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Similar decrease in spontaneous morphine abstinence by methadone and RB 101, an inhibitor of enkephalin catabolism

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1 The dual inhibitor of enkephalin degrading enzymes, RB 101, is able to block endogenous enkephalin metabolism completely, leading to potent antinociceptive responses potentiated by blockade of CCK_B receptors. In this study we have investigated the effects induced by RB 101 given alone, or with the CCK_B antagonist, PD-134,308, on a model of spontaneous morphine withdrawal and substitutive maintenance in rats.

2 Animals were chronically treated with morphine for 7 days followed, 36 h after the interruption of drug administration, by a maintenance treatment for 5 days with methadone (2 mg kg^{-1}, i.p.), clonidine (0.025 mg kg^{-1}, i.p.), RB 101 (40 mg kg^{-1}, i.p.), PD-134,308 (3 mg kg^{-1}, i.p.) or a combination of RB 101 plus PD-134,308. Several behavioural observations were made during this period in order to evaluate the acute effects as well as the consequence of chronic maintenance induced on spontaneous withdrawal by the different treatments.

3 Methadone was the most effective compound in decreasing the spontaneous withdrawal syndrome after acute administration. Both, methadone and RB 101 had similar effectiveness in reducing opiate abstinence during the period of substitutive treatment. PD-134,308 did not show any effect when administered alone and did not modify the effect of RB 101.

4 Naloxone (1 mg kg^{-1}, s.c.) failed to precipitate any sign of withdrawal when injected at the end of the chronic maintenance treatment suggesting that, under the present conditions, methadone and RB 101 did not induce significant physical opiate-dependence.

5 The mildness of the side effects induced by chronic RB 101, suggests that systemically active inhibitors of enkephalin catabolism could represent a promising treatment in the maintenance of opiate addicts.

Keywords: Spontaneous morphine withdrawal; RB 101; methadone; endogenous enkephalins; clonidine; PD-134,308; CCK_B receptors

Introduction

Drug-dependence is defined as the need for continued drug exposure to avoid a withdrawal syndrome, which is characterized by physical and/or psychological disturbances when the drug is withdrawn. Molecular and cellular adaptations in specific brain regions occur during opiate exposure in an attempt to maintain homeostasis. Thus, biochemical studies have demonstrated up-regulation of the adenosine 3':5'-cyclic monophosphate (cyclic AMP) system in response to chronic opiate exposure in several regions of the central nervous system (Makman *et al.*, 1988; Crain & Shen, 1990; Terwilliger *et al.*, 1991). Similarly, chronic opiate treatment increases levels of the active subunits of G-proteins (Nestler *et al.*, 1989), cyclic AMP-dependent protein kinase (Nestler & Tallman, 1988), and a number of cyclic AMP-regulated phosphoproteins such as tyrosine-hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines (Guitart *et al.*, 1990). These adaptations are responsible for electrophysiological and behavioural disturbances during opiate withdrawal (review in Nestler, 1992). One way to restore the homeostatic state is to re-establish the level of opioid receptor occupancy by exogenous or endogenous agonists. Accordingly, the opioid agonist, methadone, has been extensively used as substitute treatment in the rehabilitation of opiate addicts, but it maintains a degree of opiate dependence (review in O'Brien, 1993). A novel and more physiological approach to the treatment of the opioid dependence consists of enhancing the effects of the endogenous opioid system by protecting the natural opioid peptides, enkephalins, from their catabolism (review in Roques & Noble, 1995). This latter process is ensured *in vivo* by two zinc ectopeptidases, neutral endopeptidase 24.11 (NEP) (Malfroy *et al.*, 1978) and aminopeptidase N (APN) (Hambrook *et al.*, 1976; Waksman *et al.*, 1985). The appartenance of both enzymes to the same group of metallopeptidases has resulted in the concept of dual inhibitors, able to inhibit completely enkephalin inactivation (Fournié-Zaluski *et al.*, 1984; Waksman *et al.*, 1985; Bourgoin *et al.*, 1986; review in Roques *et al.*, 1993). A potent attenuation of the withdrawal syndrome was observed after central administration of dual inhibitors, such as kelatorphan or RB 38A (Maldonado *et al.*, 1989). As expected, the administration of selective inhibitors of NEP produce a weaker, but significant, reduction in the severity of naloxone-precipitated abstinence (Dzoljic *et al.*, 1986; 1992; Haffmans & Dzoljic, 1987; Livingston *et al.*, 1988). Recently RB 101, a new dual inhibitor of enkephalin catabolism, able to cross the blood brain barrier, has been synthesized (Fournié-Zaluski *et al.*, 1992). This compound induces potent antinociceptive responses after systemic administration (Noble *et al.*, 1992) and almost completely eliminates the behavioural expression of naloxone-precipitated morphine withdrawal in rats (Maldonado *et al.*, 1995).

On the other hand, the peptide cholecystokinin (CCK) has been shown to play an opposite role to that of opioids in the control of several central nervous system functions. Thus, selective antagonists of the CCK_B receptor, which is the predominant form in the central nervous system (Moran *et al.*, 1986), were reported to facilitate the antinociceptive responses induced by morphine (Panerai *et al.*, 1987; Dourish *et al.*, 1990; Xu *et al.*, 1992) or by endogenous opioids protected from inactivating enzymes by RB 101 (Maldonado *et al.*, 1993; Noble *et al.*, 1993; Valverde *et al.*, 1994).

