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Endomorphin-2 and Endomorphin-1 Promote the Extracellular Amount of Accumbal Dopamine via Nonopioid and Mu-Opioid Receptors, Respectively

Hiroko Okutsu[1,6], Shu Watanabe[2], Ichiro Takahashi[3], Yuri Aono[2], Tadashi Saigusa*[,2,4], Noriaki Koshikawa[2,4] and Alexander R Cools[5]

[1]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda-shi, Chiba, Japan; [2]Department of Pharmacology, Nihon University School of Dentistry, Chiyoda-ku, Tokyo, Japan; [3]Department of Dental Anaesthesiology, Nihon University School of Dentistry, Chiyoda-ku, Tokyo, Japan; [4]Division of Oral and Craniomaxillofacial Research, Dental Research Centre, Nihon University School of Dentistry, Chiyoda-ku, Tokyo, Japan; [5]Department of Psychoneuropharmacology, University of Nijmegen, Nijmegen, The Netherlands

Activation of mu-opioid receptors in the nucleus accumbens (NAc) is known to increase accumbal dopamine efflux in rats. Endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2; EM-2) and endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2; EM-1) are suggested to be the endogenous ligands for the mu-opioid receptor. As the ability of EM-2 and EM-1 to alter the accumbal extracellular dopamine level has not yet been studied in freely moving rats, the present study was performed, using a microdialysis technique that allows on-line monitoring of the extracellular dopamine with a temporal resolution of 5 min. A 25 min infusion of either EM-2 or EM-1 into the NAc (5, 25, and 50 nmol) produced a dose-dependent increase of the accumbal dopamine level. The EM-2 (50 nmol)- and EM-1 (25 and 50 nmol)-induced dopamine efflux were abolished by intra-accumbal perfusion of tetrodotoxin (2 μM). Intra-accumbal perfusion of the mu-opioid receptor antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH_2; 3 nmol) failed to affect the EM-2 (50 nmol)-induced dopamine release, whereas it significantly inhibited the EM-1 (25 and 50 nmol)-induced dopamine release. The EM-1 (50 nmol)-induced accumbal dopamine efflux was significantly reduced by the systemic administration of the putative mu1-opioid receptor antagonist naloxonazine (15 mg/kg, intraperitoneally (i.p.), given 24 h before starting the perfusion). Systemic administration of the aspecific opioid receptor antagonist naloxone (1 mg/kg, i.p., given 10 or 20 min before starting the perfusion) also failed to affect the EM-2 (50 nmol)-induced dopamine efflux, whereas it significantly inhibited the EM-1 (25 and 50 nmol)-induced dopamine efflux. The present study shows that the intra-accumbal infusion of EM-2 and EM-1 increases accumbal dopamine efflux by mechanisms that fully differ. It is concluded that the effects of EM-2 are not mediated via opioid receptors in contrast to the effects of EM-1 that are mediated via mu1-opioid receptors in the NAc.

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INTRODUCTION

Endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2; EM-2) and endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2; EM-1) are isolated from bovine (Zadina *et al*, 1997) as well as human brains (Hackler *et al*, 1997) and considered to be the endogenous ligands for the mu-opioid receptor (Goldberg *et al*, 1998; Zadina *et al*, 1997). The immunoreactivity of EM-2 and EM-1 is prominently present in regions in which mu receptors are concentrated. Thus, large amounts of EM-2-like immunoreactivity (Schreff *et al*, 1998) and EM-1-like immunoreactivity (Martin-Schild *et al*, 1999) have been found in the nucleus accumbens (NAc) that contains various subtypes of opiate receptor (mu-, kappa- and delta-opioid receptors). Pharmacologically, the two endomorphins are potent analgesics and show similar properties (Goldberg *et al*, 1998). Both EM-2 and EM-1 produce short acting, naloxone-sensitive antinociception in the tail flick test (Stone *et al*, 1997). However, pharmacological intervention studies have also revealed that the mechanisms of

*Correspondence: Dr T Saigusa, Department of Pharmacology, Nihon University School of Dentistry, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan, Tel: +81 3 3219 8126, Fax: +81 3 3219 8136, E-mail: saigusa@dent.nihon-u.ac.jp

[6]Current address: Pharmacology Laboratories, Institute for Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan

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the action of the two endomorphins are not completely identical. Thus, the antinociceptive effect of EM-2 is more sensitive to naloxonazine than that of EM-1 (Sakurada *et al*, 1999, 2000, 2001, 2002). The studies of Sakurada and co-workers have suggested that EM-2 acts predominantly as a mu1-opioid receptor agonist and EM-1 as a mu2-opioid receptor agonist.

The activation of mu-opioid receptors in the NAc induces a large and rapid increase of accumbal dopamine efflux. Thus, fentanyl, a mu receptor agonist, increases the naloxone-reversible accumbal dopamine efflux (Yoshida *et al*, 1999). The ability of the EM-2 and EM-1 to alter the extracellular amount of accumbal dopamine in freely moving rats has not yet been studied. Therefore, we analyzed the effects of EM-2 and EM-1 on the extracellular amount of dopamine in the NAc of freely moving rats, using the recently improved microdialysis technique that allows on-line monitoring of dopamine with a temporal resolution of 5 min instead of 20 min (Saigusa *et al*, 2001; cf. Fusa *et al*, 2002, 2005; Murai *et al*, 1994; Saigusa *et al*, 1997, 1999; Takada *et al*, 1993; Tomiyama *et al*, 1993, 1995).

