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Differential conditioned place preference responses to endomorphin-1 and endomorphin-2 microinjected into the posterior nucleus accumbens shell and ventral tegmental area in the rat

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Running title: mesolimbic place preference responses to endomorphins

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CPA, conditioned place aversion; CPP, conditioned place preference; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; Dyn, Dynorphin A (1-17); EM-1, endomorphin-1; EM-2, endomorphin-2; NRS, normal rabbit serum.

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Abstract

An unbiased conditioned place preference (CPP) paradigm was used to evaluate the reward effects of endogenous μ-opioid receptor ligands, endomorphin-1 (EM-1) and endomorphin-2 (EM-2) from the mesolimbic posterior nucleus accumbens (Acb) shell and the ventral tegmental area (VTA) in CD rats. EM-1 (1.6-8.1 nmol) microinjected into posterior Acb shell produced CPP, while EM-2 (8.7-17.5 nmol) given into the same Acb shell produced conditioned place aversion (CPA). EM-1 (1.6-16.3 nmol) microinjected into the VTA produced CPP, while EM-2 (8.7 and 17.5 nmol) given into the same VTA site did not produce any effect, but at a high dose (35 nmol) produced CPP. EM-1 (3.3 nmol) or EM-2 (17.5 nmol) microinjected into the nigrostriatal substantia nigra was not significantly different from vehicle-injected groups. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) at 94.13 pmol or 3-methoxynaltrexone at 0.64 pmol microinjected into the posterior Acb shell blocked EM-1-induced CPP and EM-2-induced CPA. At a higher dose, CTOP (941.3 pmol) and 3-methoxynaltrexone (6.4 pmol) produced CPA and CPP, respectively. Co-administration with antiserum against dynorphin A(1-17) (Dyn) (10 μg) microinjected into the posterior Acb shell blocked EM-2-induced CPA. However, it did not affect EM-1-induced CPP. It is concluded that EM-1 and EM-2 produce site dependent CPP and CPA, respectively, by stimulation of different subtypes of μ-opioid-receptors; stimulation of one subtype of μ-opioid-receptor at the posterior Acb shell and VTA by EM-1 induces CPP, while stimulation of another subtype of μ-opioid receptor at the posterior Acb shell, but not the VTA by EM-2 induces the release of Dyn to produce CPA.

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The newly discovered endogenous opioid peptides, endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) activate μ-opioid receptors with high affinity and selectivity (Zadina et al., 1997; Tseng et al., 2000). In the opioid receptor binding assay, both EM-1 and EM-2 selectively compete for μ-opioid receptor sites with high affinity and have no appreciable affinities for δ- and κ_1- opioid receptors (Goldberg et al., 1998; Monory et al., 2000). Both EM-1 and EM-2 also activate μ-opioid receptor-mediated G-protein activation by increasing the binding of [^{35}S]GTPγS, which is selectively blocked by the μ-opioid receptor antagonists βfunaltrexamine (β-FNA) and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), but not by δ antagonist naltrindole or κ antagonist nor-binaltorphimine (nor-BNI) (Narita et al., 1998, 2000). In addition, neither EM-1 nor EM-2 produces any G-protein activation in the membrane preparation obtained from μ-opioid receptor clone (MOR-1) knockout mice (Mizoguchi et al., 1999; Narita et al., 1999). The specific action of EM-1 and EM-2 in stimulating the μ-opioid receptor found *in vitro* is consistent with the *in vivo* antinociceptive studies in mice. For example, EM-1 or EM-2 given intracerebroventricularly or intrathecally produces antinociception, which is blocked by the pretreatment with CTOP or β-FNA (Goldberg et al., 1998; Tseng et al., 2000; Ohsawa et al., 2001). EM-1 or EM-2 does not produce any antinociception in MOR-1 knockout mice (Mizoguchi et al., 1999). These findings strongly indicate that the μ-opioid receptors play an essential role in mediating EM-1- and EM-2-induced antinociception.

However, recent studies indicate that different subtypes of μ-opioid receptors are involved in antinociception induced by EM-1 and EM-2. Like morphine or DAMGO,

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EM-1 stimulates one subtype of μ-opioid receptors, while EM-2 stimulates another subtype of μ-opioid receptors that involve the release of dynorphin acting on κ-opioid receptors for producing antinociception (Tseng et al., 2000; Ohsawa et al., 2001). This view is supported by the findings that pretreatment with the μ_1-receptor antagonist naloxonazine or 3-methoxynaltrexone, blocks the antinociception induced by EM-2 more effectively than EM-1 (Sakurada et al., 2000, 2001). Spinal pretreatment with antisense oligodeoxynucleotides (ODNs) against exon-1, -4, or -8 of MOR-1 to knockdown different isoforms of the μ-opioid receptor, differentially attenuates the antinociception induced by EM-1 and EM-2 (Wu et al., 2002). In addition, mice or rats made antinociceptive tolerant to EM-1 exhibit no cross-tolerance to EM-2, while mice or rats made tolerant to EM-2 exhibit a cross-tolerance to EM-1 to produce antinociception (Wu et al., 2001; Hung et al., 2002). These findings strongly indicate that different subtypes of μ-opioid receptors are involved in the pharmacological actions produced by EM-1 and EM-2.