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Currently the most common method for studying physical opiate dependence in animals is to precipitate a withdrawal syndrome by administration of an opiate antagonist. This procedure was previously used to study the effects of the inhibitors of enkephalin catabolism and their interaction with selective CCK_B antagonists such as PD-134,308 in opiate-dependence (Maldonado *et al.*, 1989; 1995). However, in a clinical situation, this syndrome is usually found when opiate addicts interrupt drug consumption. Therefore, we have developed in this study an experimental model of spontaneous opiate abstinence and substitutive maintenance treatment in rats allowing the responses triggered by a spontaneous morphine abstinence to be evaluated. This was achieved by disrupting the opiate agonist administration and by studying the behaviour of the rats submitted to different treatments. In these conditions, closer to those encountered with opiate addicts, the effects of RB 101 given alone or associated with the CCK_B antagonist, PD-134,308, were evaluated. A procedure allowing the spontaneous morphine abstinence undergone by the same rat to be evaluated at different times was developed. This experimental model was then used to evaluate the effects induced by RB 101, with or without the CCK_B antagonist, and to compare the responses to those produced by compounds currently used to reduce opiate dependence in human subjects, such as clonidine and methadone.

Methods

Animals

Male albino Sprague-Dawley rats (Depré), ranging in weight from 200 to 220 g at the start of the experiments, were used in this study. Animals were placed in cages with water and food available *ad libitum*, and maintained in a controlled environment.

Drugs

Morphine HCl was obtained from Francopia. Methadone HCl, naloxone HCl, and clonidine HCl were purchased from Sigma laboratories. RB 101, the dual inhibitor of enkephalin-degrading-enzymes (Fournié-Zaluski *et al.*, 1992), and PD-134,308 ([R-(R*,R*)]-4-[[2-[[3-(1H-indol-3-yl)2-methyl-1-oxo-2-[[(trycyclo[3.3.1.1.^{37}]dec-2-yloxy)carbonyl]amino]propyl]-amino-1-phemylethyl]amino]-4-oxobutanoic acid), the CCK_B antagonist (Hughes *et al.*, 1990), were synthesized in our laboratory as described. All the drugs were administered intraperitoneally (i.p.) in a volume of 1 ml kg^{-1}, except naloxone which was injected subcutaneously (s.c.) in the same volume. The doses of RB 101 (N-[(R,S)-2-benzyl-3[(S)(2-amino-4-methyl-thio)butyldithio]-1-oxo-propyl]-L-phemyl-alamine benzyl ester) (40 mg kg^{-1}, i.p.) and PD-134,308 (3 mg kg^{-1}, i.p.) were selected from a previous study aimed at measuring their antinociceptive responses on the rat tail-flick test (Valverde *et al.*, 1994) and their effects on naloxone-precipitated morphine withdrawal syndrome (Maldonado *et al.*, 1995).

Induction of physical dependence and spontaneous morphine withdrawal

Rats were divided into different groups ($11 < n < 17$, per group) corresponding to morphine treated and saline control groups. The protocol used to induce morphine dependence and to evaluate spontaneous withdrawal is summarized in Figure 1.

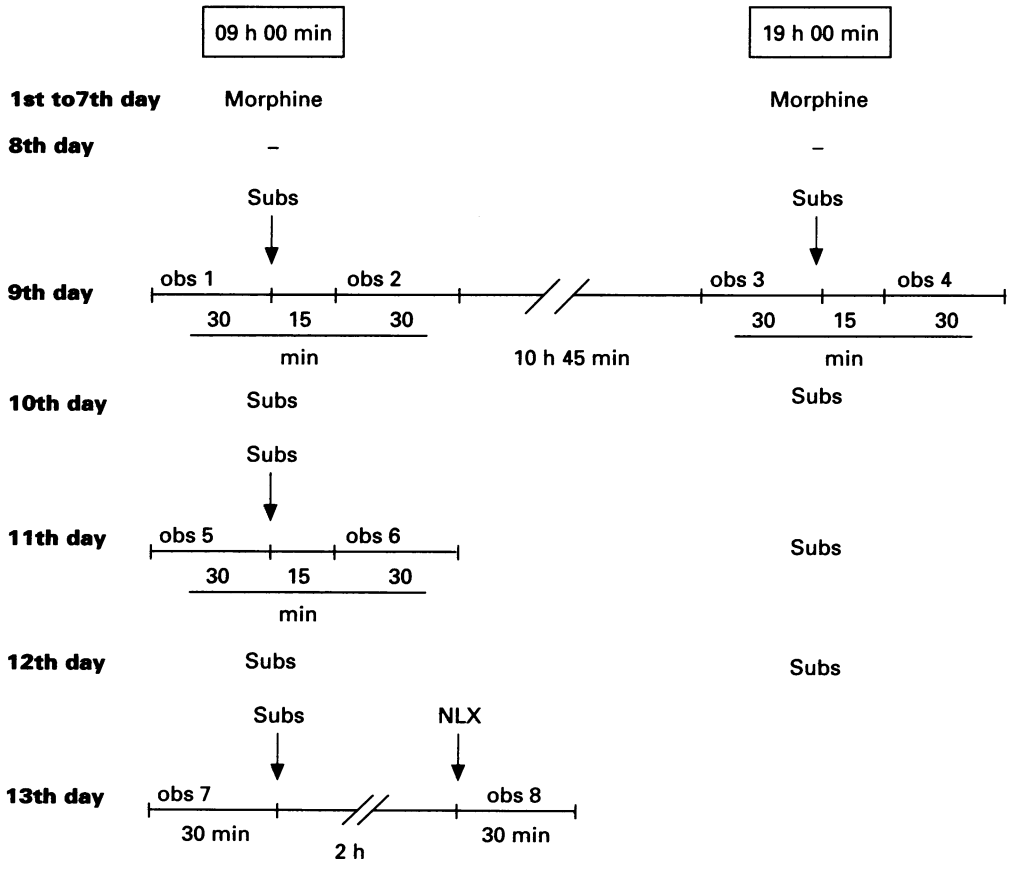

Figure 1 Experimental protocol used to induce morphine-dependence (days 1st to 7th), and to evaluate the effects of different substitutive treatments on spontaneous morphine abstinence (days 9th to 13th). Morphine = morphine injection, NLX = naloxone injection, obs = observation session, Subs = substitutive treatment administration: saline, methadone (2 mg kg^{-1}), clonidine (0.025 mg kg^{-1}), PD-134,308 (3 mg kg^{-1}), RB 101 (40 mg kg^{-1}), or PD-134,308 plus RB 101.

