0022-3565/90/2521-0235$00.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 252, No. 1

Printed in U.S.A.

Respiratory and Locomotor Stimulation by Low Doses of Dermorphin, a Mu_1 Receptor-Mediated Effect[1]

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Accepted for publication September 29, 1989

ABSTRACT

The selective opioid *mu* receptor agonist dermorphin increased the locomotor activity of rats dose dependently at 10 to 100 pmol/kg i.c.v. Respiratory rate, relative tidal volume and respiratory minute volume also increased unrelated to changes in locomotor activity. Higher doses, on the other hand, produced catalepsy and respiratory depression. Pretreatment of the rats with the mu_1-selective antagonist naloxonazine (10 mg/kg i.v.) blocked the stimulant locomotor and respiratory effects of low doses of dermorphin (10–100 pmol/kg), but potentiated the respiratory depressant effect of a high dose (10 nmol/kg) of dermorphin. The selective benzodiazepine antagonist flumazenil (5 mg/kg), which has been shown previously to antagonize catalepsy and respiratory depression produced by relatively high doses of dermorphin, did not antagonize the respiratory or locomotor stimulant effect of dermorphin. The data suggest that mu_1-opioid receptors are responsible for the low dose stimulant effects of dermorphin on locomotor activity and respiration whereas mu_2 receptors mediate the respiratory depressant effect of dermorphin.

Low doses of morphine or opioid peptides increase locomotor activity, whereas higher doses induce a cataleptic state, characterized by the absence of movement and muscular rigidity (Tortella *et al.*, 1978; Browne *et al.*, 1979; Brady and Holtzman, 91981; Havemann *et al.*, 1983; Locke and Holtzman, 1986). Locke and Holtzman (1986) suggested that the depressant effect of opioids on locomotion might be mediated by *mu*-opioid and stimulant effect by *delta*-opioid receptors. In accordance with this concept, a potent and selective *mu* receptor agonist dermorphin (Rossi *et al.*, 1986; Krumins, 1987) induced catalepsy in the rat when administered i.c.v. at doses above 0.3 nmol/kg (Paakkari and Feuerstein, 1988).

The well known respiratory depressant effect of opioids (for review see Borison 1977) was also demonstrated for dermorphin in conscious rats (Paakkari *et al.*, 1988). However, small doses of opioids can also induce an increase in ventilatory frequency (Hassen *et al.*, 1982; Hurle *et al.*, 1985) or mean instantaneous minute ventilation (Haddad *et al.*, 1984; Schaeffer and Haddad, 1985).

The objective of the present study were to investigate whether low doses of dermorphin would also induce locomotor and respiratory stimulation and, if so, would the respiratory stimulation be secondary to the increase in locomotor activity. Furthermore, the role of mu_1-opioid receptors in mediating the stimulatory effect of dermorphin was examined in rats treated with mu_1 receptor antagonist naloxonazine (Hahn *et al.*, 1982). In addition, because the cataleptic and ventilatory depressant effect of dermorphin were antagonized by the benzodiazepine antagonist flumazenil (Ro 15-1788) (Paakkari and Feuerstein, 1988; Paakkari *et al.*, 1988), we found it of interest to examine whether the benzodiazepine/GABAergic system is also associated with dermorphin-induced locomotor and respiratory stimulation.

Methods

Animals. Conscious male Sprague-dawley rats (250–330 g, Taconic Farms, Germantown, NY) were used. The animals received standard rat pellets and water *ad libitum* and were kept at 22°C and 12 hr/hr light dark cycle.

Procedure for i.c.v. and i.v. injections. Rats were anesthetized with an i.m. injection of ketamine (130 mg/kg) and acepromazine (1.3 mg/kg). A stainless-steel guide cannula was inserted stereotaxically (David Kopf Instruments, Tujunga, CA) through the skull into the right lateral brain ventricle (i.c.v.) and fixed with glue (Eastman 910 adhesive). Coordinates for the lateral ventricle were: AP, −0.8 mm and L, 1.2 mm (1967). After the operation the rats were allowed to recover for at least 3 days in single cages, with food and water *ad libitum*. A group of rats was implanted also with PE-50 tubing, filled with heparinized (100 U/ml) saline, in the external jugular vein for naloxonazine injections. The tubing was threads s.c. to the posterior neck, exited through a small incision, then cut to a length of about 5 cm and sealed

Received for publication April 19, 1989.
[1] This work was supported by United States Army Medical Research and Development command protocol G39228.