The evidence that different subtypes of μ-opioid receptor are involved in pharmacological actions of EM-1 and EM-2 can also be obtained from the conditioning place preference (CPP) studies. Like other μ-opioid receptor agonists such as morphine or DAMGO, EM-1 given intracerebroventricularly in mice produces CPP, while EM-2 produces conditioned place aversion (CPA) (Narita et al., 2001, 2002). Since the nucleus accumbens (Acb) and ventral tegmental area (VTA) represent two key structures of the mesolimbic dopaminergic system for the reinforcing properties of opioids (Herz and Spanagel, 1995; Watson et al., 1989; Wise and Rompre, 1989), the present study was then undertaken to investigate the differential reward effects of CPP and CPA of EM-1

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and EM-2 microinjected into the mesolimbic dopaminergic posterior Acb shell and VTA in rats. The possible mechanisms for EM-1 and EM-2 to produce opposite CPP and CPA responses were discussed based on the hypothesis that EM-1 and EM-2 stimulate different subtypes of μ-opioid receptors.

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Methods and Materials

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 ± 0.5°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 (5mg/kg, intraperitoneal), anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal) and were mounted in a stereotaxic apparatus (David Kopf Instrument, Tujinga, 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. Unilateral as opposed to bilateral placement of the cannula was chosen for the reason that bilateral injections are more difficult to perform symmetrically at the intended injection site, and previous reports suggest unilateral injection is sufficient to produce CPP and CPA (Bals-Kubik et al., 1993; Suzuki et al., 1997; Zangen et al., 2002). The coordinates for the placement of the cannula were AP 0.7-1.00 mm posterior to bregma and 1.00 mm lateral to the midline for posterior Acb shell (N = 204); AP 2.70-2.96 mm anterior to interaural point and 0.5 mm lateral to the midline for VTA (N = 81); AP 3.70 mm anterior to interaural and 1.9 mm lateral to the midline for substantia nigra (N = 31) (Paxinos and Watson, 1997). After a

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recovery period of at least 5 days, animals that were without motor defects and with normal pre-conditioning were used for the experiments.

Conditioned Place Preference (CPP). The place conditioning experiment consisted of pre-conditioning, conditioning and post-conditioning phases. Injections of vehicle or drug were only done during the conditioning phase. A two-compartment box (60 x 29.2 x 29.2 cm) with a transparent Plexiglas front separated by a gray cylinder platform (10.3 cm in diameter and 12 cm in height) was used. One compartment was white with a textured floor and the other was black with a smooth floor. For pre-conditioning, rats were initially placed on the neutral cylinder gray platform and allowed to step down off of the platform to either the white or black compartment. A sliding wall was then put down on the platform and the rat was free to access either compartment through two openings (9.5 x 12 cm each) on each side of the platform. The amount of time spent in the black or white compartment was manually measured for 15 min. Rats which spent less than 5 min 50 s in either the white or black compartment were considered not to be neutral in preference for either side and were excluded from further study (less than 5 % of rats). This initial preference was measured once in the morning and once in the afternoon for each rat and the values of two measurements were averaged.

The place conditioning session (2^{nd} phase) was carried out on days 2 to 4. The box was divided into two equal-sized compartments by putting down the sliding wall after the removal of the gray cylinder platform. Conditioning session was conducted twice daily, morning and afternoon, and repeated for 3 days. Rats were placed in either the black or white compartment immediately following the injection and left in that compartment for

40 min. Forty min is in agreement with previous studies (Bals-Kubik et al., 1993; Shippenberg and Heidbreder, 1995). Four of 8 rats from each group were confined to either the black or white compartment after injection of EM-1 or EM-2 in the morning session of each day, and were confined to the opposite compartment after the injection of saline vehicle for the afternoon session. The other 4 of 8 rats were confined to either the black or white compartment after injection with saline vehicle for the morning session and were confined to the opposite compartment after injection of EM-1 or EM-2 for the afternoon session of the place conditioning. Animals receiving vehicle in both sessions served as controls. Drug treatment consisted of EM-1or EM-2 microinjected into the posterior Acb shell, VTA, or substantia nigra. The morning session was carried out at 9-10 AM and the afternoon session was carried out at 3-4 PM of the day.

The post-conditioning session (the 3rd phase) was carried out on day 5 and was exactly the same as the pre-conditioning. The scores for the drug-paired place were then calculated by subtracting the pre-conditioning score from post-conditioning score. A positive score represents CPP, while a negative score represents CPA.