Briefly, saline and morphine were injected i.p. twice daily. The morphine dose was progressively increased from 7 mg kg^{-1} to 50 mg kg^{-1} over a period of 7 days. The first and second number inside the parentheses represent the dose of morphine (mg kg^{-1}) injected at 09 h 00 min and 19 h 00 min, respectively on consecutive days: 1st day (7, 10), 2nd day (15, 20), 3rd day (25, 30), 4th day (35, 40), 5th day (45, 50), 6th day (50, 50) and 7th day (50, 50). Morphine treatment was disrupted on day 7 in order to induce a spontaneous opiate abstinence. In a preliminary study, the withdrawal syndrome was evaluated at different time points (24, 36 and 48 h) after the disruption of morphine administration. The maximum severity of abstinence was observed 36 h after the last dose of morphine. The first observation of the withdrawal syndrome was therefore performed at this time.

To evaluate the withdrawal syndrome, the rats were placed individually into test chambers consisting of round boxes (30 cm diameter × 35 cm height). Several behavioural signs of morphine abstinence were evaluated during a 30 min period. The number of bouts of teeth chattering, mastication, wet dog shakes, yawning and paw tremor were counted. Ptosis, eye twitch, piloerection and diarrhoea were evaluated over 2.5 min periods with one point being given for the presence of each sign during each period, and the number of periods showing the sign were then counted (highest score = 12). Body weight was determined before and after each observation.

The protocol used in this study to evaluate the spontaneous morphine abstinence was first validated (Figure 1). For this purpose, the withdrawal syndrome was observed at six different time points. The first observation was made 36 h after the interruption of chronic saline or morphine treatment. Rats were injected with saline at the end of this observation and 15 min later were observed for a second time. The same procedure was repeated 12 and 48 h later (48 and 84 h respectively after disruption of chronic treatment) in order to perform the third, fourth, fifth and sixth observations.

The evolution of the withdrawal syndrome under the influence of different substitutive treatments was then studied for five days (Figure 1). A total number of eight observations were carried out. The four first observations were made on the ninth day, using the same procedure as in the first experiment. The fifth and sixth observations were made on the 11th day, before and 15 min after the substitutive treatment, respectively. Finally, the seventh and eighth observations were carried out on the 13th day, before and 2 h after the substitutive treatment. An injection of naloxone (1 mg kg^{-1}, s.c.) was made immediately before the eighth observation, to evaluate the possible presence of a residual withdrawal syndrome induced by the different chronic substitutive treatments.

In order to summarize the results obtained from the different observations, a global withdrawal score was individually calculated for each rat. To obtain this global value and to give all the signs a proportional weighting, the score obtained from each sign was multiplied by a constant (Maldonado *et al.*, 1992). These points were added to obtain the individual global score as previously reported. The value of this global withdrawal score in the first observation session (control observation) was around 11.

Statistical analysis

In the first study, performed to validate the experimental protocol, the values obtained on withdrawal signs and global scores are expressed as absolute values. In the second part of the work, the results of the different observation sessions are expressed as percentages of the values obtained for each different group of treatment in the first session.

In both experiments, individual comparisons between the different groups of animals were made by using a two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were treatment (between subjects) and observation session (within subjects). If a significant effect was observed, one-way ANOVA was used to determine the significance at each point. In the first experiment, post-hoc comparisons were made using Student's unpaired t test in order to determine differences between chronic saline- and morphine-treated groups in each session. In the second experiment, assessment of differences in each session between the saline group and the groups receiving the different treatments were made by means of Dunnett's test between subjects. Newman-Keul's test within subjects was used to evaluate the differences in each group of animals between each one of the six first observation sessions. The last two observations (seventh and eight) were excluded from this comparison, in that they were performed for a different purpose, i.e. to evaluate the development of dependence by the substitutive treatment, and that animals received an additional treatment (naloxone injection) before the last session. Differences between the results observed in each group before and after naloxone (seventh and eighth sessions) were compared by means of Student's paired t test.

Results

Spontaneous withdrawal syndrome validation

In the first experiment, two-way analysis of variance revealed a significant treatment effect for mastication ($F_{(1,23)} = 9.083$, $P < 0.01$), teeth chattering ($F_{(1,23)} = 12.342$, $P < 0.005$), eye twitch ($F_{(1,23)} = 5.775$, $P < 0.05$), ptosis ($F_{(1,23)} = 13.461$, $P < 0.001$) and piloerection ($F_{(1,23)} = 9.276$, $P < 0.01$). A significant effect of time was observed only in the case of mastication ($F_{(5,115)} = 2.780$, $P < 0.05$), teeth chattering ($F_{(5,115)} = 3.168$, $P < 0.01$), and eye twitch ($F_{(5,115)} = 3.551$, $P < 0.005$). One-way ANOVA within subjects revealed that this time-dependent variation was significant in the morphine-treated group (mastication, $F_{(5,80)} = 3.512$, $P < 0.01$; teeth chattering, $F_{(5,80)} = 5.277$, $P < 0.0005$; eye twitch, $F_{(5,80)} = 5.097$, $P < 0.0005$; and piloerection, $F_{(5,80)} = 2.899$, $P < 0.05$), but not in saline-treated animals. Control animals chronically treated with saline showed a low incidence of some behavioural signs of opiate withdrawal. However, morphine-treated animals showed a high frequency of these behavioural signs that was significant, when compared with saline-treated animals in all the cases (Student's t test) (Figure 2).