ABBREVIATION: DAMGO, D-Ala^2-Me-Phe^4-Gly-ol^5-enkephalin.

by heat. The rats received a single i.v. injection of naloxonazine (10 mg/kg, as the free base) or saline in a volume of 1 ml/kg 22 to 24 hr before dermorphin injections.

On the day of the experiment, a 7.5 mm long 30 g cannula was inserted into the ventricular space through the guide cannula. The injection cannula was connected *via* polyethylene tubing to a Hamilton microliter syringe and the drug solution was injected over a period of 20 sec in a volume of 10 μl. Each rat received a single dermorphin injection. The proper position of the i.c.v. cannula was ascertained at the end of the experiment by inspection of the brain ventricles after an injection of methylene blue (10 μl).

Analgesia testing. Analgesia was measured by the tail-flick test (D'Amour and Smith, 1941). A cut-off time of 12 sec was used to avoid tissue damage. Tail-flick response times were represented as percentage of maximum possible effect (% of MPE) according to the equation:

$$\%\ \text{of MPE} = \frac{\text{post drug response latency} - \text{predrug response latency}}{\text{cut-off time} - \text{predrug response latency}} \times 100$$

Locomotor activity monitoring. Locomotor activity was monitored from 1 hr before until 2 hr after the drug administration. Digiscan Optical Digital Sensor Activity Monitors equipped with Datalogger 8000 data collection devices (Omnitech Electronics Inc., Columbus, OH) were used to measure activity. The data was collected in counts per 5 min. During the experiment the animals were placed in the Oxymax Plexiglas chambers (see Respiratory recording) set inside the activity monitors, which made the simultaneous recording of respiration and locomotor activity possible.

Recording of respiration. Ventilation rate and relative ventilation tidal volume were monitored by the Oxymax 85 system (Columbus Instruments, Columbus, OH). The recording system consists of two sealed Plexiglas test chambers and one reference chamber (internal dimensions: 112 × 197 × 297 mm, volume: 6.55 l). Room air is pumped through each chamber at the constant rate of 2 liters/min. Recording of the respiration rate and relative tidal volume is based on the minute pressure changes in the chamber caused by the subject's breathing. The tidal volume information is presented as a series of pulses whose frequency is proportional to the pressure changes within the chamber. Totalizing these pulses with a computer allows for the presentation of an index tidal volume. Relative tidal volume and the calculated minute volume are therefore expressed in arbitrary units.

The respiratory data were gathered at 2-min intervals for 60 min before and 60 to 120 min after the injections. The data were stored and processed by an IBM PC computer using the program Dataquest provided by the manufacturer. Thereafter the output files were imported into the spreadsheet (Framework II, Ashton-Tate) for calculation of maximum effects. The results are expressed as percentage of changes (means ± S.E.) from the base line during a 10-min time period, at 50 to 60 min after the injection, at which time the respiratory effects were maximal. The base lines were calculated from the values during the last 20 min before the drug injection. In all parameters the differences between the basal values of the groups were statistically not significant. Differences between means were analyzed with one-way analysis of variance followed by Tukey's test. For data with unequal variances the Kruskall-Wallis test followed by Mann-Whitney *U* tests were used. A significant difference was obtained at $P < .05$.

Chemicals. Dermorphin (Sigma Chemical Co., St. Louis, MO) and naloxonazine (a generous gift from Dr. G. W. Pasternak, Memorial Sloan-Kettering Cancer Center, New York, NY) were dissolved in 0.9% saline, naloxonazine with a few drops of glacial acetic acid (final pH 7.0). Flumazenil (gift from W. Haefely, Hoffman-La Roche Inc., Basel, Switzerland) was dissolved in 10% Emulphur 620 (GAF Corp., Grasseli, NJ)-ethanol mixture (1:1), warmed and sonicated immediately before administration. Flumazenil or saline were given i.p. in a volume of 1 ml/kg, 20 min before the dermorphin injections. Naloxonazine (10 mg/kg i.v.) was given 20 to 24 hr before dermorphin.

Results

Locomotor effects of dermorphin. Dermorphin was tested at doses of 3, 10, 30 and 100 pmol/kg. The three highest doses increased locomotor activity, with 30 pmol/kg having the maximal effect (Fig. 1). Increases in total (60 min) locomotor activity produced by 100 pmol/kg of dermorphin did not reach statistical significance ($P < .19$). However, the increase in locomotor activity during the first 10 min after the injection was significant (fig. 2).

