6 – 12.6) pmol and 6.6 (3.5 – 19.6) pmol, respectively. Neither antisera had any effect on the response to i.t. morphine. Intracerebroventricular (i.c.v.) [Dmt^1]DALDA was not affected by prior i.c.v. administration of anti-Dyn or anti-ME. Pretreatment with norbinaltorphimine (norBNI) or naltriben also attenuated the antinociceptive response to i.t., but not i.c.v., [Dmt^1]DALDA. These data suggest that i.t. [Dmt^1]DALDA causes the release of dynorphin and [Met^5]enkephalin-like substances that act at κ and δ receptors, respectively, to contribute to the extraordinary potency of [Dmt^1]DALDA.

Of the three subtypes of opioid receptors (μ, δ and κ), μ appears to be most important in analgesia. [Dmt[1]]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2',6'-dimethyltyrosine) is a dermorphin analog with extraordinary affinity (Kd ~ 150 pM) and selectivity for μ receptors (Schiller et al., 2000). [Dmt[1]]DALDA was reported to be ~14,000 and ~150 times more selective for μ than δ and κ, respectively (Schiller et al. 2000). Surprisingly, the *in vivo* analgesic potency of [Dmt[1]]DALDA far exceeded its affinity and potency at μ receptors. Compared to morphine, [Dmt[1]]DALDA was 30-200 times more potent after intracerebroventricular (i.c.v.) administration, and 1000-5000 times more potent after intrathecal (i.t.) administration (Neilan et al., 2001; Shimoyama et al., 2001; Zhao et al., 2002; Riba et al., 2002). In contrast, the affinity of [Dmt[1]]DALDA for the μ receptor was estimated to be only 7-fold greater compared to morphine (Schiller et al. 2000)

The extraordinary potency of intrathecal [Dmt[1]]DALDA suggested that mechanisms other than activation of μ receptors must be involved in its analgesic action in the spinal cord. Previous studies using selective μ, δ and κ antagonists concluded that the antinociceptive action of [Dmt[1]]DALDA was mediated via μ receptors, and that [Dmt[1]]DALDA was interacting with the same population of spinal μ receptors as DAMGO (H-Tyr-D-Ala-Gly-NMePhe-Gly-ol) (Riba et al. 2002). However, [Dmt[1]]DALDA exhibited little to no cross-tolerance in morphine-tolerant animals, which led to the suggestion that [Dmt[1]]DALDA and morphine may be acting at different subtypes of μ receptors (Neilan et al. 2001; Riba et al. 2002). Differences in response to [Dmt[1]]DALDA and morphine in different mice strains and with antisense oligodeoxynucleotides targeting against specific exons of the mouse μ opioid receptor gene support the suggestion that [Dmt[1]]DALDA and morphine act at different subtypes of μ receptors (Neilan et al. 2001). However, repeated systemic administration of [Dmt[1]]DALDA resulted in

profound tolerance to both [Dmt^1]DALDA and morphine in the spinal cord, but with little supraspinal tolerance (Zhao et al. 2002). This recent finding raises the possibility that [Dmt^1]DALDA might act via different mechanisms in the spinal cord versus brain.

It was recently reported that endomorphin-1 and endomorphin-2, two opioid peptides isolated from mammalian brain, act via different mechanisms after i.t. administration. Both endomorphin peptides demonstrate high affinity and selectivity for μ receptors in radioligand binding assays (Zadina et al., 1997). However, an additional component of the antinociceptive response to i.t. endomorphin-2 is due to the release of dynorphin A(1-17) and [Met^5]enkephalin which subsequently act on κ and δ receptors, respectively, in the spinal cord (Ohsawa et al., 2001; Sakurada et al., 2001). Activation of δ and κ receptors can potentiate the action of μ opioid agonists in the spinal cord (Porreca et al., 1992; He and Lee, 1998). In this study, we examined whether spinal dynorphin and [Met^5]enkephalin may play a role in the spinal action of [Dmt^1]DALDA.

Materials and Methods

Animals. Male CD-1 mice (25-30 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in a temperature-controlled room maintained on a 12-h light/dark cycle. Food and water were available *ad lib* until the time of the experiment. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain and approved by the Institution for the Care and Use of Animals at Weill Medical College of Cornell University.

Drugs and Chemicals. [Dmt^{1}]DALDA and [^{3}H][Dmt^{1}]DALDA (47 Ci/mmol) were synthesized by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, Montreal, Quebec, Canada) according to methods described previously (Schiller et al., 1989; Schiller et al. 2000; Zhao et al. 2002). [^{3}H]DPDPE (H-Tyr-D-Pen-Gly-Phe-D-Pen; 42 Ci/mmol), [D-Ala^{2}] deltorphin II and morphine sulfate were provided by the National Institute on Drug Abuse (Rockville, MD). [^{3}H]U69,593 ((5α,7α,8β)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl)-benzeneacetamide) (59 Ci/mmol) and [^{35}S]GTPγS (1000-1200 Ci/mmol) were purchased from Amersham Biosciences Corp. (Piscataway, NJ). All other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Antisera. Rabbit antisera against dynorphin A (1-17) and [Met^{5}]enkephalin were obtained from Peninsula Laboratories/Bachem (San Carlos, CA). According to the manufacturer, the antiserum against dynorphin A (1-17) showed 0.43% cross-reactivity with dynorphin A (1-13), but does not cross-react with dynorphin A (1-8), dynorphin B or [Leu^{5}]enkephalin. The antiserum against [Met^{5}]enkephalin showed cross-reactivity against [Met^{5}]enkephalin-Arg-Phe (0.1%), [Leu^{5}]enkephalin (3%), and β-endorphin (0.1%), but not against dynorphin A (1-17).

MOR, DOR and KOR membranes. Membranes prepared from either CHO-K1 cells transfected with hMOR or hDOR, or HEK293 cells transfected with hKOR were purchased from Perkins Elmer Life Sciences, Boston, MA.