Drugs and Drug Administrations. EM-1 and EM-2 were obtained from Calbiochem (La Jolla, CA). The peptides were dissolved in 10% hydroxypropyl-beta-cyclodextrin in sterile saline solution (0.9% NaCl solution) for microinjection into the forebrain sites. CTOP was obtained from BACHEM (Torrance, CA) and dissolved in 0.9% NaCl solution containing 0.01% Triton X-100. 3-Methoxynaltrexone hydrochloride was obtained from Sigma (St. Louis, MO) and dissolved in sterile 0.9% NaCl solution. The antiserum against dynorphin A (1-17) (Dyn) was produced by immunization of male New Zealand white rabbits according to the method described in previous publication,

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and the potencies and cross-immunoreactivity of this antiserum have been characterized (Tseng and Collin, 1993; Wu et al., 2002). The injection volume for each microinjection was 0.5 µl. Injections were made by hand with a 30 gauge injection needle attached to a microsyringe via polyethylene tubing and administered over a 30 s period. The injection needle was left in place for an additional 60 s to ensure complete distribution. The stereotaxic coordinates of the intended injection sites were AP 0.7-1.00 mm posterior to bregma, 1.00 mm lateral to the midline and 7.4 mm down from the surface of the skull for posterior Acb shell; AP 2.70-2.96 mm anterior to interaural point, 0.5 mm lateral to midline and 8.4 mm down from the surface of the skull for VTA; AP 3.70 mm anterior to interaural, 1.9 mm lateral to the midline and 8.2 mm down from the surface of the skull for substantia nigra (Paxinos and Watson, 1997).

Histological Identification. At the end of the experiments, 0.5 µl of methylene blue solution (2% in saline) was injected into the same sites, which had been injected with EM-1, EM-2 or vehicle. The rats were then sacrificed by placing in a CO_2 (100 %) chamber 10 to 20 min after injection. The brains were removed, frozen and sectioned sagittally for microscopic examination of the injection sites. The stereotaxic atlas of rats by Paxinos and Watson (1997) was used as a guide for the identification of anatomic structure.

Statistical Analysis. Conditioning scores are expressed as means ± S.E.M. The mixed factorial two-way analysis of variance (ANOVA) was used to analyze the differences of the score between pre-conditioning and post-conditioning of each group of rats. Student's t-test was used to test the differences between groups and one-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to compare the

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difference between drug treated groups and the vehicle treated group. The accepted level of significance was $p < 0.05$. GraphPad Prism software was used to perform the statistics (version 3.0; GraphPad Software, Inc., San Diego, CA).

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Results

Histological identification of injection sites The coronal sections ranging from bregma 0.48 to 1.20 mm for rats microinjected with EM-1 (Fig. 1A) and EM-2 (Fig 1C) into the posterior Acb, 3.40 to 4.20 mm anterior to interaural point for rats microinjected with EM-1(Fig 1B) and EM-2 (Fig. 1D) into the VTA, and 2.70-3.40 mm anterior to interaural point for rats microinjected with EM-1 and EM-2 (Fig 2) into substantia nigra are shown. Histological examination verified that all the injection sites intended for the posterior Acb shell, VTA and substantia nigra were within the intended regions of the brain sites.

Effects of EM-1 and EM-2 microinjection into the posterior Acb shell on the place preference Groups of rats were microinjected with different doses of EM-1, EM-2 or saline vehicle into the posterior Acb shell for place conditioning. EM-1 at doses from 1.6 to 8.1 nmol given into the posterior Acb shell dose-dependently produced CPP. The CPP produced by EM-1 reached a maximum at 3.3 nmol and at a higher dose (8.1 nmol) produced no further increase of CPP (Fig. 3A; $F_{(3, 44)}$=2.80; $p < 0.05$). In contrast, EM-2 at doses from 8.7 to 17.5 nmol given into the same posterior Acb shell dose-dependently produced CPA (Fig. 3B; $F_{(3, 43)}$=4.28; $p < 0.01$). Microinjection of the vehicle did not produce any significant effect on the CPP or CPA.

Effects of EM-1 and EM-2 microinjection into the VTA on the place preference Groups of rats were microinjected with different doses of EM-1, EM-2 or saline vehicle into the posterior VTA for place conditioning. EM-1 at doses from 1.6 to 16.3 nmol dose-dependently produced CPP. The CPP response to EM-1 reached a maximum at a dose of 8.1 nmol and at a higher dose (16.3 nmol) produced no further

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increase of CPP (Fig 4A; $F_{(3, 42)}$=3.34; $p < 0.03$). EM-2 at doses of 8.7 and 17.5 nmol given into the VTA did not produce any effect. However, EM-2 at a higher dose (35.0 nmol) produced CPP (Fig 4B; $F_{(3, 39)}$=1.77; $p < 0.03$). Microinjection of vehicle produced no effect on the place preference.

Effects of EM-1 and EM-2 microinjection into the substantia nigra on the place preference Groups of rats were microinjected with EM-1 (3.3 nmol), EM-2 (17.5 nmol) or vehicle into the substantia nigra for place conditioning (Fig. 2). EM-1, EM-2 or vehicle given into the substantia nigra did not produce any significant CPP or CPA (Data not shown).

