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Involvement of δ-opioid receptors in the effects induced by endogenous enkephalins on learned helplessness model

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Abstract

Pharmacological, neurochemical and behavioural findings support a possible role of endogenous opioids in clinical depression. There is evidence from animal studies that δ-opioid receptors are involved in several behavioural responses to opioids, including motivational activities. In the present study, the mixed enkephalin catabolism inhibitor, RB 101 (*N*(*R,S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthiobutyl-dithio]-1-oxopropyl)-L-phenylalanine benzyl ester) (1.25, 2.5 and 5 mg/kg), induced a dose-dependent antidepressant-like effect in a learned helplessness model. Thus, RB 101 reversed escape deficits in rats previously subjected to inescapable shocks, suggesting the involvement of endogenous enkephalins in depression. Similar effects were observed after administration of the selective δ-opioid receptor agonist, BUBU (Tyr-D.Ser-(*O-tert*-butyl)-Gly-Phe-Leu-Thr(O-Tet-butyl-OH) (1 and 2 mg/kg). Moreover, RB 101 effects were antagonized by administration of naltrindole (NTI) (0.1 mg/kg), which points to a preferential involvement of δ-opioid receptors in this enkephalin-controlled behaviour. As RB 101 has been reported to be almost devoid of opiate-related side-effects, it could represent a promising alternative in the treatment of depressive patients who are unresponsive to, or intolerant of, classical antidepressants.

Keywords: RB 101; BUBU (Tyr-D.Ser-(*O-tert*-butyl)-Gly-Phe-Leu-Thr(*O*-Tet-butyl-OH); Naltrindole; Learned helplessness; Enkephalin catabolism inhibitor; δ-Opioid receptor

1. Introduction

The possible role of opioids in the pathogenesis of psychiatric disorders and particularly depressive illness continues to arouse a considerable interest (Naber, 1993). Several pharmacological, neurochemical and behavioural findings support a role for endogenous opioids in affective disorders. Thus, endogenous opioid peptides and their receptors are found in large concentrations in regions of the limbic system associated with the regulation of mood and behaviour (see Mansour et al., 1988). Mice with deletion of the preproenkephalin gene were shown to exhibit abnormal behavioural responses and aggressivity (König et al., 1996). Moreover, depressed patients were reported to display a deficiency, and manic patients an excess, of endogenous opioid activity (Pickar et al., 1980). In addition clinical trials indicate that opioid compounds such as β-endorphin (Kline et al., 1977; Darko et al., 1992) and buprenorphine (Bodkin et al., 1995) have antidepressant effects. Some of the effects of electroconvulsive treatment, an effective alternative for refractory depression, may be also mediated by opioid peptides (Belenky and Holaday, 1979). This therapy increases the plasma level of the endogenous opioid peptide β-endorphin (Emrich et al., 1979; Inturrissi et al., 1982; Ghadirian et al., 1988).

Previous work in our laboratory has shown that activation of the opioid peptidergic system by administration of either enkephalin catabolism inhibitors (RB 38A or RB 38B) or opioid receptor agonists ([Met^5]enkephalin, [Leu^5]enkephalin and morphine) reverses the escape deficit in a model of depression, the so-called learned helplessness model (Tejedor-Real et al., 1993, 1995). Other in-

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hibitors of enkephalin degrading enzymes, such as thiorphan and bestatin, also showed antidepressant-like responses in the conditioned suppression of motility test (Nabeshima et al., 1987).

Pharmacological observations support the view that antidepressant drugs also have a marked effect on endogenous opioid systems (Biegon and Samuel, 1980; Isemberg and Cicero, 1984; Hamon et al., 1987). Opiates are also able to modify the effects of antidepressants (De Felipe et al., 1989; Tejedor-Real et al., 1993). Moreover, a common pathway in the analgesic effects of both tricyclic antidepressants and opioids has been described (Valverde et al., 1994).

It has been recently suggested that mostly μ-opioid receptors are involved in analgesia, reward behaviour and physical dependence produced by morphine administration (Matthes et al., 1996). Nevertheless, δ-opioid receptors could play an important role in several opioid-mediated behavioural responses, including motivational and rewarding effects (Shippenberg et al., 1987; Daugé et al., 1988).