Radioligand Binding Assay. The binding affinity of [Dmt^1]DALDA to μ, δ and κ receptors was determined by competitive displacement binding using membranes expressing hMOR, hDOR and hKOR, as described above. All binding assays were carried out using 400 μg protein of membranes. For binding to μ receptors, hMOR membranes were incubated with 100 pM [^{3}H][Dmt^1]DALDA and graded concentrations of unlabeled [Dmt^1]DALDA for 60 min at 25^{o}C. Non-specific binding was determined using 1 μM [Dmt^1]DALDA. For binding to δ receptors, hDOR membranes were incubated with 2 nM [^{3}H]DPDPE and graded concentrations of [Dmt^1]DALDA for 120 min at 25^{o}C, and non-specific binding was determined using 8 μM unlabeled DPDPE. For binding to κ receptors, hKOR membranes were incubated with 0.8 nM [^{3}H]U69,593 and graded concentrations of unlabeled [Dmt^1]DALDA for 80 min at 25^{o}C, and non-specific binding was determined with 10 μM naloxone. Free radioligand was separated from bound radioligand by rapid filtration through GF/B filters (Brandel, Gaithersberg, MD) with a cell harvester (Brandel). Filters were washed 3 times with 3 ml of Tris-HCl buffer. Radioactivity was determined by liquid scintillation counting. All binding experiments were carried out in triplicate, and the results represent mean ± S.E. from 4-6 experiments. IC_{50} was determined from the displacement curves using nonlinear regression (Graphpad^{TM}, San Diego, CA). Ki values were calculated from the IC_{50} values by means of the Cheng and Prusoff equation, $Ki = IC_{50}/(1+L/Kd)$, where L and Kd are the concentration and affinity of the radiolabeled ligand in the assay (Cheng and Prusoff, 1973). The Kd values for [^{3}H][Dmt^1]DALDA, [^{3}H]DPDPE and [^{3}H]U69,593 in the MOR, DOR and KOR membranes

were previously determined to be 154 ± 10 pM, 1.95 ± 0.20 nM, and 0.694 ± 0.08 nM, respectively (data not shown).

[^{35}S]GTPγS Binding Assay. Activation of [^{35}S]GTPγS binding by increasing concentrations of [Dmt^{1}]DALDA in hMOR, hDOR and hKOR membranes was used to ascertain the potency and intrinsic activity of [Dmt^{1}]DALDA at μ, δ and κ receptors, respectively. Aliquots of membrane homogenates (8 μg protein) were incubated with 80 pM [^{35}S]GTPγS and 30 μM GDP in 1 ml Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 0.1%BSA, pH 7.4) in the presence of varying concentrations of [Dmt^{1}]DALDA for 60 min at 30^{o}C. Nonspecific binding was determined using 10 μM unlabelled GTPγS. Free radioligand was separated from bound radioligand by rapid filtration. All experiments were carried out in triplicate, and the results represent data from 4-6 experiments. Potency (EC_{50}) and intrinsic activity (Emax) were determined using nonlinear regression (Graphpad™, San Diego, CA). The EC_{50} values are presented with 95% confidential intervals.

Drug Administration. Drugs were administered i.t., i.c.v. or subcutaneously (s.c.) to mice. Intrathecal injection was carried out according to the method described by Hylden and Wilcox (Hylden and Wilcox, 1980). The needle (30 gauge) was inserted from the side of the L5 or L6 spinous process and the injection volume was 4 μl/mouse. For i.c.v. injections, mice were lightly anesthetized with isoflurane and an incision made over the scalp to expose the bregma. The injection (4 μl) was delivered 2 mm lateral and caudal to the bregma to a depth of 3 mm (Haley and McCormick, 1957). Groups of 10-20 mice were used for each dose and each mouse was only used once. Potency was determined at 30 min after i.t. and i.c.v. injection.

Antisera and Antagonists Pretreatment. Antisera against dynorphin A (1-17) or [Met^{5}]enkephalin were administered either i.t. or i.c.v , in an injection volume of 4 μl , 30 min

prior to [Dmt[1]]DALDA administration. The κ antagonist, norbinaltorphimine (norBNI), was administered s.c. 24 h prior to [Dmt[1]]DALDA. The δ antagonist, naltriben, was administered s.c. 30 min prior to [Dmt[1]]DALDA.

Antinociceptive Assay. The radiant heat tail-flick assay was used for antinociceptive tests in mice. The light intensity was adjusted such that the baseline latencies ranged between 2.5 – 3.5 s. In order to avoid tissue damage, a cut-off of 10 s was used. Antinociceptive activity for each animal was expressed as percent of maximal possible effect (% MPE) which was calculated as (P2-P1)/(10-P1) x 100, where P1 and P2 are pre-drug and post-drug response time, respectively. For dose-response analyses, the percentage of analgesic responders was calculated and the quantal dose-response curves analyzed using probit analysis (PharmTools Pro, McCary Group Inc., Elins Park, PA). Antinociception was defined as a latency response of greater than two times the baseline latency for an individual animal. Data are presented as ED_{50} with 95% confidence intervals (CI). Statistical comparisons of dose-response curves were performed by analysis of variance with F-statistic (PharmTools Pro).

Results

Radioligand binding assay. The affinity of [Dmt[1]]DALDA for μ, δ and κ receptors was originally ascertained by displacement of [^{3}H]DAMGO and [^{3}H]DSLET binding from rat brain membranes, and displacement of [^{3}H]U69,593 binding from guinea pig brain membranes, respectively (Schiller et al. 2000). Because brain tissues express more than one subtype of opioid receptors and the specificity of these radioligands are not absolute, we have evaluated the selectivity of [Dmt[1]]DALDA by determining its affinity in pure populations of μ, δ and κ receptors. Competitive displacement binding resulted in Ki of 156 ± 26 pM for hMOR membranes (n = 6), 1.67 ± 0.04 μM for hDOR membranes (n = 4), and 4.4 ± 1.7 nM for hKOR membranes (n = 4). This resulted in μ:δ selectivity of 10,700, and μ:κ selectivity of 27.