The spontaneous locomotor activity of the resting rats was near zero during the 10 min before drug injections.

Respiratory effects of dermorphin. Low doses of dermorphin (10 and 30 pmol/kg) increased the respiratory minute volume (fig. 3, lower panel). Respiration rate was increased significantly after the dose of 30 pmol/kg (fig. 3, upper panel). Higher doses (3 and 10 nmol/kg) decreased both respiration rate and minute volume. The corresponding changes in relative tidal volume were 3 ± 6% (saline), 6 ± 10% (3 pmol/kg), 86 ± 74% (10 pmol/kg), 179 ± 128% (30 pmol/kg), −3 ± 9% (100 pmol/kg), 23 ± 18 (3 nmol/kg) and −9 ± 7% (10 nmol/kg). The changes in relative tidal volume were not statistically significant.

Time course of locomotor and respiratory stimulation. The peak increase in locomotor activity was observed within 15 min from the injection, whereas the respiratory stimulation was maximal 50 to 60 min after the injection (fig. 2). The effects of the dose of 100 pmol/kg at 10 min further shows that

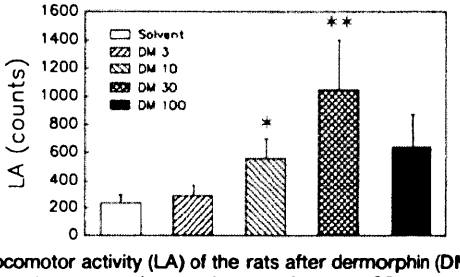

Fig. 1. Locomotor activity (LA) of the rats after dermorphin (DM, 3-100 pmol/kg i.c.v.), expressed as total counts from the 60-min period after drug injection. *P < .05; **P < .01 (drug *vs.* solvent; $n = 6$ in each group).

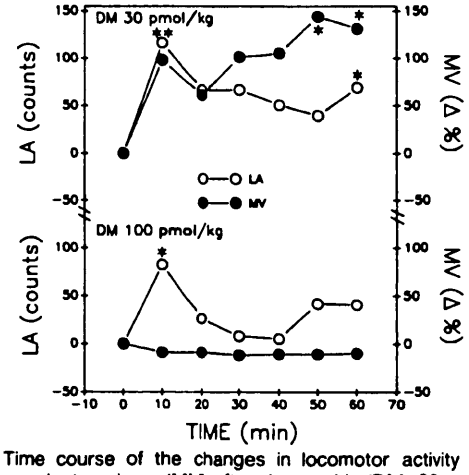

Fig. 2. Time course of the changes in locomotor activity (LA) and respiratory minute volume (MV) after dermorphin (DM, 30 or 10 pmol/kg). The counts show the difference between test and control animals (the effect of solvent is subtracted from that of drug). *P < .05; **P < .001; dependent *t* test, $n = 6$.

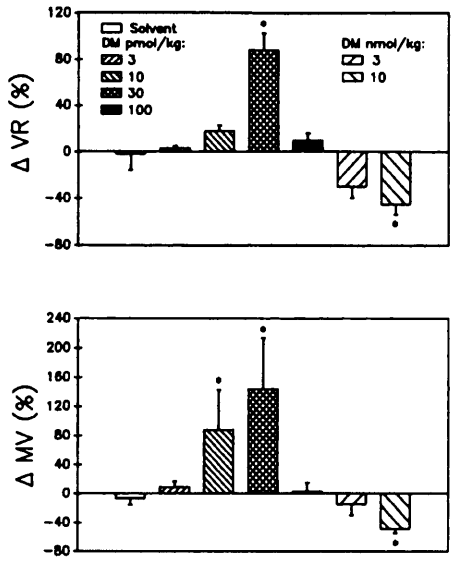

Fig. 3. Changes in ventilatory rate (VR, upper panel) and minute volume (MV, lower panel) after dermorphin (DM, 3 pmol–10 nmol/kg). *P < .05; **P < .001; n = 6 in each group.