When the global withdrawal scores were calculated, the expression of the abstinence was more reliable in morphine-treated animals (Figure 3). Thus, two-way ANOVA revealed a significant effect of treatment ($F_{(1,23)} = 21{,}124$, $P < 0.0001$) and time ($F_{(5,115)} = 5.051$, $P < 0.005$). One-way ANOVA within subjects indicated a variation between the different observation sessions in the saline group ($F_{(5,35)} = 2.949$, $P < 0.05$) and in the morphine group ($F_{(5,80)} = 7.278$, $P < 0.0001$). Post-hoc comparisons between subjects (Student's t test) revealed significant differences between saline and morphine groups in all the observation sessions (Figure 3).

Substitutive treatments efficacy

The effects induced by several substitutive treatments on this model of spontaneous morphine abstinence were compared during the different observation sessions in a second experiment. Two-way analysis of variance revealed a significant effect of treatment, time (observation session) and interaction between treatment and time for mastication, eye twitch and ptosis (Table 1). In the case of piloerection, time and treatment were also significant, but no interaction was observed between these two factors. A significant effect of time and interaction, without effect of treatment was revealed in the case of teeth chattering. Two-way analysis of variance of the global withdrawal values showed a significant effect of treatment ($F_{(5,67)} = 3.680$, $P < 0.005$), time ($F_{(5,335)} = 60.250$, $P < 0.0001$) and interaction between treatment and time ($F_{(25,335)} = 3.432$, $P < 0.0001$). One-way ANOVA between subjects revealed a significant effect of the treatment in the second ($F_{(5,67)} = 4.329$,

$P<0.0005$), third ($F_{(5,67)}=6.566$, $P<0.0001$), fourth ($F_{(5,67)}=4.899$, $P<0.001$), fifth ($F_{(5,67)}=4.845$, $P<0.001$) and sixth ($F_{(5,67)}=5.196$, $P<0.0005$) observation sessions. Post-hoc comparisons (Dunnett's test) showed that methadone significantly decreased the severity of withdrawal when compared with the saline group in the third, fourth and sixth sessions; clonidine in the fourth session, and RB 101 in the third, fourth, fifth and sixth sessions (Figure 4). When PD-134,308 was administered alone no significant effect, compared to the saline group, was observed in any session, and its association with RB 101 did not potentiate the antiwithdrawal effect. One-way ANOVA within subjects revealed a decrease in the expression of morphine abstinence with all the treatments: saline ($F_{(5,80)}=7.257$, $P<0.0001$), clonidine ($F_{(5,50)}=21.139$, $P<0.0001$), methadone ($F_{(5,55)}=25.248$, $P<0.0001$), PD-134,308 ($F_{(5,50)}=7.377$, $P<0.0001$), RB 101 ($F_{(5,50)}=18.285$,