[^{35}S]GTPγS binding. The functional activity of [Dmt[1]]DALDA was previously determined using the guinea pig ileum and mouse vas deferens assays (Schiller et al. 2000). Since both guinea pig ileum and mouse vas deferens contain more than one subtype of opioid receptors, we have compared the potency and intrinsic activity of [Dmt[1]]DALDA in [^{35}S]GTPγS binding using hMOR, hDOR and hKOR membranes. The results are summarized in Fig. 1. [Dmt[1]]DALDA increased [^{35}S]GTPγS binding in all three membranes, with EC_{50} being 17 (8.8 – 33) nM for hMOR (n =4), 2.0 (1.2 – 3.2) μM for hDOR (n = 5), and 124 (15 – 1000) nM for hKOR (n = 4).

Effects of Pretreatment with norBNI on i.t. Administration of [Dmt[1]]DALDA. To determine if the κ opioid receptor plays a role in the intrathecal action of [Dmt[1]]DALDA, mice were pretreated with the κ antagonist, norbinaltorphimine (norBNI) (13.6 μmol/kg, s.c.), 24 h prior to i.t. administration of [Dmt[1]]DALDA (4.1 pmol). The antinociceptive response to i.t. [Dmt[1]]DALDA was significantly reduced in mice pretreated with norBNI (Fig. 2A) and the ED_{50}

of i.t. [Dmt^1]DALDA was increased from 1.22 (0.59 – 2.34) pmol to 11.6 (3.09 – 44.8) pmol ($P<0.05$) (Fig. 2B).

Effects of Pretreatment with NorBNI on i.c.v Administration of [Dmt^1]DALDA. Mice were pretreated with norBNI (13.6 µmol/kg, s.c.) 24 h prior to i.c.v. administration of [Dmt^1]DALDA (10.2 pmol). Fig. 3 shows that, in contrast to i.t. [Dmt^1]DALDA, the antinociceptive response to i.c.v. [Dmt^1]DALDA was not affected by norBNI.

Effects of Pretreatment with Antiserum against Dynorphin A (1-17) on Tail-Flick Inhibition induced by i.t. and i.c.v. Administration of [Dmt^1]DALDA. To determine if spinal dynorphin contributes to the antinociceptive response to i.t. [Dmt^1]DALDA, groups of mice were pretreated with increasing dilutions of antiserum against dynorphin A (1-17) 30 min before i.t. administration of an ED_{80} dose of [Dmt^1]DALDA (4.1 pmol). Fig. 4A shows the reduction in antinociceptive response to i.t. [Dmt^1]DALDA following i.t. dynorphin A (1-17) antiserum pretreatment. The maximal reduction in antinociceptive response (30% MPE) was observed with 1:200 of the antiserum. Pretreatment with this dilution of dynorphin A (1-17) antiserum increased the ED_{50} of [Dmt^1]DALDA from 1.22 pmol to 6.2 (3.6 – 12.6) pmol ($P<0.05$) (Fig. 4B). In contrast, i.c.v. pretreatment with the same dilution of dynorphin A (1-17) antiserum had no effect on the antinociceptive response to i.c.v. [Dmt^1]DALDA (10.2 pmol) (data not shown).

Effects of Pretreatment with Antiserum against [Met^5]enkephalin on i.t. and i.c.v. Administration of [Dmt^1]DALDA. To determine if [Met^5]enkephalin also contributes to the antinociceptive response to i.t. [Dmt^1]DALDA, groups of mice were pretreated i.t. with varying dilutions of [Met^5]enkephalin antiserum 30 min before i.t. administration of an ED_{80} dose of [Dmt^1]DALDA (4.1 pmol). This antiserum does not show cross-reactivity to dynorphin peptides. Fig. 5A shows the reduction in antinociceptive response to i.t. [Dmt^1]DALDA

following i.t. [Met^5]enkephalin antiserum pretreatment. The maximal reduction in antinociceptive response (30% MPE) was observed with 1:50 dilution of the antiserum. Pretreatment with this dilution of [Met^5]enkephalin antiserum (1:50) increased the ED_{50} of [Dmt^1]DALDA from 1.22 pmol to 6.6 (3.5 – 19.6) pmol ($P<0.05$) (Fig. 5B). In contrast, i.c.v. pretreatment with the same dilution of [Met^5]enkephalin antiserum had no effect on the antinociceptive response to i.c.v. [Dmt^1]DALDA (10.2 pmol) (Fig. 6A).

Effects of Naltriben Pretreatment on i.t. [Dmt^1]DALDA. To establish a role for the δ opioid receptor in the spinal action of [Dmt^1]DALDA, mice were pretreated with naltriben (3 mg/kg, s.c.) 30 min prior to administration of [Dmt^1]DALDA (4.1 pmol, i.t,). This dose of naltriben was chosen because it significantly reduced the antinociceptive response to i.t. deltorphin and increased its ED_{50} from 7.42 (4.0-15.2) to 20.8 (12.1-40.9) nmol (Fig. 6B). This dose of naltriben also significantly reduced the antinociceptive response to i.t. [Dmt^1]DALDA (Fig. 6B).

Effects of i.t. Pretreatment with Antisera against Dynorphin A (1-17) or [Met^5]enkephalin on i.t. Morphine. To determine if the additional contribution of dynorphin and [Met^5]enkephalin on spinal antinociception was unique to [Dmt^1]DALDA, we also examined the effect of dynorphin antiserum and [Met^5]enkephalin antiserum on i.t. morphine. Mice were administered an ED_{80} dose of morphine (2.63 nmol) 30 min after i.t. administration of either dynorphin antiserum (1:200) or [Met^5]enkephalin antiserum (1:50). Neither antiserum had any effect on the antinociceptive response to i.t. morphine (Fig. 7).