Learned helplessness is a well validated and widely used model of depression (Willner, 1990). Uncontrollable and aversive events induce neurochemical (Weiss et al., 1981), cognitive (Jackson et al., 1980), motivational (Drugan and Maier, 1982) and emotional deficits (Maier et al., 1972) called learned helplessness effects, which are claimed to resemble the symptoms found in depressed patients (Weiss et al., 1981). Initial attempts to train animals to become helpless were only partially successful as a significant proportion of rats do not respond in this way after inescapable shocks. In previous studies, we found that rats expressing high emotivity in open-field test were more likely to acquire the learned helplessness response (Tejedor-Real et al., 1990). Consequently, animals were preselected, in a preliminary experiment, with an open-field test. Only rats showing a high emotivity in this test were used to acquire learned helplessness.

Based on previous literature, the present work was designed to investigate in the learned helplessness model the potential antidepressant effect of RB 101 (*N*(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthiobutyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester), an enkephalin catabolism inhibitor able to cross the blood–brain barrier and to protect completely the endogenous enkephalins being metabolized by inactivating the ectopeptidases, aminopeptidase N (EC 3.4.11.2) and neutral endopeptidase (EC 3.4.24.11) (Fournié-Zaluski et al., 1992).

A second aim was to evaluate the possible involvement of δ-opioid receptors in the effect of endogenous enkephalins on learned helplessness. For this purpose, animals were treated with naltrindole (NTI), a selective δ-opioid receptor antagonist (Portoghese et al., 1988), together with RB 101, to study its ability to antagonize the effect of the enkephalin catabolism inhibitor. We also addressed this possibility by administering BUBU (Tyr-D.Ser-(*O*-*tert*-butyl)-Gly-Phe-Leu-Thr(*O*-Tet-butyl-OH) (Gacel et al., 1988), a systemically active specific δ-opioid receptor agonist resistant to peptidase degradation (Delay-Goyet et al., 1988).

2. Materials and methods

2.1. Animals

Male Wistar rats (Central Animal Service of the University of Cádiz) weighing 200–250 g at the beginning of each experiment, were used. They were individually housed and exposed to a 12 h/12 h light/dark cycle in a room at constant temperature (21 ± 1°C) and humidity with noise kept to a minimum. Food and water were available ad libitum.

The experiments were conducted according to the international ethical guidelines (Zimmermann, 1983), and the experimental protocol was approved by the Local Ethical Committee of Animal Experimentation for the Faculty of Medicine of the University of Cádiz (License no. 079604).

2.2. Apparatus

The open field used to select emotional animals was 50-cm high and 1 m in diameter. The walls and the floor were made of white material. The floor was divided into three concentric circles, the outer and the medium ones, but not the inner one, were divided into 12 squares by 12 radial lines. The open field was placed in a light-proof, sound-proof room, and was brightly illuminated with a 100-W single light source located 60 cm above the centre of the apparatus (800 lx).

Inescapable footshocks were delivered in Plexiglass chambers (walls and covers $20 \times 10 \times 10$ cm) with a stainless-steel grid floor consisting of rods spaced 1.5 cm apart.

Escape and avoidance training was evaluated in automated two-way shuttle-boxes ($52 \times 22 \times 29$ cm). Each shuttle-box was divided into two equal-sized compartments by a partition with a gate (7×7 cm) that provided access to the adjacent compartment. The floors were stainless steel grids (1.5 cm apart). Static load-cells connected to the grid floor monitored escape and avoidance responses and the position of the animal on any given trial. Both pieces of apparatus were provided with a scrambler.

2.3. Surgery

Animals receiving drugs by i.v. route were anesthetized with equitesin (5 ml/kg = 212.5 mg/kg chloral hydrate + 48.6 mg/kg pentobarbital; i.p.) and a polyethylene cannula was implanted in the external jugular vein. Correct location of the cannulae was checked in each animal at the end of every experiment by an inflow of pentobarbital.

2.4. Drug administration

RB 101 solutions were prepared in a mixture of EtOH/cremophor El/H_2O (1:1:8). RB 101 and BUBU were administered i.v. in a volume of 0.1 ml/100 g body weight 10 min before the escape and avoidance test. NTI was administered i.p. in a volume of 0.5 ml/100 g body weight 15 min before the shuttle-box test. Control animals received the same volumes of either vehicle or saline according to each experiment.

2.5. Treatment groups

Animal groups received at random an acute treatment during the 3 days of the escape and avoidance test, according to one of the following protocols.

2.5.1. Experiment 1

RB 101 (1.25, 2.5 and 5 mg/kg, i.v.) or vehicle. A non-shocked group was included in this experiment.