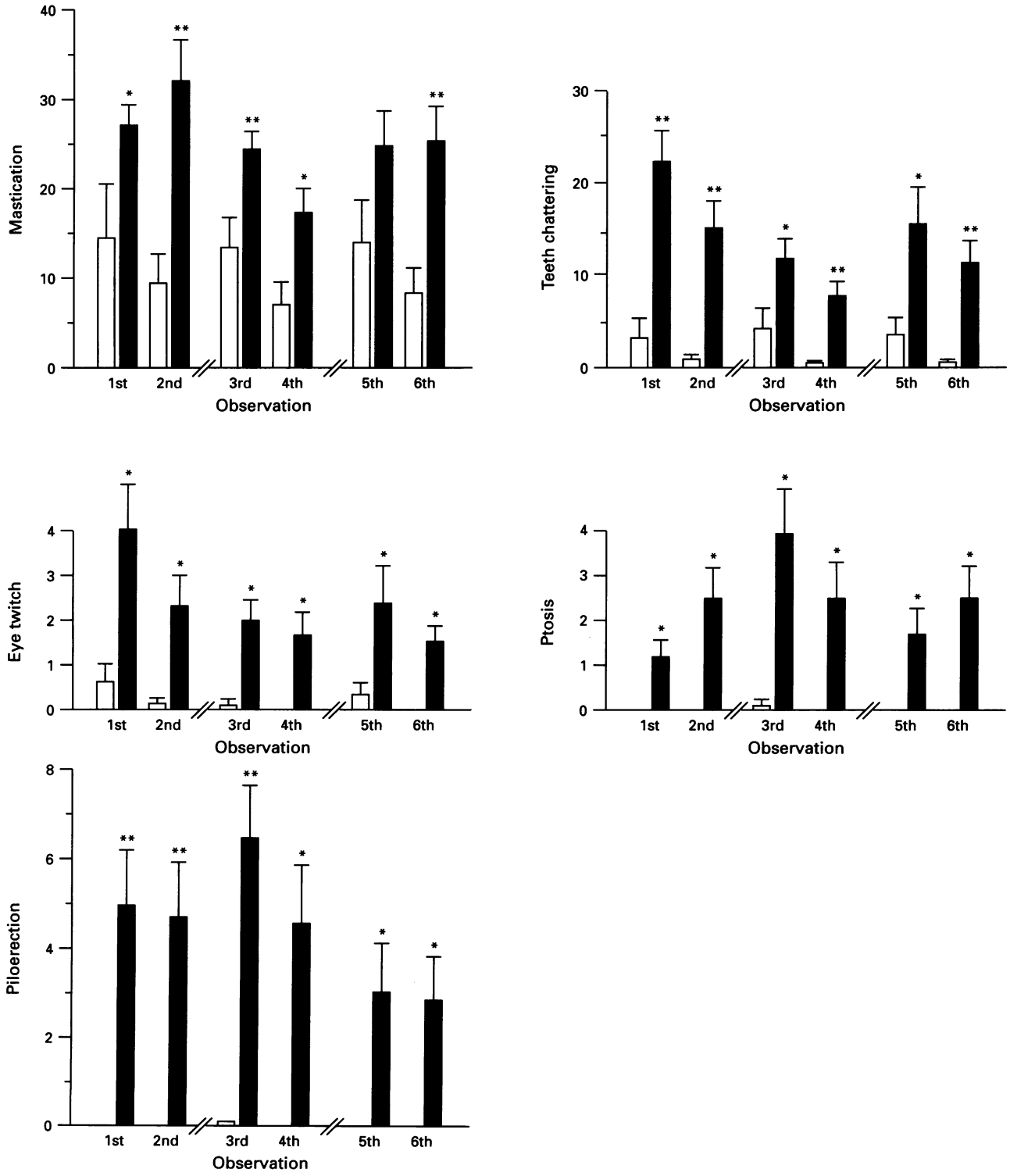

Figure 2 Somatic signs of spontaneous morphine abstinence (mastication, teeth chattering, eye twitch, ptosis and piloerection) in animals chronically treated with saline (open column) and morphine (solid column) during the six observation sessions. First, third and fifth observations were performed 36, 48 and 84 h respectively after the disruption of chronic treatment. Second, fourth and sixth observations were made 15 min after the former session and acute saline administration. Values are means ± s.e.mean. $n=8$ animals in saline and 17 in morphine-treated group. $^{*}P<0.05$; $^{**}P<0.01$ vs saline group (Student's unpaired t test).

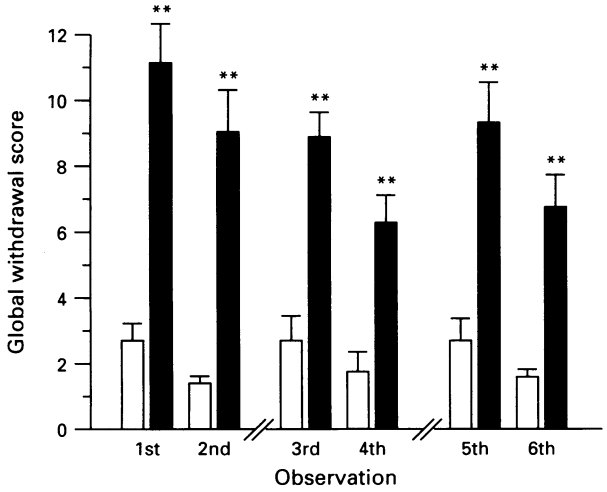

Figure 3 Global withdrawal scores of spontaneous morphine abstinence in animals chronically treated with saline (open column) and morphine (solid column) during the six observation sessions. First, third and fifth observations were performed 36, 48 and 84 h respectively after the disruption of chronic treatment. Second, fourth and sixth observations were made 15 min after the former session and acute saline administration. Values are means ± s.e.mean. $n=8$ animals in saline and 17 in morphine-treated group. $^{**}P<0.01$ vs saline group. (Student's unpaired t test).

Table 1 Summary of two-way ANOVA of different signs of withdrawal syndrome during chronic substitutive treatment: the factors of variation were treatment (t) and observation session (o)

	ANOVA F	P
Mastication		
(t)	(5,67) = 2.43	< 0.05
(o)	(5.335) = 28.34	< 0.0001
(t-o)	(25,335) = 3.46	< 0.0001
Teeth chattering		
(t)	(5,67) = 2.18	NS
(o)	(5,335) = 44.12	< 0.0001
(t-o)	(25,335) = 3.13	< 0.0001
Eye twitch		
(t)	(5.67) = 2.70	< 0.05
(o)	(5,335) = 34.68	< 0.0001
(t-o)	(25,335) = 1.64	< 0.05
Ptosis		
(t)	(5.67) = 2.83	< 0.05
(o)	(5,335) = 5.58	< 0.0001
(t-o)	(25,335) = 1.77	< 0.05
Piloerection		
(t)	(5,67) = 3.88	< 0.005
(o)	(5.335) = 7.42	< 0.0001
(t-o)	(25,335) = 1.50	NS

$P<0.0001$), and RB 101 plus PD-134,308 ($F_{(5,50)}=5.179$, $P<0.001$). Post-hoc comparisons within subjects (Newman-Keuls) showed significant differences for all the treatments between the value obtained in the first observation session and the other sessions. Thus, saline induced a decrease of withdrawal in the fourth and sixth sessions, clonidine and PD-134,308 in the second, fourth and sixth sessions, and methadone, RB 101 and RB 101 plus PD-134,308 decreased morphine abstinence in all the sessions (Figure 4).

Failure of substitutive treatments to induce dependence

In a preliminary experiment, the effect induced by the chronic injection of different doses of methadone (2 and 5 mg kg^{-1}, i.p.) as a substitute treatment was studied by following the same protocol (data not shown). The injection of naloxone (1 mg kg^{-1}, s.c.) at the end of the substitutive treatment (13th day) induced a significant increase in the expression of morphine abstinence only when methadone was administered at the highest dose (5 mg kg^{-1}), revealing the induction of physical dependence in this case (data not shown). Since RB 101 has been shown to be unable to induce dependence in a similar chronic situation (Noble *et al.*, 1992), the dose of methadone that did not develop opiate-dependence (2 mg kg^{-1}) was chosen for the present study.