Effects of Pretreatment with naloxonazine on i.t. [Dmt^1]DALDA. The different responses of [Dmt^1]DALDA and morphine to dynorphin antiserum and [Met^5]enkephalin antiserum suggested the possibility that they may be acting on different subtypes of μ opioid receptor.

Pretreatment with naloxonazine (μ_1 antagonist; 48.3 μmol/kg, s.c.) 24 hours before did not significantly alter the ED_{50} to i.t. or i.c.v. [Dmt^1]DALDA (data not shown).

Discussion

Previous studies revealed that the intrathecal potency of [Dmt[1]]DALDA far exceeded its affinity and potency at the μ opioid receptor. This discrepancy led us to postulate that additional mechanisms other than activation of μ opioid receptor must be involved in the spinal action of [Dmt[1]]DALDA. Our present study confirms the extraordinary selectivity of [Dmt[1]]DALDA for the μ opioid receptor. In the original study, the selectivity of [Dmt[1]]DALDA was based on displacement of [^{3}H]DAMGO and [^{3}H]DSLET binding to mouse brain membranes, and displacement of [^{3}H]U69,593 binding to guinea pig brain membranes (Schiller et al. 2000). With the availability of pure populations of transfected MOR, DOR and KOR, and the recent synthesis of [^{3}H][Dmt[1]]DALDA (Zhao et al. 2002), we were able to more accurately determine the affinity of [Dmt[1]]DALDA for the three opioid receptor subtypes. The affinity of [Dmt[1]]DALDA, as determined from saturation binding with [^{3}H][Dmt[1]]DALDA and MOR membranes, was found to be 154 ± 10 pM, and this is in excellent agreement with the Ki reported previously (143 pM) (Schiller et al. 2000). The μ:δ selectivity of [Dmt[1]]DALDA was found to be ~10,000, which is also similar to the earlier estimate (14,700) obtained from brain membranes (Schiller et al. 2000) and is similar to that reported for endomorphin-1 and endomorphin-2 (Zadina et al. 1997). [Dmt[1]]DALDA has modest affinity for KOR, and the MOR:KOR selectivity determined using pure receptor populations (27) is less than that previously determined in mouse brain membranes with selective radioligands (156) (Schiller et al. 2000).

The present study provides the first report on the potency and intrinsic activity of [Dmt[1]]DALDA at the three opioid receptors using GTPγS binding as a measure of G protein activation. [Dmt[1]]DALDA stimulated [^{35}S]GTPγS binding to membranes expressing MOR,

DOR and KOR in a concentration-dependent manner. The maximal stimulation of [35S]GTPγS binding by [Dmt1]DALDA was 90% compared to DAMGO at hMOR, 123% compared to deltorphin II at hDOR, and 46% compared to U50,488H ([trans-(±)-3,4-dichloro-N-methyl-[2-(1-pyrolidinyl)-cyclohexyl] benzeneacetamide) at hKOR (G.-M. Zhao and H.H. Szeto, unpublished data). These data suggest that [Dmt1]DALDA is a full agonist at hMOR and hDOR, but a partial agonist at hKOR.

The involvement of δ and κ opioid receptors in the spinal action of [Dmt^1]DALDA is most likely not due to direct action of [Dmt^1]DALDA on these two receptors, especially when the same antagonist treatment had no effect on supraspinal [Dmt^1]DALDA, and the μ:δ selectivity of [Dmt^1]DALDA is in excess of 10,000. We propose that [Dmt^1]DALDA causes the release of dynorphin-like and [Met^5]enkephalin-like substances in the spinal cord that can then subsequently act on κ and δ receptors, respectively, to contribute to the overall antinociceptive action of intrathecal [Dmt^1]DALDA. This idea is supported by the finding that the antinociceptive response to intrathecal [Dmt^1]DALDA was significantly attenuated by intrathecal administration of antisera to dynorphin A (1-17) or [Met^5]enkephalin. The dynorphin antiserum used in the study was raised against dynorphin A(1-17), and shows only 0.43% cross-reactivity against dynorphin A(1-13), and none against [Leu^5]enkephalin or dynorphin B. Likewise, the [Met^5]enkephalin antiserum only has 2.8% cross-reactivity against [Leu^5]enkephalin and none against dynorphin A(1-17). These antisera had no effect on the antinociceptive response to intrathecal morphine, and i.c.v. administration of these antisera had no effect on supraspinal [Dmt^1]DALDA. This is consistent with the lack of effect of norBNI or naltriben on supraspinal [Dmt^1]DALDA. It was also noted in an earlier paper that norBNI and

naltrindol (another δ antagonist) had no effect on supraspinal [Dmt^1]DALDA; however, their effect on spinal [Dmt^1]DALDA was not examined (Neilan et al. 2001).

The enkephalin peptides mediate antinociception supraspinally and spinally by interacting with delta$_1$ and delta$_2$ receptors, respectively (Takemori and Portoghese, 1993). In our study, the response to i.t. [Dmt^1]DALDA was attenuated by pretreatment with naltriben (delta$_2$ antagonist), while naltriben had no effect on supraspinal [Dmt^1]DALDA. There is evidence that enkephalins and other δ agonists can potentiate the action of μ agonists in the spinal cord (Vaught and Takemori, 1979; Lee et al., 1980; He and Lee, 1998), and the release of endogenous [Leu^5]enkephalin by swim-stress potentiated the antinociceptive potency of morphine (Vanderah et al., 1993). Thus the release of enkephalin by [Dmt^1]DALDA can be expected to potentiate the action of [Dmt^1]DALDA at μ receptors in the spinal cord.