First, we examined the effects of the sodium channel blocker tetrodotoxin (TTX) on the EM-2- and EM-1-induced increase of extracellular dopamine in order to establish whether the effects are dependent on neural activity. Next, we analyzed whether mu-opioid receptors are involved in the effects of EM-2 and EM-1. For that purpose, CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Phe-Thr-NH_2), a mu-opioid receptor antagonist, together with EM-2 or EM-1 was locally infused into the NAc. In order to investigate the putative involvement of mu1-opioid receptors on the EM-1-induced accumbal dopamine efflux, the effects of the systemically administered mu1-opioid receptor antagonist naloxonazine (Pasternak and Wood, 1986) were also studied. Given the finding that CTOP did not antagonize the effects of EM-2, it became necessary to establish the opioid nature of the effects of EM-2. For that purpose, an additional experiment on naloxone, an opioid receptor antagonist that acts at all opioid receptor subtypes, although with a greater affinity for mu-opioid receptors, was included. Naloxone was chosen, because studies on antinociceptive behavior (Stone *et al*, 1997; Goldberg *et al*, 1998), motor behavior (Mehta *et al*, 2001), electrophysiological studies (Chapman *et al*, 1997; Wang *et al*, 2000), and systemic blood pressure studies (Czapla *et al*, 1998; Champion *et al*, 1997) have revealed that EM-2 and EM-1 produce naloxone-sensitive effects. The pretreatment by naloxone was carried out 20 min before and 10 min before starting the intra-accumbal infusion of the EM-1 or EM-2. The 20 min paradigm was chosen, since it has been found to be effective in antagonizing the increase of accumbal dopamine efflux induced by the selective mu-opioid receptor agonist fentanyl (Yoshida *et al*, 1999). As this schedule turned out to be ineffective in antagonizing the effects of EM-2, it was tried to enhance the efficacy of naloxone by reducing its metabolism and, accordingly, increasing its availability in the brain: for that reason, the 10 min schedule was included. Increasing the dose of naloxone was rejected as strategy, because high doses of this drug produce aspecific effects (Feigenbaum and Howard, 1997).

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (NRC Haruna, Japan) weighing between 200 and 220 g at the start of the experiment were used. These were housed in a temperature-controlled environment on a 12-h light–dark cycle (light period 0700–1900 h) with free access to food and water.

Surgery

Rats were anesthetized with sodium pentobarbitone (50 mg/kg, intraperitoneally (i.p.)). The anesthetized animals were placed in a stereotaxic apparatus, and a guide cannula was implanted just above the left NAc (AP 10.6 mm, ML 1.5 mm, DV 4.0 mm from interaural line; Paxinos and Watson, 1998) according to previously described procedures (Fusa *et al*, 2002, 2005; Saigusa *et al*, 1997, 1999, 2001). To avoid the ventricular system, cannulae directed at the NAc were angled 18° from the mid-sagittal plane. After completion of surgery, rats were allowed to recover for 7–10 days before experiments were carried out; guide cannulae were kept patent by stainless-steel inserts. Each animal was used only once. The experiments were performed in accordance with institutional, national, and international guidelines for care and welfare of animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Dialysis and Neurochemical Measurements

A commercially available I-shaped removable-type dialysis probe (2 mm length cellulose membrane, 0.22 mm o.d., 50 000 mol. wt. 'cutoff', Eicom A-I-8-02 type, Kyoto, Japan) was used. The experiment was started by removing the stylet from the guide cannula and inserting the dialysis probe, of which just the dialysis tubing protruded from the tip. The probe was secured to the guide cannula by a screw. Each rat was then placed in a Plexiglas box ($30 \times 30 \times 35$ cm), and inlet and outlet tubes were connected to a swivel located on a counterbalanced beam to minimize discomfort. The probe was perfused at a rate of 2.0 μl/min with modified Ringer solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.1 mM; pH 7.4) and the outflow connected by Teflon tubing to a high-performance liquid chromatography system (HTEC-500; Eicom, Kyoto, Japan). Dopamine was separated on an Eicompak PP-ODS column (particle size, 2 μm, 4.6×30 mm; Eicom, Kyoto, Japan) maintained at 25°C, using phosphate buffer (0.1 M) containing decanesulfonic acid (2.0 mM), EDTA (0.13 mM), and 1% methanol (pH 6.0) as the mobile phase at a flow rate of 0.5 ml/min. Compounds were quantified by electrochemical detection using a glassy carbon working electrode set at + 400 mV against a silver–silver chloride reference electrode (WE-3G; Eicom, Kyoto, Japan), giving a detection limit for dopamine of about 0.02 pg/sample at a 2:1 signal-to-noise ratio. The probes had an *in vitro* recovery of approximately 12% for dopamine, but the reported concentrations were not adjusted for recovery *in vivo* because these estimations are inaccurate (Benveniste *et al*, 1989; Lindefors *et al*, 1989). Previous experiments in which we have used the same technique and procedure have shown that the dopamine efflux is more or less stabilized 16 h after probe insertion,

and that the release seen at that time is largely dependent on neuronal release as more than 70% of the release is TTX sensitive (Saigusa *et al*, 2001). Perfusate samples were taken every 5 min for quantification of dopamine. Drugs were administered either i.p. or intracerebrally through the dialysis probe, at least 20 h after the probe insertion: baseline levels of dopamine were the mean of the last 12 samples before the drug administration.