The injection of naloxone (1 mg kg^{-1}, s.c.) just before the eighth observation session (five days after the beginning of the substitutive treatment) revealed that chronic administration of the different substitutive treatments did not induce dependence. Thus, no significant increase in the expression of the global, withdrawal score was observed in any group of animals (data not shown).

Discussion

The interest of substitutive treatments with methadone or other opiate agonists or partial agonists for heroin addicts have been extensively investigated in clinical conditions (review in O'Brien, 1993). However, no well-defined animal models have been reported, that are able to measure the efficacy of new compounds on this paradigm. Therefore, in this study we have validated a behavioural model in rats, allowing the evaluation of both spontaneous morphine abstinence and the evolution of the withdrawal behaviour during administration of different chronic substitutive treatments. The main advantage of this approach is the possibility of inducing an opiate withdrawal syndrome relatively close to that encountered in human subjects. The protocol used induced a high degree of opiate-dependence, shown by the presence of a spontaneous abstinence when the chronic morphine administration was disrupted. Thus, during the different observation sessions, morphine-treated rats had almost all the behavioural signs of abstinence. However, one of the major signs of opiate withdrawal, the jumping behaviour, did not appear in spontaneous abstinence when using this protocol. In agreement with this observation, jumping is usually not reported when measuring spontaneous abstinence to opiates (Shearman *et al.*, 1980; Van der Laan *et al.*, 1991; Harris & Aston-Jones, 1993). In morphine-dependent mice, jumping increases occurred only when naloxone was given after 2 h, and the response was shown to be maximal at 3 h and declined thereafter (El-Kadi & Shariff, 1994). Another criterion validating the proposed experimental protocol was the strong attenuation of the spontaneous abstinence syndrome when opiate exposure was re-established by administration of the opioid agonist, methadone. In order to reduce the high variability currently found in the evaluation of the individual signs of spontaneous abstinence, a global score was calculated. This global score is a more reliable and constant measure of abstinence, and was used in the second part of the study (Maldonado *et al.*, 1992).

The administration of saline to control animals decreased the expression of the spontaneous abstinence in all the experiments. This response was presumably due to a conditioned effect induced by injection. In agreement with this hypothesis, previous studies showed that after a sufficient number of pairings with morphine injection, a simple neutral environmental stimulus was able to produce behavioural effects resembling those of morphine itself in blocking the disruptive withdrawal syndrome (Tye & Iversen, 1975). In our experiments, it is possible that the repeated injection of morphine led to the development of a conditioned response. When the animal receives a vehicle injection under the same conditions and by the same route (i.p.) as the opiate agonist, this stimulus associated with the rewarding effects of morphine could be enough to produce an abstinence decrease. The relevance of these conditioned stimuli in opiate-dependence has been sug-