The role of dynorphin in pain modulation is highly controversial. Whereas low doses of dynorphin produce antinociception, higher doses produce a long-lasting allodynia (Vanderah et al., 1996; Laughlin et al., 1997). Many studies support an antinociceptive function of dynorphin in the spinal cord by negatively modulating transmission of nociceptive information. Dynorphin can inhibit substance P release (Zachariou and Goldstein, 1997) and inhibit synaptic transmission of nociceptive neurons in the spinal cord (Randic et al., 1995) via activation of κ receptors. On the other hand, dynorphin can exert pronociceptive effects via non-opioid mechanisms that involve N-methyl-D-aspartate receptors (Bakshi et al., 1992; Laughlin et al. 1997; Vanderah et al., 2000). Recent data obtained with *dream*$^{-/-}$ mice clearly support an antinociceptive role for endogenous dynorphin. DREAM (downstream regulatory element antagonistic modulator) is a putative transcriptional repressor for the prodynorphin gene (Carrion et al., 1999). Mice lacking DREAM had elevated levels of prodynorphin mRNA and dynorphin A peptides in the spinal

cord and displayed reduced responses in models of acute thermal, mechanical and visceral pain (Cheng et al., 2002).

Activation of spinal release of dynorphin was reported to contribute to the intrathecal action of endomorphin-2, but not endomorphin-1(Ohsawa et al. 2001; Sakurada et al. 2001). The antinociceptive response to intrathecal endomorphin-2 was blocked by intrathecal pretreatment with antiserum against dynorphin A(1-17) or norBNI. However, spinal dynorphin does not appear to play a role in the spinal action of endomorphin-1 nor DAMGO (Sakurada et al. 2001). We also found no effect of dynorphin antiserum or norBNI on intrathecal morphine action. As the endomorphins and DAMGO are also highly selective μ agonists, these findings suggest that only certain μ agonists can elicit the release of dynorphin from the spinal cord. There is less evidence to support a role for endogenous enkephalins in the spinal action of other μ agonists. It was reported that [Met^5]enkephalin antiserum blocked the effect of intrathecal endomorphin-2 in one study (Sakurada et al. 2001) but not in another (Ohsawa et al. 2001).

It has been proposed that these diverse μ agonists may activate different subtypes of μ receptors in the spinal cord (Sakurada et al., 1999; Sakurada et al., 2000). Autoradiographic studies have demonstrated the presence of both μ_1 and μ_2 receptors in the brain and spinal cord (Moskowitz and Goodman, 1985). Pretreatment with naloxonazine, the μ_1 antagonist, partially blocked the action of intrathecal endomorphin-2 but not endomorphin-1 or DAMGO, suggesting that activation of μ_1 receptors in the spinal cord leads to dynorphin release (Sakurada et al. 1999; Sakurada et al. 2001). However, in another study, the authors reported that the same dose of naloxonazine blocked all three agonists, although endomorphin-2 was more sensitive compared to endomorphin-1 and DAMGO (Sakurada et al. 2000). In general, available evidence suggests that μ_1 is involved in supraspinal but not spinal analgesia. In the present

study, naloxonazine had no significant effect on either i.t. or i.c.v. [Dmt^1]DALDA, and this is consistent with findings reported earlier (Neilan et al. 2001). In view of the confusion in the literature, and the lack of a molecular correlate for the pharmacologically-defined μ_1 receptor, it is probably premature to speculate on μ receptor subtypes and mechanisms of spinal analgesia.

In summary, intrathecal [Dmt^1]DALDA causes the release of dynorphin-like and [Met^5]enkephalin-like substances in the spinal cord that subsequently act on κ and δ receptors, respectively, to contribute to the potency of intrathecal [Dmt^1]DALDA.

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Footnotes

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Figure Legends

Fig. 1 The stimulation of [^{35}S]GTPγS binding by [Dmt^{1}]DALDA to membranes prepared from CHO-K1 cells transfected with hMOR or hDOR, and HEK293 cells transfected with hKOR. Membranes were incubated with 80 pM [^{35}S]GTPγS and 30 μM GDP in the presence of varying concentrations of [Dmt^{1}]DALDA for 60 min at 30^{o}C. Nonspecific binding was determined using 10 μM unlabelled GTPγS. [Dmt^{1}]DALDA increased [^{35}S]GTPγS binding in all three membranes, with EC_{50} being 17 (8.8 – 33) nM for MOR (n=4), 2.0 (1.2 – 3.2) μM for DOR (n=5), and 124 (15 – 1000) nM for KOR (n=4).

Fig. 2 Effects of norBNI on tail-flick inhibition induced by i.t. [Dmt^{1}]DALDA in CD-1 mice. A. Time-course of antinociceptive response to i.t. [Dmt^{1}]DALDA (4.1 pmol) in the absence and presence of norBNI. B. Dose-response curves for i.t. [Dmt^{1}]DALDA in the absence and presence of norBNI. Mice were pretreated with norBNI (13.6 μmol/kg, s.c.) 24 h prior to i.t. administration of [Dmt^{1}]DALDA. Pretreatment with norBNI increased the ED_{50} of i.t. [Dmt^{1}]DALDA from 1.22 pmol to 11.6 pmol ($P<0.05$).

Fig. 3 Effects of pretreatment with norBNI on i.t. and i.c.v. [Dmt^{1}]DALDA in CD-1 mice. NorBNI (13.6 μmol/kg) was administered s.c. 24 h prior to [Dmt^{1}]DALDA (4.1 pmol i.t. or 10.2 pmol i.c.v.). The antinociceptive response to i.t. [Dmt^{1}]DALDA was significantly attenuated by i.t. pretreatment with norBNI ($P < 0.001$; t-test). NorBNI had no effect on i.c.v. [Dmt^{1}]DALDA. % MPE, percent of maximal possible effect as defined in **Materials and Methods**.