Effects of different doses of CTOP microinjection into the posterior Acb shell on the place preference Groups of rats were microinjected with different doses of CTOP or vehicle into the posterior Acb shell for the place conditioning. Microinjection of CTOP at doses of 47.07 and 94.13 pmol given into the posterior Acb shell did not produce any significant CPP or CPA. However, CTOP at a higher dose (941.3 pmol) produced CPA (Fig. 5A; $F_{(3, 45)}$=10.60; $p < 0.001$). A dose of 94.13 pmol of CTOP was then chosen for determining the effect on the EM-1-induced CPP and EM-2-induced CPA.

Effects of CTOP microinjected into the posterior Acb shell on the EM-1-induced CPP and EM-2-induced CPA Groups of rats were microinjected with EM-1 (3.3 nmol) or EM-2 (17.5 nmol) alone or co-administered with CTOP (94.13 pmol) into the posterior Acb shell and the effects of CTOP on EM-1-induced CPP or EM-2-induced CPA were studied. Administration of CTOP significantly blocked EM-1-induced CPP and EM-2-induced CPA (Fig. 5B).

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Effects of different doses of 3-methoxynaltrexone microinjected into the posterior Acb shell on the place preference Groups of rats were microinjected with different doses of 3-methoxynaltrexone or vehicle into the posterior Acb shell for place conditioning. 3-Methoxynaltrexone at a dose of 0.64 pmol microinjected into the posterior Acb shell did not produce any significant CPP or CPA. However, a higher dose (6.4 pmol) of 3-methoxynaltrexone given into the posterior Acb shell produced CPP (Fig. 6A; F=4.62). A dose of 0.64 pmol of 3-methoxynaltrexone was then chosen for determining the effect on EM-1-induced CPP and EM-2-induced CPA.

Effects of 3-methoxynaltrexone microinjected into the posterior Acb shell on EM-1-induced CPP and EM-2-induced CPA Groups of rats were microinjected with EM-1 (3.3 nmol) or EM-2 (17.5 nmol) alone or co-administered with 3-methoxynaltrexone (0.64 pmol) into the posterior Acb shell and the effects of 3-methoxynaltrexone on the EM-1-induced CPP and EM-2-induced CPA were studied. 3-Methoxynaltrexone significantly blocked both EM-1-induced CPP and EM-2-induced CPA (Fig. 6B).

Effects of NRS or antiserum against Dyn co-administered with EM-1 and EM-2 microinjected into the posterior Acb shell on EM-2-induced CPA and EM-1-induced CPP Groups of rats were co-administered NRS (10 μg) or antiserum against Dyn (10 μg) with EM-1 (3.3 nmol) or EM-2 (17.5 nmol) microinjected into the posterior Acb shell and the effects on the EM-2-induced CPA and EM-1-induced CPP were studied. Co-administration of antiserum against Dyn with EM-2 microinjected into the posterior Acb shell completely blocked the EM-2-induced CPA. In contrast, the same treatment with antiserum against Dyn did not affect EM-1-induced CPP. NRS

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microinjected into the posterior Acb shell did not produce any significant effect on EM-2-induced CPA (Fig. 7). NRS or antiserum against Dyn did not cause CPP or CPA when administered alone.

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Discussion

EM-1 given into the mesolimbic posterior Acb shell or VTA produces CPP

Previous studies have demonstrated that like other μ-opioid agonists such as morphine and DAMGO, EM-1 given intracerebroventricularly in mice produce CPP (Suzuki et al., 1991; Narita et al., 2001). The CPP induced by EM-1 is blocked by pretreatment with μ-opioid receptor antagonist β-FNA, indicating that the effect is mediated by the stimulation of μ-opioid receptors (Narita et al., 2001). The VTA and Acb represent two key structures of the mesolimbic dopaminergic system for the reinforcing properties of opioids (Watson et al., 1989; Wise and Rompre, 1989). The microinjection technique was then used to identify the sites sensitive to EM-1 involved in the CPP or CPA. We found in the present studies that EM-1 given into either the posterior Acb shell or VTA produced CPP, suggesting that both the posterior Acb shell and VTA are the sites sensitive to EM-1 for producing CPP. The EM-1-induced CPP from the posterior Acb shell was blocked by μ-opioid receptor antagonist CTOP indicating that the effect of EM-1 is mediated by the stimulation of μ-opioid receptors.

Other μ-opioid receptor agonists such as morphine or DAMGO given into the VTA also produce CPP (Bals-Kubit et al., 1993; Olmstead and Franklin, 1997). However, they also report that DAMGO or morphine given into the Acb shell produces no CPP (Bals-Kubit et al., 1993; Olmstead and Franklin, 1997). Zangen and colleagues (2002) report that EM-1 given into the VTA, but not the Acb shell produces CPP. The reason that DAMGO, morphine or EM-1 microinjected into the Acb shell does not produce a significant CPP in their studies is not clear at this time.