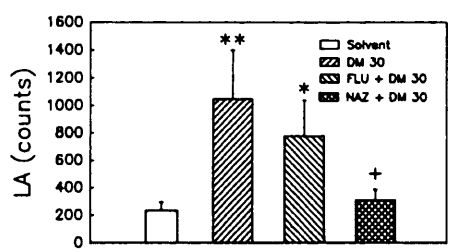

Fig. 4. Influence of naloxonazine (NAZ, 10 mg/kg i.p.) and flumazenil (FLU, 5 mg/kg i.p.) pretreatments on the locomotor effect of dermorphin (DM, 30 pmol/kg). *P < .05; **P < .001 when compared to solvent, +P < .05 when compared to DM (30 pmol/kg; n = 6 in each group).

respiratory and locomotor stimulation were unrelated to each other, as the minute volume remained unchanged although locomotor activity increased significantly (fig. 2).

Effects of dermorphin after pretreatment with naloxonazine or flumazenil. Naloxonazine (10 mg/kg i.v.) was given 20 to 24 hr before dermorphin. The dose of naloxonazine and the time for pretreatment was chosen to obtain maximal *mu*$_1$-selectivity (Hahn *et al.*, 1982; Ling *et al.*, 1986). Naloxonazine antagonized both the locomotor (fig. 4) and respiratory (fig. 5) stimulation produced by dermorphin, 30 pmol/kg, the dose which had the maximal effect in the dose-response study. Flumazenil (5 mg/kg i.p. 20 min before dermorphin) did not significantly attenuate the locomotor and respiratory stimulant effects of dermorphin (figs. 4 and 5).

To confirm the selectivity of naloxonazine antagonism on dermorphin-induced respiratory stimulation, one group of naloxonazine-treated rats was given a respiratory depressant dose of dermorphin, 10 nmol/kg. Respiratory depression was potentiated by naloxonazine pretreatment (fig. 6).

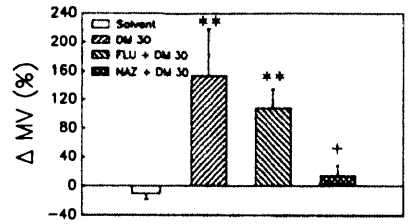

Fig. 5. Influence of naloxonazine (NAZ) and flumazenil (FLU) pretreatments on the respiratory stimulant effect of dermorphin (DM, 30 pmol/kg). **P < .01 when compared to solvent; +P < .05 when compared to DM; n = 6 in each group.

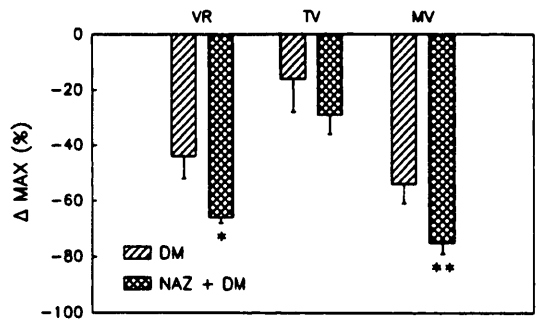

Fig. 6. Potentiating effect of naloxonazine (NAZ) pretreatment on respiratory depression induced by a high dose of dermorphin (DM, 10 nmol/kg). *P < .05; **P < .01 (DM *vs.* NAZ + DM, n = 6 in both groups). VR, ventilatary rate; TV, ventilatary tidal volume; MV, ventilatary minute volume; max. maximal.

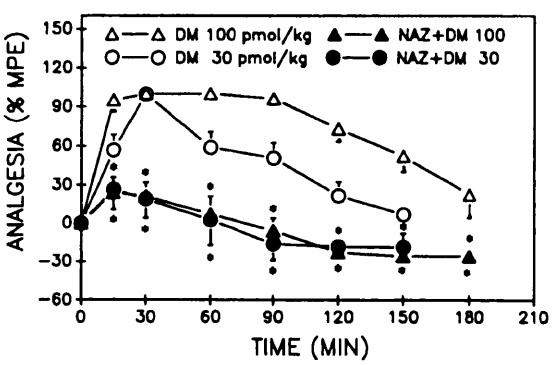

Fig. 7. Analgesia induced by dermorphin (DM, 0.1 and 0.03 nmol/kg). The filled symbols represent the effect of the same doses of DM in naloxonazine (NAZ)-treated animals. *P < .05, n = 5 in all groups. MPE = maximum possible effect.

Analgesic effect of dermorphin. Dermorphin, 0.03 and 0.1 nmol/kg, induced analgesia, which was antagonized by naloxonazine (fig. 7).