Drugs

Drug used were EM-2 (Peptide Institute Inc., Osaka, Japan), EM-1 (Peptide Institute Inc., Osaka, Japan), TTX (Sigma-RBI, St Louis, MO, USA), CTOP (Sigma-RBI, St Louis, MO, USA), naloxone (Sigma-RBI, St Louis, MO, USA), and naloxonazine (naloxonazine dihydrocloride; Sigma-RBI, St Louis, MO, USA). The EM-2, EM-1, and CTOP were dissolved in the modified Ringer solution. The EM-2 and EM-1 were infused via dialysis membrane for 25 min, whereas the CTOP was similarly infused for 50 min (starting 25 min before EM-2 and EM-1 infusion). The sodium channel blocker TTX was also dissolved in the modified Ringer solution to be used for perfusions and was administered intracerebrally through the dialysis probe. Naloxone was dissolved in saline. Naloxonazine was suspended in 2.5% Tween-80 solution and i.p. administered 24 h before commencing EM-2 infusion. The doses used were based on the outcome of previously reported experiments (EM-2 and EM-1: Sakurada *et al*, 2001; TTX: Fusa *et al*, 2002; Saigusa *et al*, 2001; Takada *et al*, 1993; Tomiyama *et al*, 1993, 1995; CTOP: Yoshida *et al*, 1999; naloxone: Yoshida *et al*, 1999; naloxonazine: Piepponen and Ahtee, 1995). The reported doses of EM-2 and EM-1 were the amount (nmol) of compounds in 25 min perfusion liquid (50 μl) and the dose of CTOP was the amount of the compound in 50 min perfusion liquid (100 μl).

Histology

At the end of the experiment, rats were deeply anesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and perfused transcardially with 10% formaldehyde solution. The brain was removed, sectioned (50 μm), and stained with cresyl violet to permit probe location.

Statistical Analysis

All values were expressed as a percentage of baseline levels. Comparison of time-course data was performed using two-way ANOVA (analysis of variance) for repeated measures with the factors treatment and time (repeated). Statistical significance was considered to be $P<0.05$.

RESULTS

Histology

Placements of the dialysis probes in the NAc are given in Figure 1. Although the tip of the probes were found between the anterior–posterior planes 10.0 and 10.9, they all are projected in a single plane, namely A 10.6 (Figure 1). The length of the probe membrane (2 mm) prevented a lucid separation between the core and shell region; accordingly, the data are given per NAc rather than per distinct accumbal subregion. The total number of rats used was 189 and the number of rats with misplaced dialysis probes was 30. Only data of rats with correctly placed probes ($n=159$) were incorporated in the analysis.

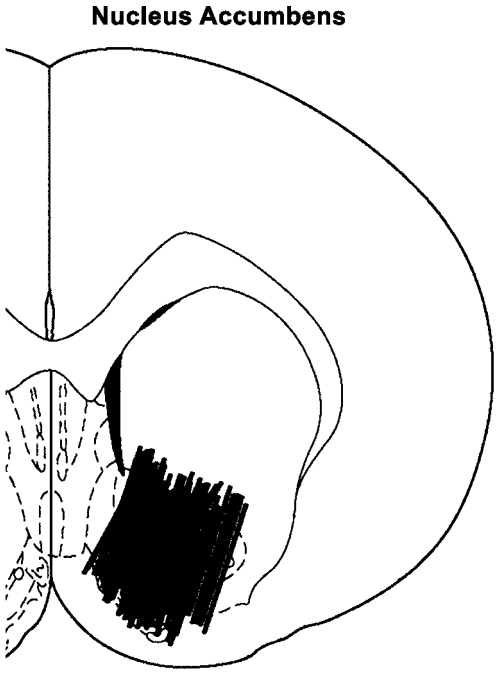


Figure 1 Schematic illustration showing locations of the probe in the NAc. The diagram is taken from the Atlas of Paxinos and Watson; the number represents anterior distance (mm) from interaural line.

Basal Extracellular Dopamine Level in the NAc

Baseline concentration of dopamine in dialysates from the NAc was 1.0 ± 0.05 pg/5 min (mean $\pm$ SEM; $n=159$).

Effects of EM-2 and EM-1 Perfusion on the Extracellular Dopamine Level in the NAc

A 25 min infusion of either EM-2 (Figure 2a; 5 nmol ($n=7$), 25 nmol ($n=6$), and 50 nmol ($n=6$)) or EM-1 (Figure 2b; 5 nmol ($n=6$), 25 nmol ($n=6$), and 50 nmol ($n=6$)) into the NAc produced a dose-dependent increase of the accumbal extracellular dopamine level: these effects lasted for about 10–35 min (EM-2—treatment $F(2,96)=84.92$, $P<0.001$; time $F(5,96)=4.77$, $P<0.001$; interaction $F(10,96)=1.14$, $P=0.34$; EM-1—treatment $F(2,90)=124.04$, $P<0.001$; time $F(5,90)=5.27$, $P<0.001$; interaction $F(10,90)=1.91$, $P=0.05$)

The Neuronal Dependence of the EM-2 and EM-1-Induced Transient Increase of Dopamine Efflux in the NAc

TTX (2 μM) infused for 2 h via the dialysis probe reduced basal levels of DA by approximately 70%. This TTX infusion almost completely abolished EM-2 (50 nmol; $n=6$; Figure 3a; treatment $F(1,60)=421.58$: $P<0.001$; time $F(5,60)=3.89$, $P<0.01$; interaction $F(5,60)=2.92$, $P<0.05$; 10–35 min)- and EM-1 (50 nmol; $n=6$; Figure 3b:

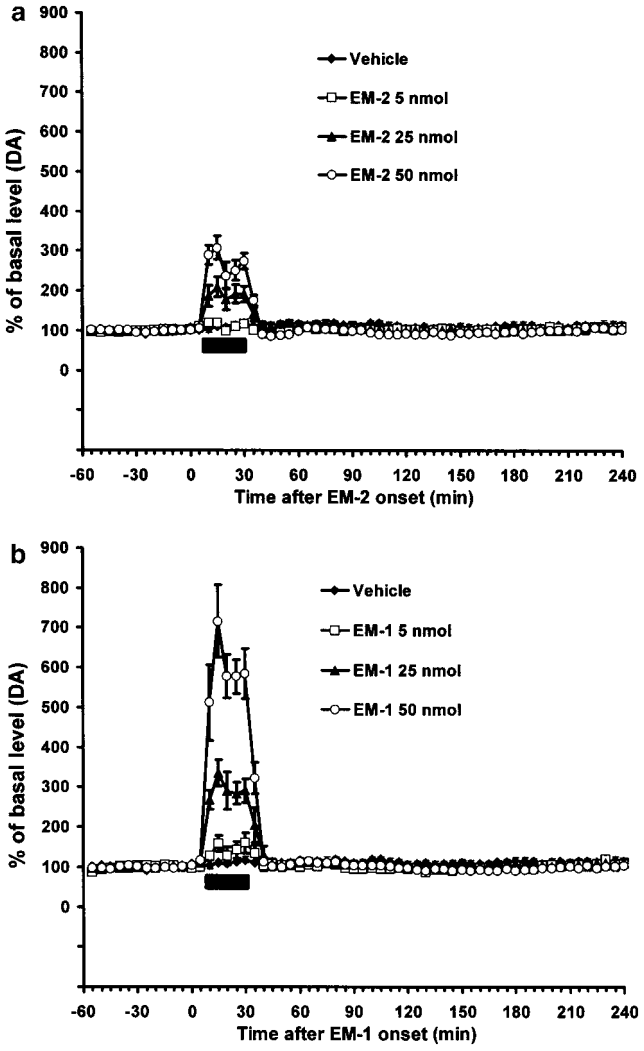


Figure 2 (a) Effects of infusion of vehicle ($n=6$, filled diamonds), 5 nmol EM-2 ($n=7$, open squares), 25 nmol EM-2 ($n=6$, filled triangles), and 50 nmol EM-2 ($n=6$, open circles) into the NAc on basal extracellular levels of dopamine (DA) in the NAc. The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-2. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of vehicle or EM-2. (b) Effects of infusion of vehicle ($n=6$, filled diamonds), 5 nmol EM-1 ($n=6$, open squares), 25 nmol EM-1 ($n=6$, filled triangles), and 50 nmol EM-1 ($n=6$, open circles) into the NAc on basal extracellular levels of dopamine (DA) in the NAc. The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of vehicle or EM-1.

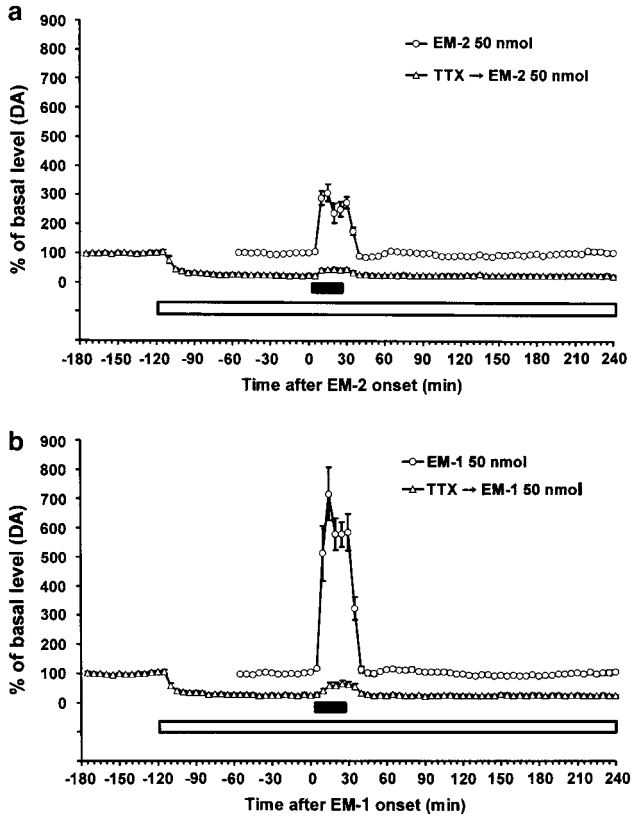


Figure 3 (a) Effects of perfusion of 2 μM TTX on a 25 min infusion of 50 nmol EM-2-induced increase in DA (dopamine) levels in the NAc ($n=6$, open triangles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-2. Vertical bars indicate SEM. The opened bar above the abscissa indicates the period of TTX perfusion that commenced 120 min before the onset of EM-2 infusion. The filled bar indicates the period of infusion of EM-2. (b) Effects of perfusion of 2 μM TTX on a 25 min infusion of 50 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=6$, open triangles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The opened bar above the abscissa indicates the period of TTX perfusion that commenced 120 min before the onset of EM-1 infusion. The filled bar indicates the period of infusion of EM-1.

treatment F $(1,60)=311.50$: $P<0.01$; time F $(5,60)=3.78$, $P<0.01$; interaction F $(5,60)=3.46$, $P<0.01$; 10–35 min)-induced enhancement of dopamine levels in the NAc. The EM-1 (25 nmol)-induced accumbal dopamine efflux was also completely abolished (data not shown).