2.5.2. Experiment 2

NTI (a selective δ-opioid receptor antagonist) (0.01, 0.1 or 1 mg/kg i.p.) or saline. NTI doses were selected according to previous studies (Kitchen and Pinker, 1990; Smadja et al., 1992; Baamonde et al., 1992).

2.5.3. Experiment 3

RB 101 (5 mg/kg i.v.) + saline (i.p.), RB 101 (5 mg/kg, i.v.) + NTI (0.1 mg/kg, i.p.), BUBU (1 and 2 mg/kg, i.v.) + saline(i.p.) or solvent(i.v.) + saline(i.p.). Taking into account the results of experiment 2, a non-effective dose of NTI was administered to antagonize RB 101.

2.6. Procedure

2.6.1. Emotional rat selection

In previous experiments it was found that rats with a high emotionality level are more susceptible to learned helplessness (Tejedor-Real et al., 1990). In order to make animals more susceptible to become helpless, before each experiment, all rats were individually subjected to two open-field sessions of 5-min duration each, one per day for two successive days. The number of faecal boluses excreted, was recorded, as a measure of emotionality, and the rats were selected according to the average number in both sessions. Animals with moderate or low defecation rates were eliminated. Only rats with an average number of five boluses or more excreted were selected. The method was performed as described previously (Tejedor-Real et al., 1990). The defecation rate in a novel environment has been reported to be directly related to the emotionality level (Broadhurst, 1960).

2.6.2. Inescapable shock pre-treatment

Sixty scrambled, randomized inescapable electric footshocks were delivered (0.8 mA, 15-s duration, intershock interval 10–90 s) (AC, 50 Hz) to the grid floor. Inescapable footshock pre-treatment was performed in the morning.

2.6.3. Conditioned avoidance training

In order to evaluate the escape and avoidance test, avoidance training was performed 48 h after the inescapable shock pre-treatment. Animals were placed singly in the shuttle-box and subjected to 30 avoidance trials, with 30 s between trials. During the first 3 s of each trial, a light signal was presented (conditioned stimulus) allowing animals to avoid the shock (Giral et al., 1988). If no avoidance response occurred within this period, a 0.8-mA shock lasting 3 s was applied via the grid floor. If there was no escape response within this period, shock and light were terminated. The response required of the rat, either avoidance or escape, was to cross the gate into the other compartment of the shuttle-box. An escape failure was when the rat failed to cross into the other compartment during shock delivery. Avoidance sessions were performed for three consecutive days in the morning, and the number of escape failures was recorded.

2.7. Statistical analysis

Values are expressed as mean number of escape failures ± S.E.M. recorded over 30 trials during each shuttle-box session. Results were statistically evaluated using the Kruskal–Wallis H test. Post-hoc comparisons between treatment groups were made using the Mann–Whitney U-test. The level of significance was $P < 0.05$. Only significant values are shown.

3. Results

3.1. Experiment 1: behavioral effects induced by RB 101 on the learned helplessness model

Control non-shocked animals and shocked animals treated with RB 101 displayed fewer escape failures than shocked non-treated animals. Kruskal–Wallis analysis revealed significant differences between the groups (saline; RB 101 1.25, 2.5 and 5 mg/kg; non-shocked + saline) during the three daily shuttle-box sessions: 1st session $H = 12.5$ ($df = 4$), $P < 0.01$; 2nd session $H = 16.27$ ($df = 4$), $P < 0.005$; 3rd session $H = 11.6$ ($df = 4$), $P < 0.05$.

3.1.1. Effects of inescapable shocks on control animals

As in previous experiments, significant differences were found in the number of escape failures between non-shocked and shocked non-treated animals. Post-hoc analysis of the data showed that differences between these

Table 1
Effect of inescapable shocks on control animals

	1st session	2nd session	3rd session
Non-shocked ($n = 7$)	8.87 ± 1.55	5.00 ± 1.48	7.40 ± 1.20
Shocked ($n = 15$)	19.80 ± 1.66^{b}	20.60 ± 2.20^{b}	22.33 ± 1.99^{a}

Mean number of escape failure ± S.E.M. during the 30 trials of the three daily shuttle-box sessions.
[a] $P < 0.005$, [b] $P < 0.001$ vs. non-shocked rats (Mann–Whitney U-test).

values reached significance during the three daily shuttle-box sessions (1st session: $U = 7$ (7–15), $P < 0.001$; $U = 3$ (6–15), $P < 0.001$; 3rd session: $U = 9$ (6–15), $P < 0.005$) (Table 1).