Discussion

We have shown previously that the central administration of the heptapeptide dermorphin produces catalepsy and respiratory depression at doses above 300 pmol/kg (Paakkari and Feuerstein, 1988; Paakkari *et al.*, 1988). This study disclosed opposite effects of the same opioid, *i.e.*, stimulation of locomotor activity and respiration when lower doses, 10 to 100 pmol/kg, were given.

The high potency of dermorphin (up to 5000 times more potent than morphine) described in analgesia studies and bioassays (Broccardo *et al.*, 1981; Stevens and Yaksh, 1986), is evident also when the effects of opioids on locomotion are compared: the doses of morphine that in previous studies (Brady and Holtzman, 1981) induced maximal locomotor activation were 10,000-fold greater than the equipotent doses of dermorphin in our study. Even the corresponding doses of the specific *mu* agonist DAMGO were still 30 to 200-fold greater than those of dermorphin in our study (Locke and Holtzman, 1987; Latimer *et al.*, 1987). The outstanding potency of dermorphin, which has been shown to have high selectivity and affinity for *mu* sites (about 3 times higher affinity than DAMGO; Krumins 1987), suggests that the locomotor activation is mediated *via mu* receptors.

To our knowledge, the respiratory changes have not been measured in previous studies in which locomotor effects of opioids were investigated. It is conceivable that an increase in locomotor activity could subsequently lead to an increase in respiration. Indeed, low doses of dermorphin consistently increased respiratory rate and relative tidal volume. However, simultaneous recording of respiration and locomotion revealed that stimulation of respiration was unrelated to the increase in locomotor activity for the following reasons: first, the peak stimulation in locomotion was observed 10 to 15 min after dermorphin injection, whereas the maximum effect on respiration was reached 50 to 60 min after dermorphin administration; second, locomotor activity returned back to basal values at time of maximal respiratory stimulation; and third, the dose of 100 pmol/kg of dermorphin increased locomotor activity significantly without respiratory stimulation.

Centrally administered *mu*-opioid agonists induced in most studies a respiratory depression (Holaday, 1982; Pfeiffer *et al.*, 1983; Pazos and Florez, 1983; Ward and Takemori, 1983; Morin-Surun *et al.*, 1984), as did dermorphin in our previous study (Paakkari *et al.*, 1988). However, local injections of small doses (3–30 pmol) of the *mu*-opioid agonist DAMGO into the nucleus tractus solitarius increased respiratory frequency, whereas a high dose (300 pmol) had a depressant effect (Hassen *et al.*, 1982). Furthermore, the *mu* agonists morphine (0.5 mg/kg) and morphiceptin analog (Tyr-Pro-NMePhe-D-Pro-NH_2, 2–125 μg/kg) increased instantaneous minute ventilation in conscious dogs upon intracisternal administration (Haddad *et al.*, 1984; Schaeffer and Haddad, 1985). Also, the *mu*-agonist D-Ala^2-Me-Phe^4-Met(O)ol^5-enkephalin (FK-33824) and the *delta*-agonist D-Ala^2-D-Leu^5-enkephalin increased respiratory frequency and decreased tidal volume when applied to the ventral surface of the medulla oblongata of the anesthetized cat, whereas when applied to the rostro-dorsal surface of the pons they depressed respiratory frequency and increased tidal volume (Hurle *et al.*, 1985).

Pharmacological, biochemical and receptor binding studies support the presence of two subtypes of *mu* receptors: the mu_1 receptor which binds both opiates and most enkephalins with similar very high affinities (K_d values < 1 nM), and the mu_2 receptor which binds morphine preferentially (Wolozin and Pasternak, 1981; Nishimura *et al.*, 1983; Lutz *et al.*, 1985; Goodman and Pasternak, 1985). The autoradiographic distribution of mu_1-sites to brain areas involved in antinociception (Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985a), and the correlation between the density of mu_1-sites and analgesic sensitivity in different mice strains (Moskowitz and Goodman, 1985b), as well as pharmacological studies with mu_1-antagonists and *delta*/mu_1-agonists (Pasternak *et al.*, 1980; Zhang and Pasternak, 1981, 1988; Bodnar *et al.*, 1988) imply that supraspinal opioid analgesia is mediated *via* mu_1 receptors. The mu_1 receptor antagonist naloxonazine selectively antagonized morphine analgesia without affecting the hypoxemia and hypercarbia produced by morphine (Ling *et al.*, 1983, 1985), suggesting that the respiratory depressant effect of opioids is mediated by mu_2 receptors. Along this hypothesis, it can be speculated that the stimulatory effects of the selective *mu*-agonist dermorphin on respiration are mediated *via* mu_1 receptors. The aforementioned respiratory stimulation by small doses of *mu*-agonists or *delta*-agonists (Hassen *et al.*, 1982; Schaeffer and Haddad, 1985; Hurle *et al.*, 1985), known to bind to mu_1 receptors with high affinity (Wolozin and Pasternak, 1981; Nishimura *et al.*, 1982; Lutz *et al.*, 1985; Itzhak and Pasternak, 1987), could also be explained assuming that the small doses would bind to high affinity mu_1 receptors only, leading to stimulation; at higher doses, the respiratory depressant mu_2-effect would dominate. In our study dermorphin at the dose of 100 pmol/kg did neither stimulate nor depress respiration; this lack of change from base line (fig. 3) may be due to a balance between the respiratory stimulant and depressant effect of this dose.