Effects of Mu-Opioid Receptor Antagonist on the EM-2- and EM-1-Induced Transient Increase of Dopamine Efflux in the NAc

Perfusion of the NAc with the mu-opioid receptor antagonist CTOP (3 nmol; $n=6$) failed to affect the intra-accumbal infusion of EM-2 (50 nmol; $n=6$)-induced increase in the extracellular amount of accumbal dopamine (Figure 4a; treatment F $(1,57)=3.28$, $P=0.08$; time F $(5,57)=5.30$, $P<0.001$; interaction F $(5,57)=0.04$, $P=1.00$; 10–35 min). As 25 nmol EM-1 produced effects more less comparable to those produced by 50 nmol EM-2, we initially tested the ability of CTOP to antagonize 25 nmol EM-1. Perfusion of the NAc with the mu-opioid receptor antagonist CTOP (3 nmol; $n=8$) highly significantly inhibited the effects of EM-1 (25 nmol; $n=6$) on the extracellular amount of accumbal dopamine (Figure 4b; treatment F $(1,72)=102.79$, $P<0.001$; time F $(5,72)=1.05$, $P=0.40$; interaction F $(5,72)=1.95$, $P=0.10$; 10–35 min). CTOP (3 nmol; $n=8$) was even more effective in inhibiting the effects of a higher dose of EM-1 (50 nmol; Figure 4c; treatment F $(1,71)=228.57$, $P<0.001$; time F $(5,71)=3.33$, $P<0.01$; interaction F $(5,71)=5.26$, $P<0.001$; 10–35 min). The systemic administration of the mu1-opioid receptor antagonist naloxonazine (15 mg/kg, 24 h before the EM-1 perfusion) reduced basal levels of DA till 0.5 ± 0.08 pg/5 min