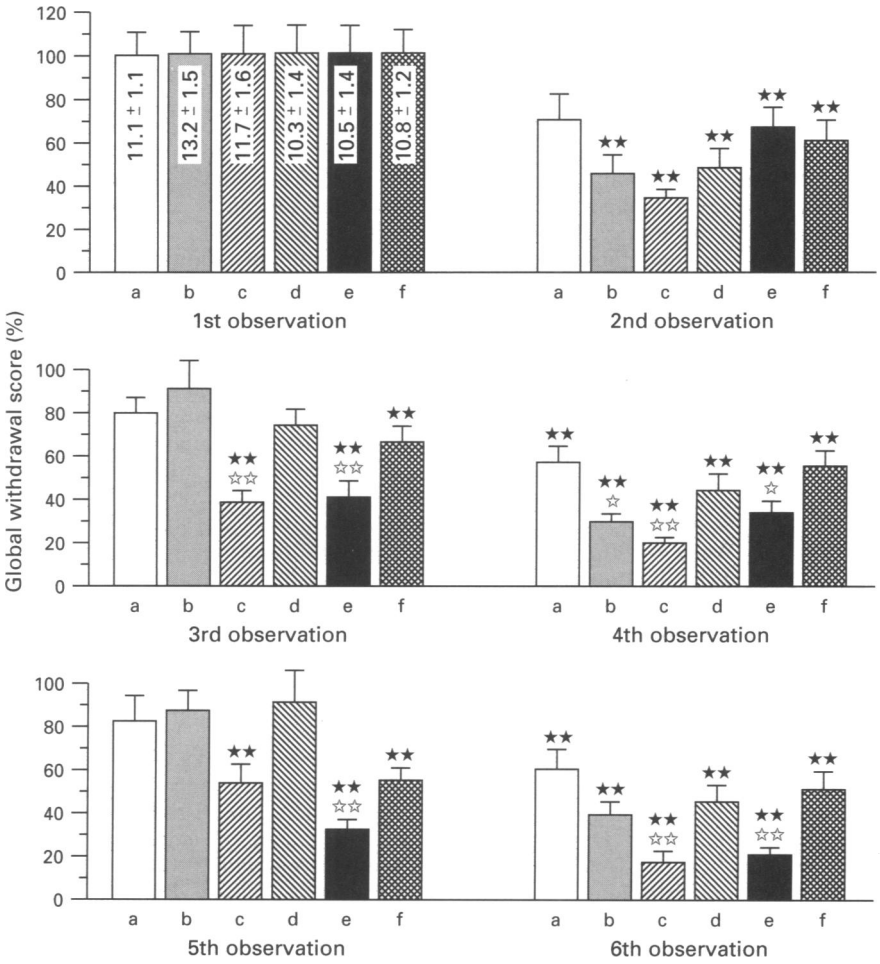

Figure 4 Global withdrawal score of spontaneous morphine abstinence. Effects of chronic treatment, twice a day, with saline (a), clonidine (0.025 mg kg^{-1}) (b), methadone (2 mg kg^{-1}) (c), PD-134,308 (3 mg kg^{-1}) (d), RB 101 (40 mg kg^{-1}) (e) and RB 101 plus PD-134,308 (f) on spontaneous abstinence. Values (percentages of the first session) are means ± s.e.mean. Numbers inside the columns of the first session express absolute values of withdrawal syndrome score. $n = 11$ in all the different treatment groups except saline ($n = 17$). ★★$P < 0.01$ vs value of the same group in the first session (Newman-Keuls test). ☆$P < 0.05$; ☆☆$P < 0.01$ vs value of saline in the same session (Dunnett's test).

gested previously in studies using different behavioural models of drug abuse. Thus, the drug-seeking behaviour seems to be maintained, at least in part, by conditioned stimuli associated with the positive reinforcing effects produced by drug ingestion (Stewart *et al.*, 1984; Wise & Bozarth, 1987). However, the conditioned response obtained in the present study was significantly less intense than that induced by several of the compounds tested.

The effect induced by the new mixed inhibitor of enkephalin catabolism, RB 101, was studied on this model of spontaneous abstinence. RB 101 induced a decrease in the expression of morphine withdrawal that was significant in the third, fourth, fifth and sixth sessions when compared to the saline group. It has been previously reported that central (Dzoljic *et al.*, 1986; Haffmans & Dzoljic, 1987) or peripheral (Livingston *et al.*, 1988; Dzoljic *et al.*, 1992) administration of selective inhibitors of NEP reduce the severity of naloxone-precipitated withdrawal. As expected, a more intense inhibition of this precipitated withdrawal syndrome was observed after central administration of the complete inhibitors of enkephalin catabolism, kelatorphan and RB 38A (Maldonado *et al.*, 1989), and after peripheral administration of RB 101 (Maldonado *et al.*, 1995). The decrease in the severity of morphine abstinence produced by the inhibitors is very likely due to increase in the extracellular levels of enkephalins (Bourgoin *et al.*, 1986). In agreement with this, the systemic administration of RB 101 inhibited [^{3}H]-diprenorphine binding *in vivo* (Ruiz-Gayo *et al.*, 1992). Several non-opioid peptides, such as substance P, cholecystokinin and neurotensin, are also degraded by NEP and/or APN *in vitro* (review in Roques *et al.*, 1993). Nevertheless, these peptides do not seem to be implicated in the decrease of the withdrawal syndrome produced by RB 101. Indeed, the extracellular levels of substance P (Yaksh *et al.*, 1991) and CCK (Butcher *et al.*, 1989) in the central nervous system did not change after the administration of neutral endopeptidase and aminopeptidase N inhibitors. Moreover, the effects of these peptides are usually opposite to those of opioids (Faris *et al.*, 1983; Kalivas *et al.*, 1984; Vaught, 1988).

The selective CCK_B antagonist, PD-134,308, did not modify the spontaneous morphine abstinence when administered alone. This result supports previous findings showing that acute administration of PD-134,308 (Maldonado *et al.*, 1995) or chronic treatment with different CCK_B antagonists (Panerai *et al.*, 1987; Dourish *et al.*, 1988; 1990; Xu *et al.*, 1992) did not modify naloxone-precipitated morphine withdrawal. In addiction, peripheral (Pournaghash & Riley, 1991) or central (Maldonado *et al.*, 1994) administration of CCK did not precipitate any sign of withdrawal in morphine-dependent rats. Taken together, these data suggest that CCK plays a minor role in the development and expression of morphine physical dependence.

In contrast to previous results observed in different pharmacological tests, PD-134,308 did not improve the effect of RB 101 in decreasing spontaneous morphine abstinence. CCK_B

antagonists have been previously reported to facilitate several pharmacological responses induced by the endogenous enkephalins, such as the antinociceptive (Noble *et al.*, 1993; Valverde *et al.*, 1994) and 'antidepressant-like' effects of RB 101 (Smadja *et al.*, 1995). A similar facilitatory response by CCK_B antagonists on RB 101-induced reduction in the severity of naloxone-precipitated morphine withdrawal has also been observed (Maldonado *et al.*, 1995). Two different mechanisms have been proposed to explain these facilitatory effects (Noble *et al.*, 1993). First, activation of CCK_B receptors could reduce the levels of endogenous enkephalins released in the extracellular space, while the reverse situation could occur by stimulation of CCK_A binding sites (Cesselin *et al.*, 1984; Hagino *et al.*, 1991; Noble *et al.*, 1994). Similarly, CCK_B antagonists might increase the release of endogenous enkephalins in some brain areas implicated in the pharmacological responses of opiates (Millington *et al.*, 1992). The second possibility is that CCK peptides could produce allosteric changes in the opioid receptors leading to a postreceptor change which counters the opioid effector system. Indeed, CCK_8 modifies the binding of μ opioid agonists to their receptors (Wang & Han, 1990). CCK_B antagonists could selectively modify the transduction mechanisms related to μ opioid receptors which are the most important in the development of opiate-dependence (Cowan *et al.*, 1988; Maldonado *et al.*, 1992). Chronic stimulation of opioid receptors by morphine has been suggested to induce a progressive compensatory increase in the activity of the central CCK system (Panerai *et al.*, 1987; Dourish *et al.*, 1988; 1990). Accordingly, increases of CCK mRNA in different brain areas following chronic administration of morphine have been shown, suggesting an increased rate of CCK biosynthesis in the central nervous system (Zhou *et al.*, 1992; Ding & Bayer, 1993). Consequently, in the case of naloxone-precipitated withdrawal syndrome, the acute administration of the opioid antagonist could immediately induce a phasic release of endogenous CCK that would participate in the expression of morphine withdrawal. The administration of a CCK_B antagonist would be able to antagonize this effect, and consequently to potentiate the anti-withdrawal responses of RB 101. In contrast, this phasic release of CCK would not appear during the spontaneous morphine abstinence. Under these conditions, the CCK_B antagonist would be unable to potentiate the responses of RB 101. In agreement with this hypothesis, the expression of spontaneous morphine abstinence was less severe than naloxone precipitated withdrawal, suggesting that probably other physiological mechanisms are involved, following naloxone administration.