Stimulation of locomotor activity may similarly be a mu_1 receptor effect, as dermorphin does not bind to *delta* receptors, formerly proposed to mediate the opioid-induced increase in locomotor activity (Locke and Holtzman, 1986). In the present study naloxonazine did antagonize the respiratory and the locomotor stimulation by dermorphin, implying that these indeed were mu_1 receptor-mediated effects. Latimer *et al.* (1987) also suggested recently that activation of dopamine neurons in the ventral tegmental region of the rat brain by morphine and enkephalin analogs, and the subsequent increase in motor activity, are mediated by *mu* receptors, perhaps the mu_1-subtype.

The analgesic effect of dermorphin was studied to characterize further its mu_1 and mu_2 receptor-mediated actions. Dermorphin-induced analgesia was antagonized by naloxonazine, corroborating the theory that mu_1 receptors are involved in this effect. After the highest dose of dermorphin the naloxonazine-treated animals were also clearly less cataleptic than in control group (ordinary catalepsy tests were, however, not performed due to the recording of respiration), which is in concordance with the suggestion that catalepsy also is mediated *via* mu_1 receptors (Ling and Pasternak, 1982).

To ascertain the selectivity of naloxonazine antagonism against mu_1 receptors, we studied its influence on the respiratory depressant effect of a higher dose of dermorphin. Instead of antagonism, there was a potentiation of respiratory depression. This is in accordance with the concept that mu_2 receptors mediate respiratory depression (Ling *et al.*, 1985) and mu_1 receptors respiratory stimulation: when the mu_1 receptors were blocked by naloxonazine, dermorphin's depressant effect on the available mu_2 receptors was unopposed and potentiated.

We have shown previously that the benzodiazepine antagonist flumazenil attenuates the cataleptic (Paakkari and Feuerstein, 1988) and respiratory depressant (Paakkari *et al.*, 1988) effect of dermorphin. It also antagonizes the analgesic (Brady *et al.*, 1984) and intestinal (Fioramonti *et al.*, 1987) effects of centrally given *mu*-agonist opioids. However, the locomotor and respiratory stimulant effects of low doses of dermorphin

TABLE 1

Putative receptors involved in various effects of dermorphin

NAZ, naloxonazine; FLU, flumazepine; BZ/GABA, benzodiazepine-γ-aminobutyric acid (GABA) receptor complex.

Effect	Dose	Antagonized by		Receptors
		NAZ	FLU	
	pmol/kg i.c.v.			
Respiratory stimulation	>10	Yes	No	Mu_1
Locomotor stimulation	>10	Yes	No	Mu_1
Analgesia	>30	Yes	Yes	Mu_1; BZ/GABA
Catalepsy	>300	Yes	Yes	Mu_1; BZ/GABA
Respiratory depression	>1000	No	Yes	Mu_2; BZ/GABA

were not antagonized by flumazenil. Hypothetically, this can be explained by postulating that very low concentrations of dermorphin would bind to high-affinity, naloxonazine-sensitive mu_1 receptors only; at higher analgesic, respiratory depressant and cataleptic doses it would interact with benzodiazepine/GABAergic pathways (table 1), suggested to modulate various effects of opioids (Cooper *et al.*, 1983; Naughton *et al.*, 1985; Zambotti *et al.*, 1987; Moreau and Pieri, 1988).

Acknowledgments

The experiments reported herein were conducted according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Medicine, National Research Council, Department of Health, Education and Welfare Publication No. (NIH) 85-23, 1985. We wish to thank Dr. James E. Barrett for his useful comments during this study.

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