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EM-2 given into the mesolimbic posterior Acb shell, but not VTA produces CPA EM-2 is another endogenous opioid peptide with high affinity and specificity for μ-opioid receptors (Narita et al., 2001; Tseng et al., 2000). Thus, microinjection of EM-2 into the posterior Acb shell or VTA would be expected to produce the same effect as that of EM-1. However, we found that EM-2 at similar potency doses as EM-1 microinjected into the posterior Acb produced CPA. EM-2, at small doses injected into the VTA, produced no effect, and only at a higher dose of EM-2 produced CPP. The finding is in line with previous studies in mice that show EM-2 given intracerebroventricularly produces CPA (Narita et al., 2002, 2001). The CPA induced by EM-2 is mediated by the stimulation of the μ-opioid receptor, because the effect of EM-2 was blocked by the co-administration of the μ-opioid receptor blocker CTOP.

We therefore propose that EM-2 stimulates a different subtype of μ-opioid receptor from that of the μ receptor stimulated by EM-1 (see Introduction for details). This novel opioid receptor of the μ type is tentatively designated as the μ'-opioid receptor to distinguish from μ receptors stimulated by EM-1, morphine or DAMGO. 3-methoxynaltrexone has been reported to be a relatively selective μ-opioid receptor blocker for the EM-2 sensitive subtype (Sakurada et al., 2000). It was found that EM-2 co-administered with 3-methoxynaltrexone completely blocked the CPA produced by EM-2. Stimulation of this EM-2 sensitive μ'-opioid receptor subtype provokes the release of Dyn acting on κ-opioid receptors to produce CPA. This view is supported by the present finding that co-administration with antiserum against Dyn, to inactivate released Dyn, completely blocked the CPA produced by EM-2 from the posterior Acb shell. In contrast, EM-1-induced CPP from the posterior Acb shell is not affected by the co-

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administration with antiserum against Dyn. These finding are in line with previous findings in mice that i.c.v. injection with antiserum against Dyn blocks the CPA induced by EM-2 given i.c.v. Furthermore, blockade of the κ-opioid receptors by systemic injection of nor-BNI also blocks the CPA induced by EM-2 given i.c.v (Narita et al., 2002, 2001).

The postulated role of the VTA versus the posterior Acb shell in mediating the different effects of EM-1 and EM-2 receives additional support from previous studies. Immunohistological studies indicate a dense staining of EM-1, but not EM-2 in the VTA. In contrast, a dense staining of both EM-1 and EM-2 were found in the Acb (Martin-Schild et al., 1999). Immunohistochemistry study shows that abundant to moderate fiber and terminal density of μ opioid receptor immunoreactivity were found in both Acb and VTA (Moriwaki et al., 1996). Autoradiographic studies indicate dense μ-opioid receptor binding in the VTA, but only very low κ binding density can be detected within the VTA, an area that receives little dynorphinergic innervation. In contrast, the Acb shows dense μ and κ receptor bindings (Falcon et al., 1990; Dilts and Kalivas, 1989; Mansour et al., 1988; Watson et al., 1989). Electrophysiological data provide some evidence for an action of μ agonists on A10 dopaminergic neurons within the VTA, whereas κ agonists are devoid of action in this region (Hakan and Henrikson, 1989; French and Ceci, 1990). The κ-opioid receptor ligand U-69593 fails to modify dopamine release following their injection into VTA (Spanagel et al., 1992). In contrast, high κ binding is evident in the medial portion of the Acb (Mansour et al., 1988; Watson et al., 1989), a region that is also densely innervated by dynorphin-containing fibers (Falcon and Ceci, 1990). These findings are in line with the present findings that EM-2 given into the VTA produces no

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significant CPA, but produces CPA when given into posterior Acb. The CPP produced by a higher dose of EM-2 given into the VTA may be mediated by the stimulation of the μ-opioid receptors, which are sensitive to EM-1 or morphine.

Tonically active opposing μ- and κ-opioid receptors in mesolimbic systems mediating conditioned place preference responses Spanagel et al. (1992) proposed the existence of tonically active and functionally opposing μ- and κ-opioid systems that regulate dopamine release in the Acb, the major terminal area of A10 dopaminergic neurons. Activation of μ-opioid receptor subtype sensitive to EM-1 from the posterior Acb shell produces CPP, while the blockade of the μ-opioid receptors with a high dose of CTOP alone given into Acb shell produces CPA. In contrast, the stimulation of the EM-2-sensitive μ'-opioid receptor subtype at the Acb produces CPA, while the blockade of the EM-2-sensitive μ' opioid receptors by 3-methoxynaltrexone alone produces CPP. A synopsis of the results is given in a model, which illustrates the modulation of the mesolimbic dopamine neurons ascending from the VTA to the Acb modulated by EM-1 and EM-2 and their respective subtypes of μ-opioid receptors (Fig. 8). Micro-application of EM-1 into the Acb increases the release of dopamine from the Acb and produces CPP. In contrast, blockade of μ-opioid receptors by CTOP decreases the basal level of dopamine and thus causes CPA. We found in our preliminary studies using a microdialysis probe that EM-1 microperfused into the posterior Acb shell increases the release of dopamine from the posterior Acb shell (Hung et al., 2003). Microinjection of other μ-opioid agonists DAMGO or morphine into the VTA increases the release of dopamine from the Acb. In contrast, μ-opioid receptor antagonist CTOP decreases the basal release of dopamine (Spanagel et al., 1992). Local application of morphine into the