Compounds classically used for clinical treatment of opiate withdrawal, such as clonidine and methadone, were also investigated using the present model of spontaneous abstinence in order to compare their effects with those of RB 101. Methadone is the opiate agonist most currently used for maintenance treatment, and the α_2-adrenoceptor agonist, clonidine, is the most effective non-opioid drug for improving some aspects of opiate withdrawal (review in O'Brien, 1993). In the present experimental model, clonidine was able to decrease the severity of spontaneous abstinence only after its acute administration (second, fourth and sixth sessions), and this effect was significant only during the fourth observation session when compared to the saline group. No modification of withdrawal was found in the observation sessions performed immediately before the next substitutive administration (third and fifth sessions). These findings are in agreement with previous results showing that clonidine does not modify the locomotor activity responses induced by spontaneous morphine withdrawal when administered twice daily during the abstinence period (Van der Laan & De Groot, 1988). The limited effect of clonidine on spontaneous abstinence could be due, at least in part, to the short duration of action (3–6 h), and the short half-life (less than 1 h) (Conway & Jarrot, 1980) of this compound in rats, leading to recurrence of withdrawal symptoms between the injections. The anti-withdrawal effects of clonidine have been reported to be due to the inhibition of the hyperactivity of the noradrenergic system that occurs during the opiate withdrawal syndrome (Aghajanian, 1978). However, some controversial results have been found, suggesting that other mechanisms, other than α_2-adrenoceptor stimulation, and possibly without involvement of the noradrenergic system, could be involved in the anti-withdrawal effects of clonidine (Tseng *et al.*, 1975; Esposito *et al.*, 1987; Gonzalvez *et al.*, 1994).

The administration of the opiate agonist, methadone, decreased spontaneous abstinence symptoms in all the observation sessions. Although the effect was significant only in the third, fourth and sixth sessions when compared to the saline group, methadone proved to be an efficient treatment, not only following drug administration but also during the total period of maintenance treatment. In agreement with this result, methadone has been reported to induce a long-lasting effect and to be able to block withdrawal symptoms during 24–36 h in human subjects (review in O'Brien, 1993). The dose of methadone used in this study (2 mg kg^{-1}) was unable to induce a significant degree of dependence after chronic administration, as shown by the absence of modification in the expression of abstinence after naloxone injection at the end of the treatment (eighth session). This allows a reliable comparison with RB 101, since the chronic administration of the peptidase inhibitor did not induce opiate-dependence under the present conditions, in agreement with previous findings (review in Roques *et al.*, 1993). However, when methadone was administered at a higher dose (5 mg kg^{-1}), a significant development of opiate dependence occurred at the end of the chronic administration, during the eighth session (data not shown).

The responses induced by RB 101 in this model of spontaneous withdrawal were similar to those induced by methadone. Indeed, RB 101 decreased opiate abstinence in all the observation sessions and this effect was particularly strong in the sessions performed before the substitutive administration (third and fifth sessions). The duration of the acute pharmacological effects of RB 101 in antinociceptive and behavioural tests was relatively short (Noble *et al.*, 1992; Baamonde *et al.*, 1992). Therefore, the responses induced by RB 101 in sessions performed 12 h after its last administration could be due to an effective interruption of the adaptive changes underlying the expression of opiate abstinence as a consequence of the potentiation of the endogenous opioid system induced by the peptidase inhibitor. This phenomenon probably did not occur when administering a compound such as clonidine, that decreases the expression of opiate withdrawal by a heterologous mechanism without directly acting on the endogenous opioid system, i.e., by decreasing noradrenergic hyperactivity. Such heterologous mechanisms are probably efficient in alleviating the symptomatology of opiate withdrawal but not in modifying the intrinsic neural mechanisms involved in the maintenance of the withdrawal response. Interestingly, the chronic maintenance with RB 101 or methadone not only avoided the manifestation of the spontaneous withdrawal but also removed the previous state of opiate dependence, since no signs of withdrawal were observed when naloxone was injected 2 h after the last substitute injection. This effectiveness in decreasing withdrawal symptoms, not only after its acute administration but also under maintenance circumstances points to the possible usefulness of RB 101 in the control of opiate-dependence (review in Roques & Noble, 1995).

In conclusion, we have validated a new protocol for studying morphine physical dependence in rats, where the animals are exposed to conditions of spontaneous opiate abstinence and substitutive treatment comparable to those currently found in human heroin addicts. RB 101 showed an effectiveness similar to methadone in inhibiting the expression of morphine withdrawal symptoms. These responses were not facilitated by the administration of a CCK_B antagonist. Taken together, these findings and those previously reporting a lack of major side-effects after chronic administration of RB

101 (review in Roques *et al.*, 1993), indicate for this compound a promising perspective in the management of opiate withdrawal syndrome in human subjects. RB 101 could be particularly interesting as a therapeutic alternative in the maintenance of opiate addicts, specially to avoid methadone-dependence risk.

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