Fig. 4 Effects of i.t. pretreatment with dynorphin A (1-17) antiserum on tail-flick inhibition induced by i.t. [Dmt^{1}]DALDA in CD-1 mice. A. Effect of dynorphin A (1-17) antiserum titration on the response to an ED_{80} dose of [Dmt^{1}]DALDA (4.1 pmol). B. Dose-response curves to i.t. [Dmt^{1}]DALDA with and without dynorphin antiserum (1:200). Mice were pretreated with i.t. dynorphin A (1-17) antiserum 30 min prior to the i.t. administration of [Dmt^{1}]DALDA. Pretreatment with dynorphin antiserum (1:200) increased the ED_{50} of [Dmt^{1}]DALDA from 1.22 pmol to 6.2 pmol ($P<0.05$). Anti-Dyn, dynorphin A (1-17) antiserum; % MPE, percent of maximal possible effect as defined in **Materials and Methods**.

Fig. 5 Effects of i.t. pretreatment with [Met^{5}]enkephalin antiserum on tail-flick inhibition induced by i.t. [Dmt^{1}]DALDA in CD-1 mice. A. Effects of [Met^{5}]enkephalin antiserum titration on the response to an ED_{80} dose of [Dmt^{1}]DALDA (4.01 pmol). B. Dose-response curves to i.t. [Dmt^{1}]DALDA with and without [Met^{5}]enkephalin antiserum (1:50). Mice were pretreated with i.t. [Met^{5}]enkephalin antiserum 30 min prior to the i.t. administration of [Dmt^{1}]DALDA. Pretreatment with [Met^{5}]enkephalin (1:50) increased the ED_{50} of [Dmt^{1}]DALDA from 1.22 pmol to 6.6 pmol ($P<0.05$). Anti-ME, [Met^{5}]enkephalin antiserum; % MPE, percent of maximal possible effect as defined in **Materials and Methods**.

Fig. 6 Effects of pretreatment with [Met^{5}]enkephalin antiserum and naltriben on i.t. [Dmt^{1}]DALDA in CD-1 mice. A. Effect of [Met^{5}]enkephalin antiserum on tail-flick inhibition induced by i.t. or i.c.v. [Dmt^{1}]DALDA. Mice were pretreated with [Met^{5}]enkephalin antiserum (1:50) using the same route of administration 30 min before the administration of i.t. [Dmt^{1}]DALDA (4.1 pmol) or i.c.v. [Dmt^{1}]DALDA (10.2 pmol). Pretreatment with

[Met^5]enkephalin antiserum significantly attenuated the response to i.t. [Dmt^1]DALDA ($P < 0.05$; t-test). B. Effect of naltriben pretreatment on tail-flick inhibition induced by i.t. [Dmt^1]DALDA or i.t. deltorphin. Mice were pretreated with naltriben (3 mg/kg) s.c. 30 min prior to i.t. [Dmt^1]DALDA (4.1 pmol) or i.t. deltorphin (19.5 nmol). The antinociceptive response to both [Dmt^1]DALDA and deltorphin was significantly attenuated by naltriben ($P < 0.05$; t-test). Anti-ME, [Met^5]enkephalin antiserum; % MPE, percent of maximal possible effect as defined in **Materials and Methods**.

Fig. 7 Effects of i.t. Pretreatment with antisera against Dynorphin A (1-17) and [Met^5]enkephalin on tail-flick inhibition induced by i.t. Morphine. Mice were pretreated with either dynorphin antiserum (1:200) or [Met^5]enkephalin antiserum (1:50) 30 min prior to i.t. administration of morphine (2.63 nmol). Neither antiserum altered the antinociceptive response to i.t. morphine. Anti-Dyn, dynorphin A (1-17) antiserum; Anti-ME, [Met^5]enkephalin antiserum; % MPE, percent of maximal possible effect as defined in **Materials and Methods**.