3.1.2. Effects induced by RB 101

Administration of RB 101 clearly dose-dependently reduced the number of escape failures. Doses of either 2.5 or 5 mg/kg clearly modified animal behaviour in this model. However, post-hoc comparisons (Mann–Whitney U-test) showed that only administration of 5 mg/kg of RB 101 was able to reduce inescapable shock effects during the three daily shuttle-box sessions in a significant way as compared to those in control rats (1st session: $U = 61.5$ (14–16), $P < 0.05$; 2nd session: $U = 53$ (15–15), $P < 0.01$; 3rd session: $U = 54.5$ (14–15), $P < 0.05$) (Fig. 1).

3.2. Experiment 2: behavioral effects induced by NTI in the learned helplessness model

The administration of NTI at doses of 0.01, 0.1 and 1 mg/kg, i.p., did not induce any effect on helpless rats. The Kruskal–Wallis test did not reveal significant differences in escape failure number between groups (saline; NTI 0.01, 0.1 and 1 mg/kg) during the three daily shuttle-box sessions (Table 2).

Table 2
Effects induced by NTI in the learned helplessness model

Treatment	1st session	2nd session	3rd session
Saline ($n = 9$)	18.30 ± 1.82	21.10 ± 2.94	22.20 ± 2.71
NTI (1 mg/kg) ($n = 9$)	17.66 ± 2.53	18.88 ± 3.90	20.33 ± 3.80
NTI (0.1 mg/kg) ($n = 10$)	16.55 ± 3.20	21.50 ± 3.20	21.60 ± 2.89
NTI (0.01 mg/kg) ($n = 9$)	18.44 ± 2.94	22.66 ± 2.80	23.88 ± 2.59

Mean number of escape failure ± S.E.M. during the 30 trials of the three daily shuttle-box sessions in shocked rats treated with saline or NTI. No significant differences were found between groups.

3.3. Experiment 3: participation of δ-opioid receptors in the behavioural responses induced on the learned helplessness model

The experiments reported below were conducted simultaneously to avoid replicating control groups, but for the sake of clarity they are presented separately in the figures.

An initial Kruskal–Wallis test indicated a significant difference in the number of escape failures between the different treatment groups (RB 101 + saline; RB 101 + NTI; BUBU 1 mg/kg + saline; BUBU 2 mg/kg + saline; solvent + saline) during the three shuttle-box sessions: 1st session: $H = 10.42$ ($df = 4$), $P < 0.01$; 2nd session: $H = 12.16$ ($df = 4$), $P < 0.01$; 3rd session: $H = 10.7$ ($df = 4$), $P < 0.05$.

3.3.1. Antagonism by NTI of RB 101-induced behavioural effects

A post-hoc Mann–Whitney U-test indicated that administration of 0.1 mg/kg of NTI antagonized the antidepressant-like effect of RB 101 (5 mg/kg) during the three daily shuttle-box sessions, suggesting a δ-opioid receptor

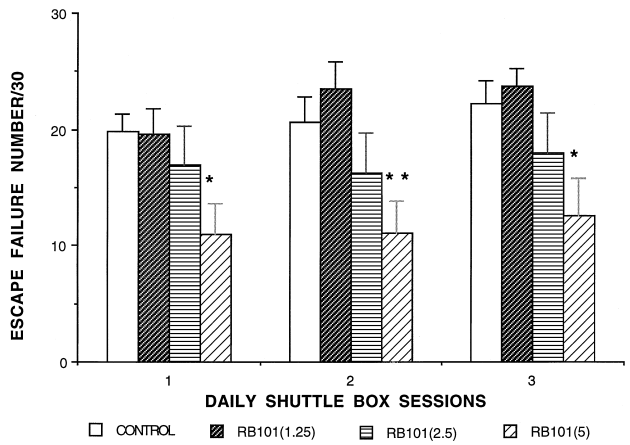

Fig. 1. Dose-dependent antidepressant-like effect of RB 101. The bars represent the mean number of escape failures (± S.E.M.) during the 30 trials of the three daily shuttle-box sessions in control rats and RB 101-treated rats (1.25, 2.5 or 5 mg/kg) after inescapable shock pretreatment. $*P < 0.05$, $**P < 0.01$ vs. control animals (Mann–Whitney U-test).