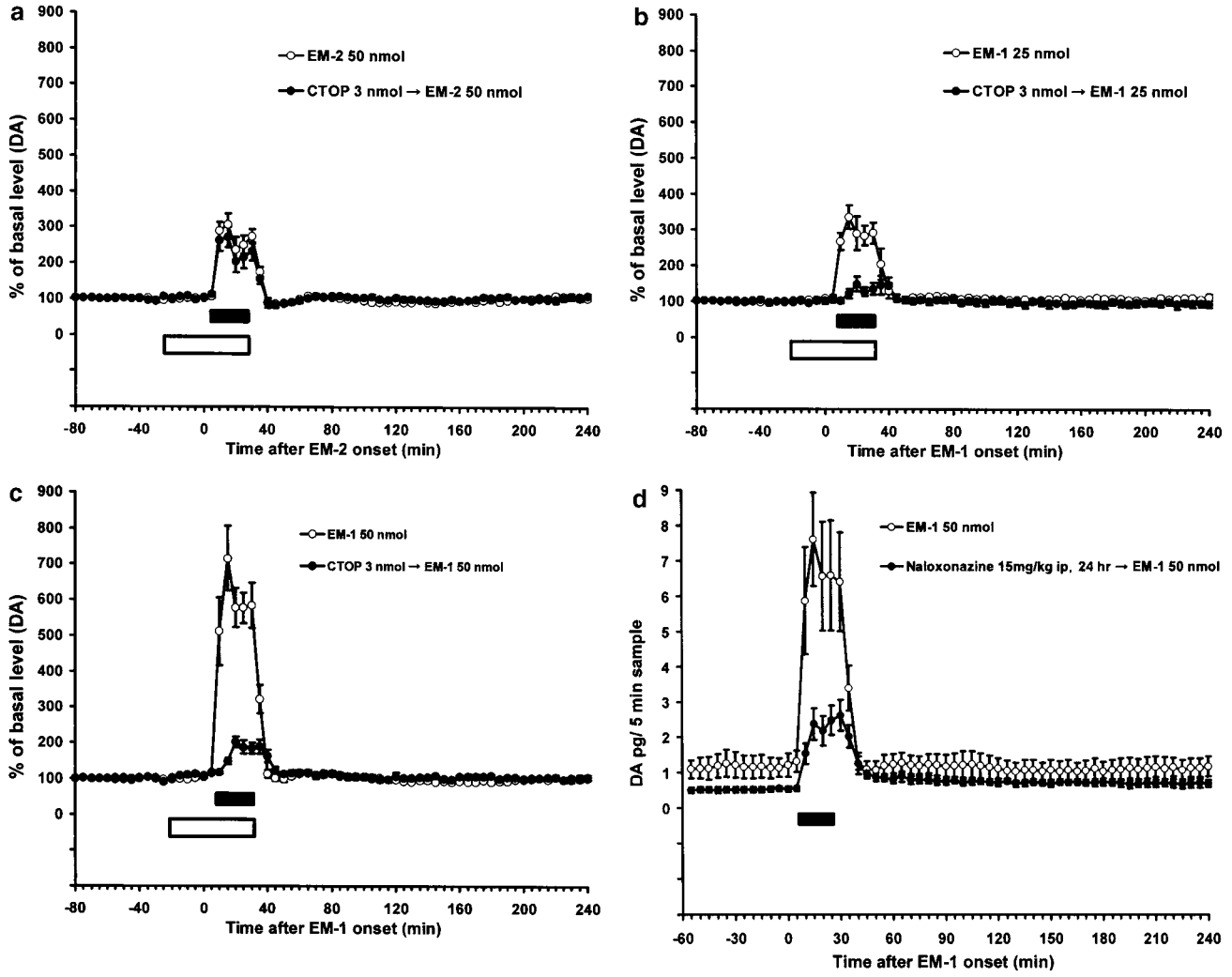


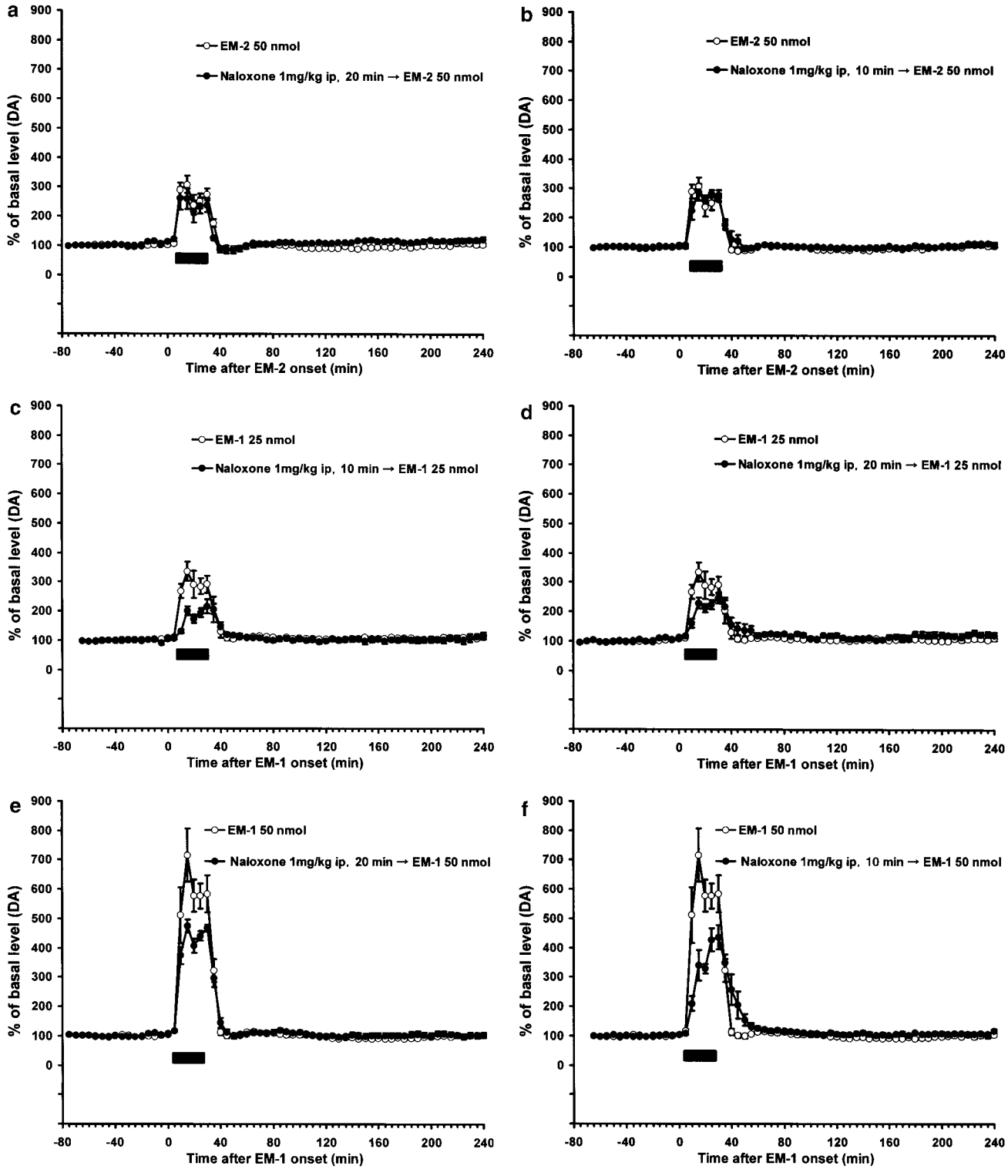
Figure 4 (a) Effects of perfusion of a 50 min infusion of 3 nmol CTOP on a 25 min infusion of 50 nmol EM-2-induced increase in DA (dopamine) levels in the NAc ($n=6$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-2. Vertical bars indicate SEM. The opened bar above the abscissa indicates the period of CTOP perfusion that commenced 25 min before the onset of EM-2 infusion. The filled bar indicates the period of infusion of EM-2. (b) Effects of perfusion of a 50 min infusion of 3 nmol CTOP on a 25 min infusion of 25 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=8$, filled circles). The data are expressed as the mean of change in 5 min observation periods after onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The opened bar above the abscissa indicates the period of CTOP perfusion that commenced 25 min before the onset of EM-1 infusion. The filled bar indicates the period of infusion of EM-1. (c) Effects of perfusion of a 50 min infusion of 3 nmol CTOP on a 25 min infusion of 50 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=8$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The opened bar above the abscissa indicates the period of CTOP perfusion that commenced 25 min before the onset of EM-1 infusion. The filled bar indicates the period of infusion of EM-1. (d) Effects of naloxonazine (15 mg/kg, i.p., 24 h before the onset of EM-1 infusion) on a 25 min infusion of 50 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=7$, filled circles). Since the basal levels between two groups showed apparent difference, the data are expressed as the mean of absolute amount of DA (pg) in the 5-min sample after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-1.