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Figure 1

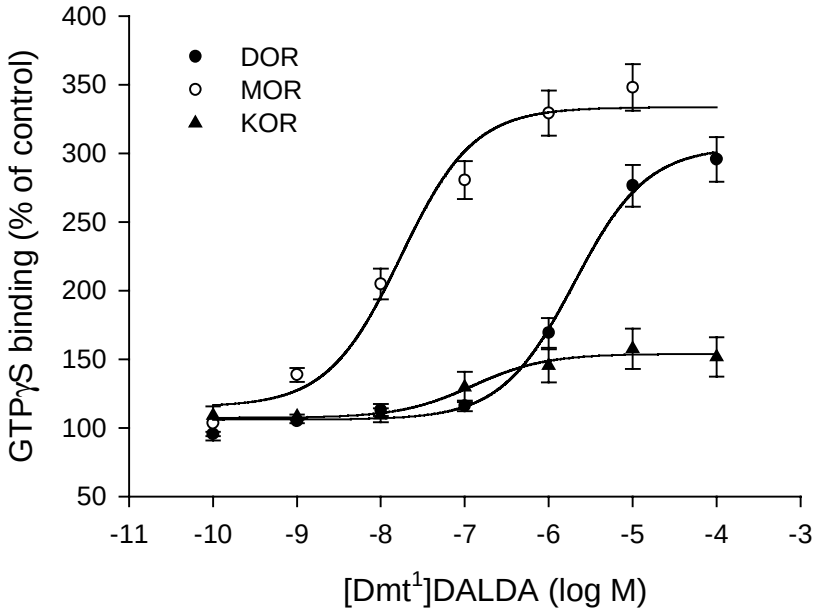

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Fig. 2

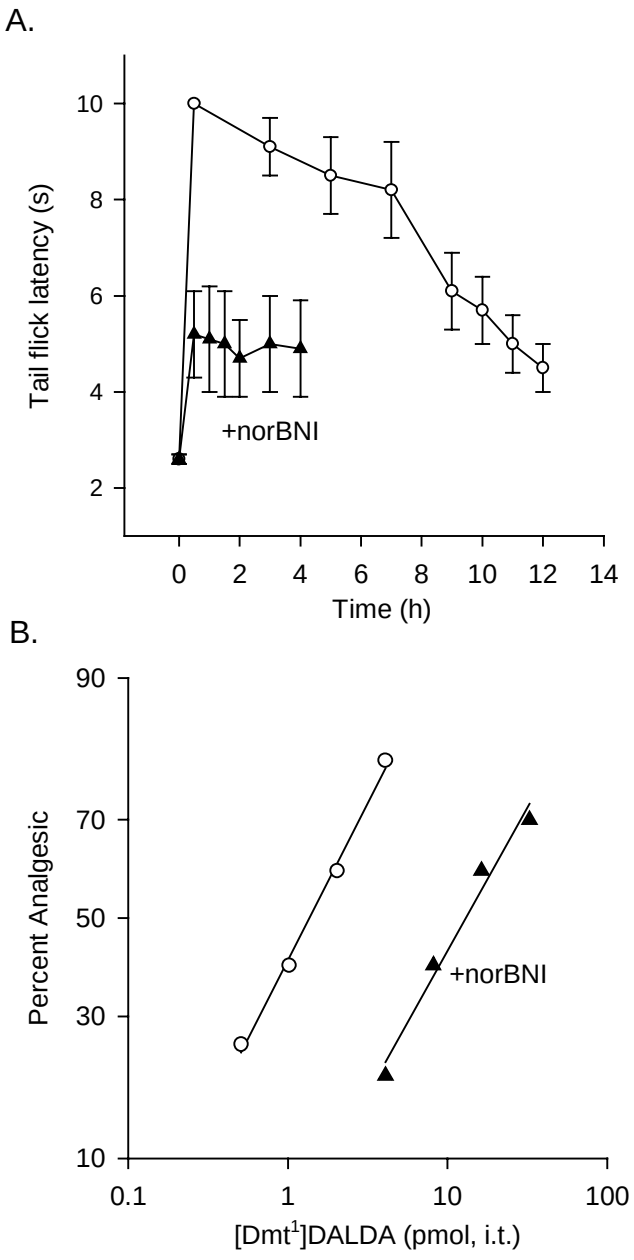

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Fig. 3

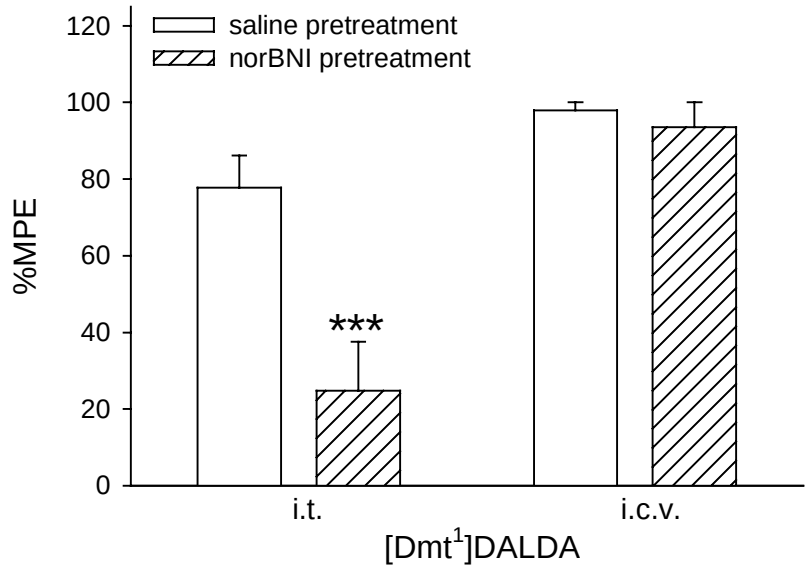