VTA increases discharge activity in the mesolimbic pathway (Gysling and Wang, 1983; Matthews and German, 1984; Latimer et al., 1987). Such an excitatory effect of opioids, which are basically inhibitory, is most easily explained by an interaction with inhibitory GABA-nergic interneurons (Kalivas et al., 1990; Johnson and North, 1992). Microinjection of EM-2 into the Acb posterior, but not the VTA, produced CPA. The EM-2-induced CPA is blocked by the co-administration with antiserum against Dyn, indicating that effect is mediated by the release of Dyn. Dyn has been proposed to be the endogenous ligand for κ-opioid receptors. Stimulation of κ-type receptors with κ receptor agonists within the Acb decreases dopamine release, whereas the blockade of the κ-opioid receptors with κ receptor antagonists markedly increases basal dopamine release (Spanagel et al., 1992). Thus, Dyn or other κ-opioid agonists may activate κ-opioid receptors and inhibit mesolimbic dopamine release, which in turn produce CPA (Shippenberg and Elmer, 1998; De Vries and Shippenberg, 2002). In any case, behavioral as well as neurochemical data indicate that the mesolimbic dopamine pathway ascending from the VTA is tonically activated by EM-1 to produce CPP and that EM-1 and EM-2 interact with two different subtypes of opioid receptors, μ and μ', for producing CPP and CPA, respectively, at the posterior Acb.

EM-1 or EM-2 given into the nigrostriatal substantia nigra produces no effect We found in the present study that EM-1 or EM-2 given into the substantia nigra did not produce any effects on CPP or CPA. Microinjection of μ- or κ-opioid receptor agonists into either the origin of the nigrostriatal substantia nigra or into its major terminal field, the nucleus caudatus-putamen, does not show either CPP or CPA (Bals-

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Kubit et al., 1993). Thus, EM-1 and EM-2 and their μ-opioid receptors in the nigrostriatal dopamine system appear not to play any important role in CPP and CPA responses.

It is concluded that EM-1 given into the posterior Acb shell and VTA produces CPP, whereas EM-2 given into the posterior Acb, but not the VTA, produces CPA. The EM-1-induced CPP and EM-2-induced CPA from the posterior Acb are blocked by the treatment with low doses of CTOP and 3-methoxynaltrexone. CTOP and 3-methoxynaltrexone at a higher dose given into posterior Acb produce CPP and CPA, respectively. The EM-2-induced CPA from the posterior Acb is abolished by the treatment with antiserum against Dyn, suggesting that the EM-2-induced CPA is mediated by the release of Dyn. Our results suggest that there is a tonically active opposing μ- and μ'-opioid receptors stimulated by EM-1 and EM-2 in the mesolimbic system mediating CPP and CPA, respectively.

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Footnotes

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Legends for figures

Fig 1. Coronal sections of the atlas of Paxinos and Watson (1997) showing the injection sites for EM-1 (A and B) and EM-2 (C and D) at the posterior Acb shell and VTA, respectively. The number of animals microinjected with EM-1 and EM-2 at the posterior Acb shell and VTA are 36-35 and 35-32, respectively. ♦=EM-1, ● = EM-2.

Figure 2. Coronal sections of the atlas of Paxinos and Watson (1997) showing the injection sites for EM-1 and EM-2 at the substantia nigra. The number of rats microinjected with EM-1 is 11 and with EM-2 is 10. ♦=EM-1, ● = EM-2.

Fig 3. Effect of the treatment with EM-1 (A) and EM-2 (B) microinjected into the posterior Acb shell on the production of place preference in rats (Fig. 1A,C:♦=EM-1, ● = EM-2). After completion of the pre-conditioning measurement on the 1st day, groups of rats were conditioned by microinjection with different doses of EM-1 (1.6, 3.3 and 8.1 nmol), EM-2 (8.7, 13.1 and 17.5 nmol) or saline vehicle into the posterior Acb shell twice a day for three days and the post-conditioning was measured on the 5th day. Each column represents the mean CPP score and the vertical bar represents the S.E.M. (N = 10 to 12 rats). † $p < 0.05$, †† $p < 0.001$ significant production of CPP or CPA of individual dose (Mixed factorial Two-way analysis of variance (ANOVA), * $p < 0.05$, ** $p < 0.01$ compared with vehicle injected group (One-way ANOVA followed by Dunnett's post-test).