($n=7$). This naloxonazine pretreatment significantly reduced EM-1 (50 nmol; $n=6$; Figure 4d; treatment F $(1,66)=51.16$, $P<0.001$; time F $(5,66)=1.36$, $P=0.25$; interaction F $(5,66)=0.98$, $P=0.44$; 10–35 min)-induced enhancement of dopamine levels in the NAc.

Effects of Naloxone on the EM-2- and EM-1-Induced Transient Increase of Dopamine Efflux in the NAc

As shown in Figure 5a, naloxone (1 mg/kg, i.p., given 20 min before the intracerebral perfusion), which itself did not significantly affect basal levels of accumbal dopamine (data not shown), failed to affect the EM-2 (50 nmol)-induced enhancement of dopamine level in the NAc ($n=6$). To enhance the putative efficacy of naloxone, naloxone was also given 10 instead of 20 min prior to the administration of EM-2; again, naloxone was completely unable to inhibit the effects of EM-2 (50 nmol) on the dopamine level in the NAc (Figure 5b, $n=9$). As mentioned above, 25 nmol EM-1 produced effects more or less comparable to those produced by 50 nmol EM-2. For that reason, we initially tested the ability of naloxone (1 mg/kg, i.p., given 10 min before the intracerebral perfusion), which itself did not significantly affect basal levels of accumbal dopamine (data not

shown), to antagonize 25 nmol EM-1. This treatment significantly reduced the EM-1 (25 nmol)-induced enhancement of dopamine levels in the NAc, especially during the initial period of 10–30 min ($n=9$; Figure 5c; treatment F $(1,78)=42.49$, $P<0.001$; time F $(5,78)=2.25$, $P=0.06$; interaction F $(5,78)=2.27$, $P=0.06$; 10–35 min). When naloxone was given 20 min before the administration of 25 nmol EM-1, it also inhibited its effects EM-1 ($n=9$; Figure 5d; treatment F $(1,78)=19.27$, $P<0.001$; time F $(5,78)=2.50$, $P<0.05$; interaction F $(5,78)=1.59$, $P=0.17$; 10–35 min). Finally, it was found that naloxone given 20 or 10 min before the administration of the higher dose of EM-1 (50 nmol) was also effective in inhibiting the effects of EM-1 (20 min interval: Figure 5e, $n=6$; treatment F $(1,60)=22.33$, $P<0.001$; time F $(5,60)=7.35$, $P<0.001$; interaction F $(5,60)=0.97$, $P=0.44$; 10–35 min; 10 min



interval: Figure 5f, $n=9$; treatment F $(1,78)=48.83$, $P<0.001$; time F $(5,78)=5.00$, $P<0.001$; interaction F $(5,78)=4.08$, $P<0.01$; 10–35 min).

DISCUSSION

Previously, we have shown that the baseline level of dopamine in the NAc is largely dependent on neuronal activity as more than 70% of the dopamine release is TTX sensitive (Saigusa *et al*, 2001). Therefore, it is assumed that the basal dopamine measured in the present study is also primarily due to the neuronal release of dopamine.

The intra-accumbal infusion of EM-2 and EM-1 dose dependently increased the dopamine levels in the dialysate from the NAc of rats. A quantitative difference between the effects of EM-2 and EM-1 on the induction of increase in accumbal dopamine efflux was observed. Thus, the ability of EM-2 to enhance the accumbal extracellular dopamine levels was far smaller than that of EM-1, when a broad range of doses (5, 25, and 50 nmol) was tested. The maximal effects caused by the infusion of the highest dose (50 nmol) of EM-2 and EM-1 were nearly fully suppressed by the intra-accumbal infusion of TTX (2 μM), indicating that, under the present experimental conditions, the EM-2- and EM-1-induced increases of accumbal dopamine levels were fully dependent on neuronal firing activity. As discussed below, the effects of EM-1 were mediated via mechanisms that fully differ from those mediating the effects of EM-2. For that reason, the differences in efficacy between EM-1 and EM-2 cannot be simply ascribed to a difference in affinity and/or intrinsic activity, but to a difference in mechanisms.