Figure 4

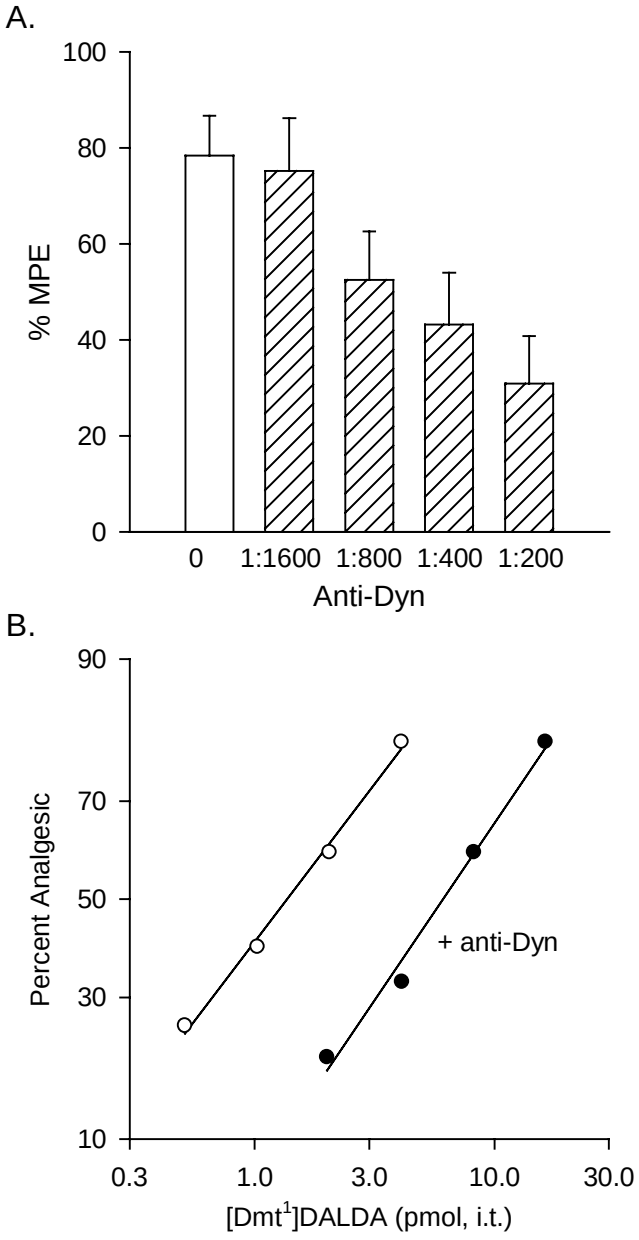

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Fig. 5

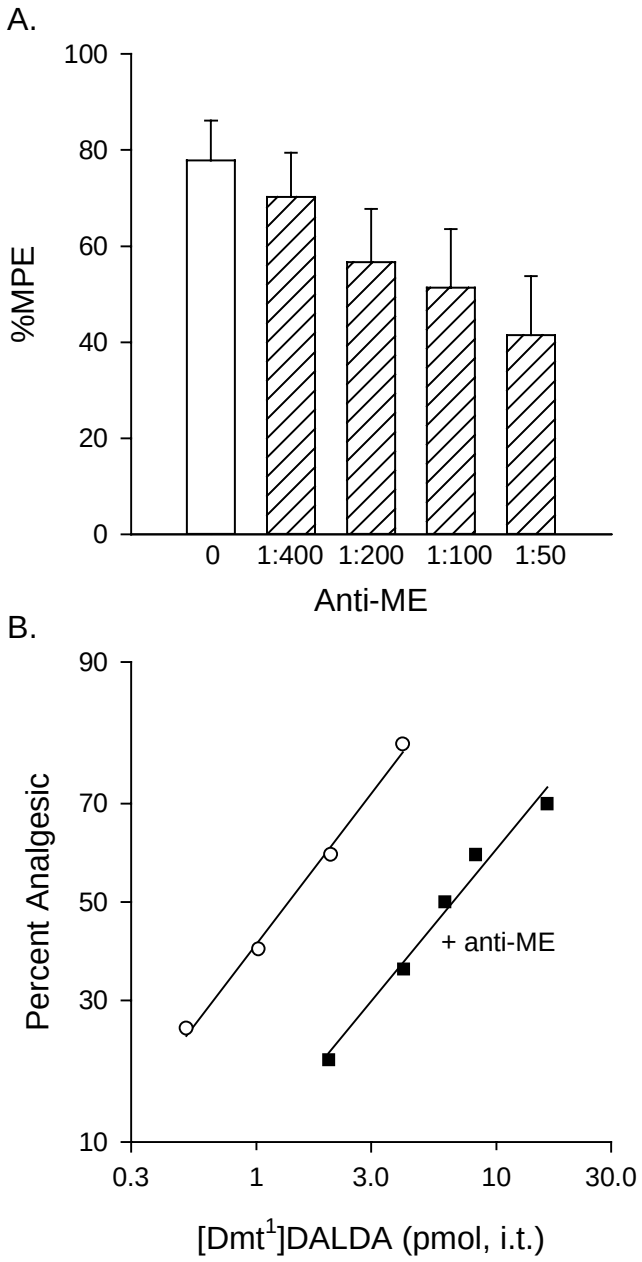

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Fig. 6

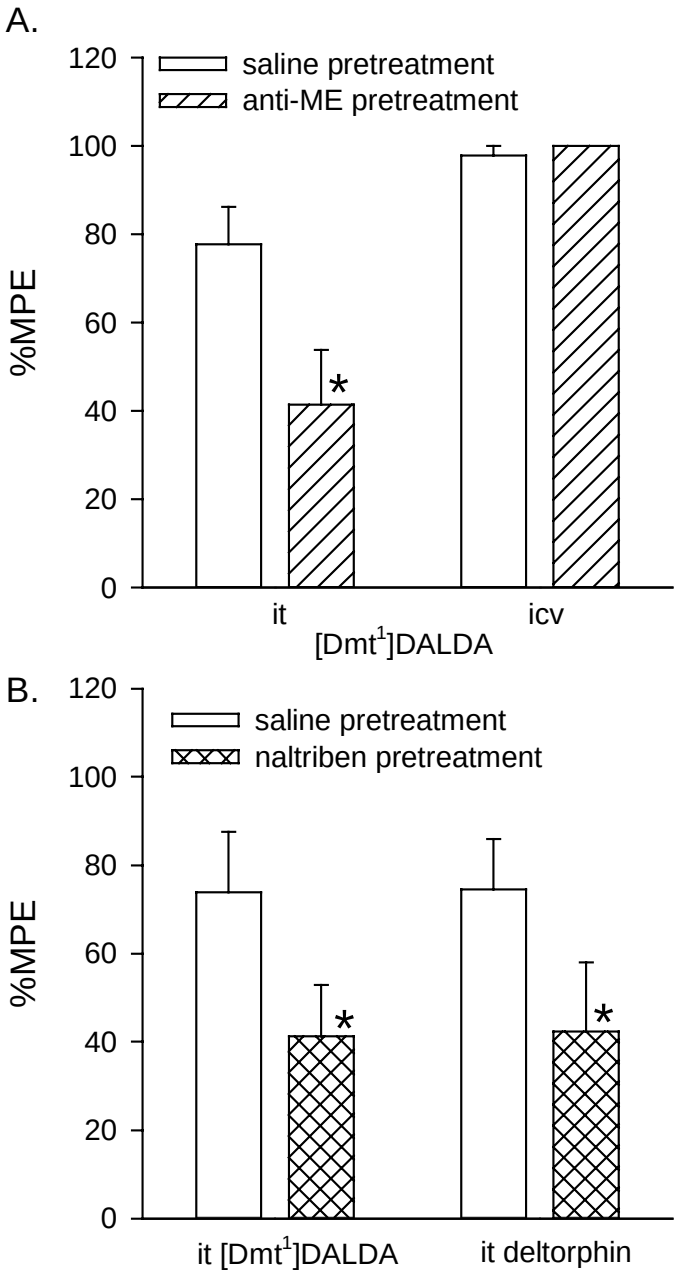

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Fig. 7