Fig 4. The effects of the treatment with EM-1 (A) and EM-2 (B) microinjected into the

VTA on the production of the place preference in rats (Fig. 1B,D: ♦=EM-1, ● = EM-2). After completion of the pre-conditioning measurement on the 1st day, groups of rats were conditioned by microinjection with different doses of EM-1 (1.6, 8.1 and 16.3 nmol), EM-2 (8.7, 17.5 and 35.0 nmol) or saline vehicle into the VTA twice a day for three days and the post-conditioning was measured on the 5th day. Each column represents the mean CPP score and the vertical bar represents the S.E.M. (N = 10 to 12 rats). † $p < 0.05$ significant place preference or aversion of individual dose (Mixed factorial two-way ANOVA), * $p < 0.05$, ** $p < 0.01$ compared with vehicle-injected groups (One-way ANOVA followed by Dunnett's post-test).

Fig 5. Effects of different doses of CTOP (A) and effect of CTOP co-administered with EM-1 or EM-2 (B) microinjected into the posterior Acb shell on the place preference in rats. Rats injected with vehicle served as control. After completion of the pre-conditioning measurement on the 1st day, groups of rats were conditioned by microinjection with different doses of CTOP (941.3, 94.13 and 47.07 pmol) or microinjection of CTOP (94.13 pmol) with EM-1 (3.3 nmol), EM-2 (17.5 nmol) or saline-vehicle into the posterior Acb shell twice a day for three days and the post-conditioning was measured on the 5th day. Each column represents the mean CPP score and the vertical bar represents the S.E.M. (N = 10 to 16 rats). † $p< 0.05$, †† $p< 0.01$ significant place preference or aversion of individual dose (Mixed factorial two-way ANOVA), ***** $p< 0.01$ compared with vehicle-injected groups (One-way analysis of variance (ANOVA) followed by Dunnett's post-test). # $p< 0.01$, # # $p< 0.001$ compared with vehicle-injected groups (Student's *t*-test).

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Fig 6. Effects of different doses of 3- methoxynaltrexone (A) and effects of 3-methoxynaltrexone co-administered with EM-1 or EM-2 (B) microinjected into the posterior Acb shell on the place preference in rats. Rats injected with vehicle served as control. After completion of the pre-conditioning measurement on the 1st day, groups of rats were conditioned by microinjection with different doses of 3- methoxynaltrexone (6.4 and 0.64 pmol) or 3- methoxynaltrexone (0.64 pmol) with EM-1 (3.3 nmol), EM-2 (17.5 nmol) or saline-vehicle into the posterior Acb shell twice a day for three days and the post-conditioning was measured on the 5th day. Each column represents the mean CPP score and the vertical bar represents the S.E.M. (N = 9 to 11 rats). † $p< 0.05$, †† $p< 0.01$ significant place preference or aversion of individual dose (Mixed factorial two-way ANOVA), ***** $p< 0.05$ compared with vehicle-injected groups (One-way ANOVA followed by Dunnett's post-test). # $p< 0.05$ compared with vehicle-injected groups (Student's *t*-test). 3-MN, 3-methoxynaltrexone.

Fig 7. Effects of antiserum against Dynorphin A (1-17) or normal rabbit serum alone, or co-administered with EM-1 or EM-2 into the posterior Acb shell on the EM-1 induced CPP and EM-2-induced CPA in rats. Each column represents the mean CPP score and the vertical bar represented the S.E.M. Each group includes 9 to 11 rats. † $p< 0.05$, †† $p< 0.01$ significant place preference or aversion of individual dose (Mixed factorial two-way ANOVA), ***** $p< 0.05$ compared with vehicle-injected groups (Student *t*-test).

Fig 8. A model for the modulation of mesolimbic A 10 neurons by EM-1 and EM-2. The

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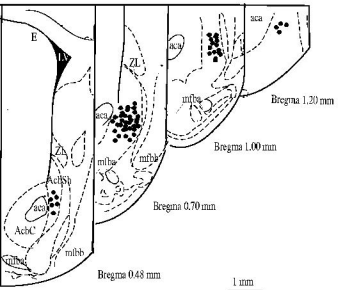
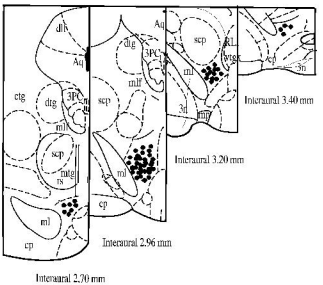
stimulation of μ-opioid receptor subtype by EM-1 in the VTA and Acb shell causes an increased release of dopamine and produces place preference. The stimulation of another subtype of opioid receptor, μ′, by EM-2 at the posterior Acb shell causes an increased release of dynorphin A(1-17) acting on κ-opioid receptors to cause a decreased basal level of dopamine and produce place aversion. The "+" represents an increased release of dopamine and "–" represents a decreased release of dopamine (see text for details). μ and μ′ are used tentatively to indicate that there are different subtypes of μ opioid receptors sensitive to EM-1 and EM-2 to produce CPP and CPA, respectively.