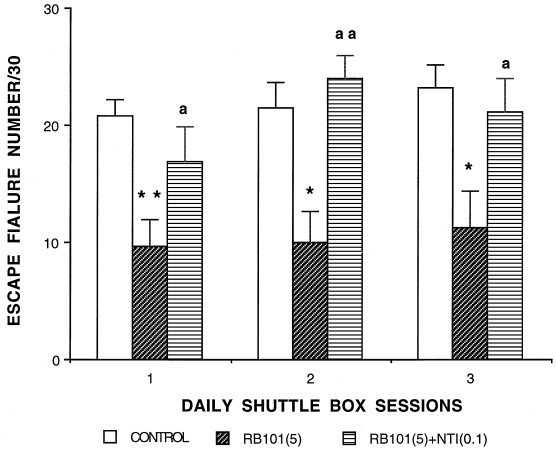

Fig. 2. Antagonism of the antidepressant-like effect of RB 101 by NTI. The bars represent the mean number of escape failures (± S.E.M.) during the 30 trials of the three daily shuttle-box sessions in control rats, RB 101 (5 mg/kg) and RB 101 (5 mg/kg) + NTI (0.1 mg/kg)-treated rats after inescapable shock pretreatment. $*P < 0.01$, $**P < 0.005$ vs. control animals; a: $P < 0.05$, aa: $P < 0.01$ vs. RB 101 (5). (Mann–Whitney U-test).

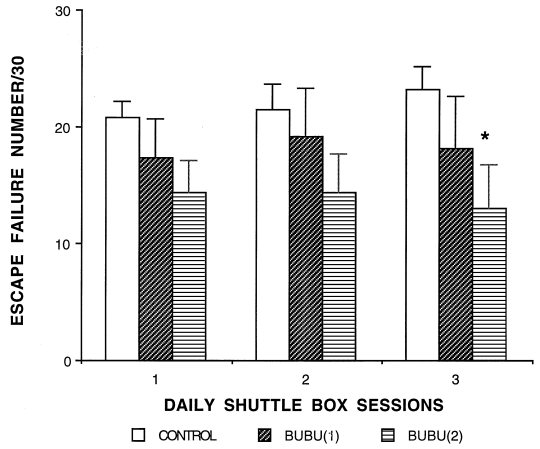

Fig. 3. Effect of BUBU administration on inescapable shock-pretreated rats. The bars represent the mean number of escape failures (±S.E.M.) in control rats and BUBU treated rats (1 or 2 mg/kg) after inescapable shock pretreatment. * $P < 0.01$ vs. control rats (Mann–Whitney *U*-test).

mediation. 1st session: $U = 20.5$ (13–7), $P < 0.05$; 2nd session: $U = 13$ (14–7), $P < 0.01$; 3rd session: $U = 21$ (13–7), $P < 0.05$ (Fig. 2). As in the first experiment, administration of RB 101 (5 mg/kg) significantly reversed the learned helplessness effects during the three shuttle-box sessions as compared to those in control animals as shown by the decrease in escape failures (1st session: $U = 28$ (13–14), $P < 0.005$; 2nd session: $U = 37$ (14–14), $P < 0.01$; 3rd session: $U = 35$ (13–14), $P < 0.01$).

3.3.2. Effects induced by BUBU

Administration of BUBU (1 and 2 mg/kg, i.v.), a selective δ-opioid receptor agonist also induced a dose-dependent antidepressant-like effect. Shock-pretreated animals receiving 2 mg/kg of BUBU before the escape and avoidance test, showed a clear reduction in the number of escape failures during the three daily shuttle-box sessions. A Mann–Whitney *U* test analysis indicated that data reached significant values during the 3rd shuttle-box sessions, as compared to control animals ($U = 24$ (9–14), $P < 0.01$) (Fig. 3).

4. Discussion

Exposure of rats to inescapable shocks induces a high rate of escape failures on subsequent testing in a shuttle-box. It has been suggested that exposure to uncontrollable stress teaches animals to be helpless (Maier and Seligman, 1976). This behavioral deficit produced by uncontrollable stress is highly sensitive to antidepressant drugs. In agreement with this, repeated administration of tricyclic antidepressants is able to reverse this behavioral response. For these reasons, this is a well validated and widely used model of depression, currently used to test antidepressant properties of compounds.

The inhibition of aminopeptidase N and/or neutral endopeptidase, the two enzymes involved in the inactivation of the endogenous enkephalins, by central administration of RB 38A (a mixed inhibitor of both enzymes) or RB 38B (a selective inhibitor of neutral endopeptidase) has been recently shown to induce antidepressant-like effects in the learned helplessness model of depression. The effectiveness of these compounds is directly related to their ability to inhibit the catabolism of endogenous enkephalins (Tejedor-Real et al., 1993, 1995). In the present work, the systemic administration of RB 101, a mixed enkephalin catabolism inhibitor, able to cross the blood–brain barrier, also induced an antidepressant-like effect on this model of depression. A similar effect has also been reported after RB 101 administration in the conditioned suppression of motility test in mice (Baamonde et al., 1992; Smadja et al., 1995) and rats (Smadja et al., 1997).