In order to investigate the role of opioid receptors in the endomorphins' action on the mesolimbic dopaminergic system, the mu-opioid receptor antagonist CTOP was directly infused into NAc to inhibit the effects of EM-2 or EM-1 on the extracellular amount of dopamine. Initially, it was attempted to counteract a drug-induced increase of extracellular dopamine that did not differ between EM-2 and EM-1; for that purpose, a dose of 50 nmol EM-2 and a dose of 25 nmol EM-1 were chosen. In contrast to the EM-2-induced effects that were not at all inhibited by CTOP, the effects of EM-1 were nicely inhibited by this compound (3 nmol), indicating that the effects of EM-1, but not EM-2, were mediated via mu-opioid receptors. The finding that 3 nmol CTOP also nearly completely inhibited the effects induced by a higher dose of EM-1 (50 nmol) confirms the above-mentioned conclusion that the EM-1-induced effects were specific of mu-opioid receptors. The additional finding that the systemic administration of the putative mu1-opioid receptor antagonist naloxonazine also inhibited the EM-1-induced accumbal dopamine efflux not only underlines the involvement of mu-opioid receptors but also suggests that the involved receptors belong to the mu1-opioid receptor subtype. Apart from this conclusion, the data suggest that the effects induced by EM-2 were not mediated via mu-opioid receptors. The experiments in which the ability of the aspecific opioid receptor antagonist naloxone was tested not only confirmed that the effects of EM-1 were mediated via the naloxone-sensitive opioid receptors but also revealed that the effects induced by EM-2 were not at all mediated via opioid receptors that are blocked by naloxone. For, neither naloxone given 20 min before EM-2 nor naloxone given 10 min before this drug was able to antagonize the effects of EM-2. This lack of inhibition cannot be ascribed to an inability of the chosen doses or treatment to reduce effects mediated via stimulation of opioid receptors, because these treatments were highly effective in attenuating the effects of EM-1 (present study); as mentioned in the Introduction, this treatment has been found to be effective in other studies as well. In other words, the present study provides the original evidence that EM-1, like fentanyl, enhances the dopamine efflux in the NAc via direct stimulation of mu-opioid receptors in this nucleus. As a final remark in this context, it has been recently reported that CTOP is less mu-opioid receptor specific than it has originally been stated: for, it can also bind to somatostatin receptors (Chieng *et al*, 1996); CTAP would be a better choice. However, the finding that both CTOP and naloxonazine inhibited the effects of EM-1 clearly shows that the involved receptors belong to the mu-opioid receptors.

The finding that EM-2-induced effects are not always mediated via opioid receptors fits in with the earlier reported fact that the inhibitory action of EM-2 upon the tachykinergic contractions of guinea-pig-isolated bronchus is insensitive of specific antagonists of the mu-, kappa-, and delta-opioid receptors, as well as insensitive of aspecific opioid receptor antagonists such as naloxone (Fischer and Undem, 1999). The present study clearly shows that this

Figure 5 (a) Effects of naloxone (1 mg/kg, i.p., 20 min before the onset of EM-2 infusion) on a 25 min-infusion of 50 nmol EM-2-induced increase in DA (dopamine) levels in the NAc ($n=6$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-2. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-2. (b) Effects of naloxone (1 mg/kg, i.p., 10 min before the onset of EM-2 infusion) on a 25 min infusion of 50 nmol EM-2-induced increase in DA (dopamine) levels in the NAc ($n=9$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-2. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-2. (c) Effects of naloxone (1 mg/kg, i.p., 10 min before the onset of EM-1 infusion) on a 25 min-infusion of 25 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=9$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-1. (d) Effects of naloxone (1 mg/kg, i.p., 20 min before the onset of EM-1 infusion) on a 25 min infusion of 25 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=9$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-1. (e) Effects of naloxone (1 mg/kg, i.p., 20 min before the onset of EM-1 infusion) on a 25 min infusion of 50 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=6$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-1. (f) Effects of naloxone (1 mg/kg, i.p., 10 min before the onset of EM-2 infusion) on a 25 min infusion of 50 nmol EM-1-induced increase in DA (dopamine) levels in the NAc ($n=9$, filled circles). The data are expressed as the mean of change in 5 min observation periods after the onset of a 25 min infusion of EM-1. Vertical bars indicate SEM. The filled bar above the abscissa indicates the period of infusion of EM-1.

also holds for effects of EM-2 in the central nervous system. Indeed, endomorphins have been found to bind to nonopioid binding sites in tissues lacking mu receptors, such as rat cerebellum or brain of homozygous transgenic MOR −/− mice (review: Wollemann and Benyhe, 2004). Remarkably, there are more agents that are known to act as opioid receptor agonists, but nevertheless produce effects that are not at all mediated via these receptors. For instance, DAMGO, a mu-opioid receptor agonist, reduces NMDA currents via a nonmu-opioid receptor mechanism (Martin *et al*, 1997), and dynorphin A, a kappa receptor agonist, induces a naloxone-insensitive reduction in dopamine uptake (Das *et al*, 1994). The most interesting finding in this respect is the discovery that the so-called delta-opioid receptor agonist (−)-TAN-67 can produce effects that are fully independent of interaction with opioid receptors: it can produce an increase of the extracellular dopamine level in the NAc via the generation of a burst of free radicals that in turn trigger a release of glutamate, which ultimately via activation of NMDA receptors enhances the release of dopamine from dopaminergic terminals in the NAc (Fusa *et al*, 2005). To what extent the effects of EM-2 described in the present study are also due to such a mechanism of action remains to be investigated in future studies. Taken together, the present study reveals that the effects of EM-2 are not mediated via opioid receptors, despite the fact that EM-2 is known to be able to stimulate directly mu-opioid receptors (see Introduction) and, indirectly, the kappa-opioid receptors (Tseng *et al*, 2000; Ohsawa *et al*, 2001).

In summary, the present study reveals that the intra-accumbal infusion of EM-2 and EM-1 increases dopamine efflux in the NAc by mechanisms that fully differ. Although the effects of both EM-2 and EM-1 are dependent on neuronal activity, the effects of EM-2 could not be blocked by an otherwise effective dose of the selective mu-opioid receptor antagonist CTOP or the aspecific opioid receptor antagonist naloxone in contrast to the effects of EM-1 that were very effectively counteracted by CTOP, naloxonazine, and naloxone. It is concluded that the effects of EM-2 are not mediated via naloxone-sensitive opioid receptors in contrast to the effects of EM-1 that are mediated via mu1-opioid receptors in the NAc.

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