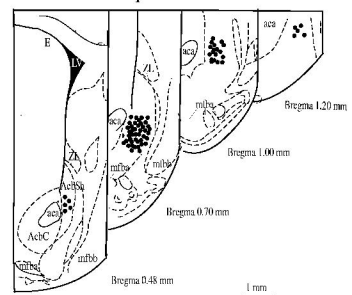
A. EM-1 at the posterior Acb shell



B. EM-1 at the VTA



C. EM-2 at the posterior Acb shell



D. EM-2 at theVTA

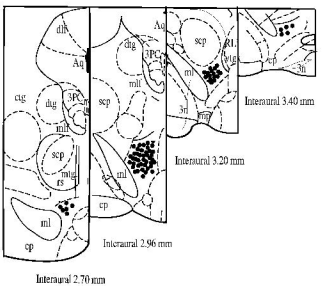
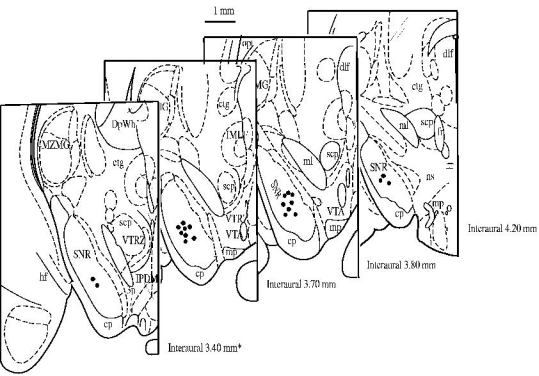
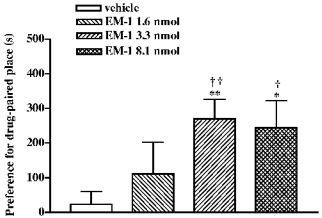


Figure 1

EM-1 and EM-2 at the substantia nigra



A) EM-1 at the posterior Acb shell



B) EM-2 at the posterior Acb shell

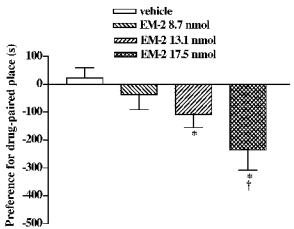
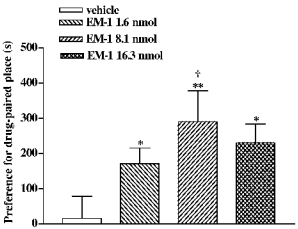


Figure 3 A, B

A) EM-1 at the VTA



B) EM-2 at the VTA

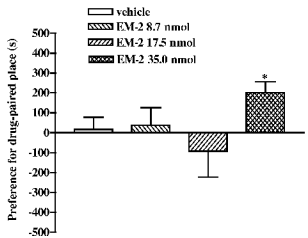
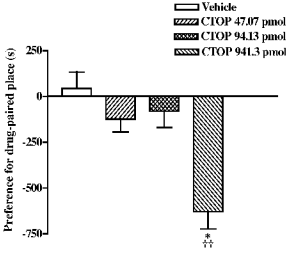


Figure 4 A, B

A) CTOP at the posterior Acb shell



B) CTOP with EMs at the posterior Acb sell

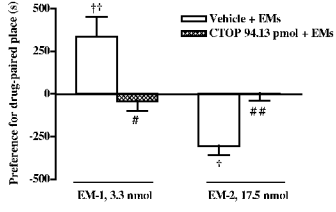
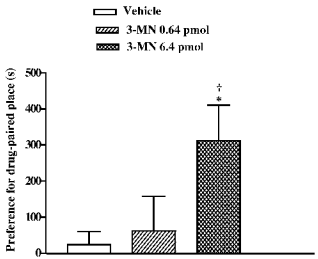


Figure 5 A,B

A) 3-MN at the posterior Acb shell



B) 3-MN with EMs at the posterior Acb shell

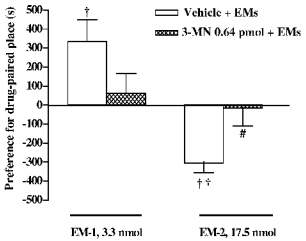


Figure 6 A, B

Dyn antiserum with EMs at the posterior Acb shell

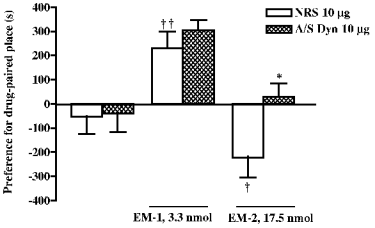


Figure 7

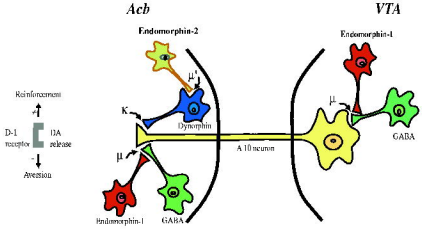

Acb
VTA
Endomorphin-2
Endomorphin-1
μ
κ
Dynorphin
μ
GABA
A10 neuron
μ
Endomorphin-1
GABA
Reinforcement
D-1 receptor
DA release
Aversion

Figure 8