The antidepressant-like effect of RB 101 is very likely due to enhancement of the extracellular concentration of endogenous enkephalins. This compound, which is devoid of affinity for opioid binding sites, nevertheless inhibits [^{3}H]diprenorphine binding to the opioid receptors in a dose-dependant manner (Ruiz-Gayo et al., 1992), presumably as a consequence of the protection of endogenous enkephalin release from the synapse. Accordingly, it has been recently reported that using in vivo microdialysis techniques, systemic administration of RB 101 increased the extracellular levels of endogenous enkephalins in restricted areas of the central nervous system (Daugé et al., 1996).

The antagonism of the antidepressant-like effects of RB 101 by the selective δ-opioid antagonist NTI supports the concept of a preferential involvement of δ-opioid receptors in these enkephalin-controlled behaviours. A similar reversal by NTI of the RB 101-induced antidepressant effect was found in the conditioned suppression of motility and swimming despair tests in mice (Baamonde et al., 1992). The antidepressant-like effects induced by the selective δ-opioid agonist BUBU in the learned helplessness model also corroborated the participation of δ-opioid receptors. Previous studies have also shown a preferential involvement of δ-opioid receptors in the behavioural responses induced in rats by kelatorphan, another mixed inhibitor of the enkephalin catabolism, microinjected into the mesolimbic and nigrostriatal pathways of rats (Daugé et al., 1988; Maldonado et al., 1990; Calenco-Choukroun et al., 1991) The presence of a high concentration of δ-opioid receptors in brain areas involved in motor/motivational control is consistent with the pharmacological effects induced by administration of δ-opioid selective compounds. Thus, a high density of δ-opioid receptors has been found in the cortex, neostriatum and nucleus accumbens (Delay-Goyet et al., 1990), brain areas preferentially involved in behavioural and motivational responses (Mogenson et al., 1980; Kalivas et al., 1983; Daugé et al., 1988). δ-Opioid receptors in these areas seem to act by modulating

dopaminergic systems (Petit et al., 1986; Waksman et al., 1987). Stimulation of δ-opioid receptors in the nucleus accumbens exerts a powerful facilitation of dopaminergic transmission, which motor stimulant effects are mediated by dopamine D_1 receptors (Longoni et al., 1991).

In addition, δ-opioid receptors have been reported to participate in the pharmacological response produced by antidepressant treatments. Thus, chronic administration of amoxapine or amitriptyline induced a reduction in the density of [^{3}H]DSTLE (δ-opioid ligand) binding sites in the hypothalamus, without affecting the characteristics of this opioid receptor in the cerebral cortex, probably as a consequence of an increase in the levels of [Met^5] and [Leu^5] enkephalin (Hamon et al., 1987).

The activation of δ-opioid receptors has been reported to increase locomotion (Gacel et al., 1990). Therefore, the improved behavioral response displayed by BUBU-treated rats could be due, in part, to this motor effect. However, this possibility seems unlikely since the administration of psychostimulants such as amphetamine or caffeine, which induce a strong increase in motor activity, did not reverse learned helplessness in using an experimental procedure similar to this study (Sherman et al., 1982). Some drugs able to increase locomotor activity, such as morphine, were effective to reverse learned helplessness effects. Nevertheless, a non-significant relationship between intertrial interval activity (locomotor activity response) and the reduction in the number of escape failures (learned helplessness response) was found in those cases (Tejedor-Real et al., 1995).

Taken together these results suggest that RB 101 produces an antidepressant-like effect in the learned helplessness model of depression. The ability of endogenous opioids, protected from their catabolism, to reverse escape deficits in rats previously subjected to inescapable shocks seems to be mediated through δ-opioid receptors. Moreover, the effects of endogenous opioids on depression could be related to an interaction with the dopaminergic system, involving preferentially dopamine D_1 receptors. The failure of RB 101 to produce the classical side-effects related to chronic opioid administration (Noble et al., 1992, 1993) demonstrates the potential interest of mixed inhibitors of enkephalin degrading enzymes as new non-addictive antidepressants. Therefore, administration of these compounds with reduced drawbacks could be interesting to treat depressive patients who are unresponsive to or intolerant of, classical antidepressants.

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