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Effects of rimonabant on the development of single dose-induced behavioral sensitization to ethanol, morphine and cocaine in mice

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ABSTRACT

Rationale: The endocannabinoid system has been implicated in the neurobiological mechanism underlying drug addiction, especially the primary rewarding dopamine-dependent processes. Therefore, endocannabinoid receptor antagonists, such as the CB1 cannabinoid antagonist rimonabant, have been proposed as candidates for preventive addiction therapies.
Objectives: Investigate the possible involvement of CB1 receptors in the development of behavioral sensitization to ethanol, morphine and cocaine in mice.
Methods: We compared the effects of different doses of rimonabant (0.3, 1, 3 and 10 mg/kg) on spontaneous locomotor activity in the open-field, hyperlocomotion induced by acute administration of ethanol (1.8 g/kg), morphine (20 mg/kg) or cocaine (10 mg/kg) and on subsequent drug-induced locomotor sensitization using a two-injection protocol in mice. We also investigated a possible depressive-like effect of an acute rimonabant challenge at the highest dose and its potential anxiogenic property.
Results: At the highest dose, rimonabant abolished ethanol- and cocaine-induced hyperlocomotion and behavioral sensitization without modifying spontaneous and central locomotor activity or inducing depressive-like behavior on the forced swim test in mice. The other doses of rimonabant also selectively blocked acute ethanol-induced central hyperlocomotion. Although rimonabant at 0.3 and 1 mg/kg potentiated the central hyperlocomotion induced by acute morphine injection, it was effective in attenuating morphine-induced behavioral sensitization at all doses.
Conclusions: Because the neural basis of behavioral sensitization has been proposed to correspond to some components of addiction, our findings indicate that the endocannabinoid system might be involved in ethanol, cocaine and morphine abuse.

1. Introduction

Psychostimulants and other drugs of abuse, such as opiates and ethanol, induce behavioral sensitization in rodents (De Vries et al., 1998; Didone et al., 2008; Masur et al., 1986; Piazza et al., 1990; Robinson and Becker, 1986), an increased behavioral response to the drug after its repeated presentation (Kalivas and Stewart, 1991; Robinson and Becker, 1986). Studies in rats and mice show that even a single exposure to drugs of abuse can induce behavioral sensitization, a model that is less influenced by variables that complicate the interpretation of behavioral responses in multiple drug exposure protocols. Indeed, a single injection of cocaine (Valjent et al., 2010), amphetamine (Chinen et al., 2006; Frussa-Filho et al., 2004), morphine (Valjent et al., 2010; Vanderschuren et al., 2001) or ethanol (Fukushiro et al., 2010) enhances the locomotor stimulation produced by subsequent injection of the respective drug given hours, days or weeks later, which is potentiated when the locomotor-stimulating effect of the priming injection is paired with the test environment (Chinen et al., 2006).

As shown by Valjent et al. (2010), the two-injection protocol of behavioral sensitization provides an excellent model for investigating

Abbreviations: Sal, saline; Veh, vehicle; Rim, rimonabant; Eth, ethanol; Mor, morphine; Coc, cocaine; VTA, ventral tegmental area; NAc, nucleus accumbens; GABA, gamma-aminobutyric acid.

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[1] The first two authors equally contributed to this study.
[2] This paper is in memory of Dr. Roberto Frussa-Filho, who dedicated his entire life to Science, because a man is alive while his name is still spoken.

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the long-lasting effects of drugs of abuse. Although evidence indicates a dissociation between locomotor sensitization and drug consumption (Ahmed and Cador, 2006; Boyson et al., 2014), the neurocircuitry that underlies behavioral sensitization and relapse to drug seeking behavior is similar in both neurochemistry and neuropharmacology (for a review see Steketee and Kalivas, 2011). Regardless of its exact correlate with human behavior, behavioral sensitization is a reliable physiopathologic model for the study of the mechanisms underlying addiction because the neural changes responsible for this phenomenon may be an important component of drug abuse (Wise and Bozarth, 1987). Of note, in the two-injection protocol, the changes in responsiveness induced by the first psychostimulant administration are revealed by the second administration. As a primary effect, most drugs that are abused by humans increase dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988), which is innervated by neurons from the mesolimbic dopaminergic system, thereby leading to hyperlocomotion in rodents (Einhorn et al., 1988; Ellinwood et al., 2000). A large body of evidence suggests that this system mediates most neuroadaptations related to the behavioral sensitization induced by distinct drugs of abuse (Costa et al., 2007; de Araujo et al., 2009; Henry and White, 1991; Wolf et al., 1994), even in a two-injection protocol (Valjent et al., 2010). Furthermore, the repeated use of addictive drugs produces incremental neuroadaptations in the mesolimbic dopamine system, characterizing drug craving in addicted individuals, which have led to the hypothesis that drug-induced neuroadaptations underlying the phenomenon of behavioral sensitization may play an important role in the induction and maintenance of the compulsive patterns of drug-seeking behaviors that characterize addiction (Robinson and Berridge, 1993).

Several lines of evidence have implicated the endocannabinoid system in behavioral responses to drugs of abuse, especially conditioned drug seeking and relapse (De Vries and Schoffelmeer, 2005; Maldonado et al., 2006). Among the two types of cannabinoid receptors, CB1 and CB2 (Mackie, 2006), CB1 has been suggested as the most important one regarding the events related to drug abuse and dependence. CB1 receptors are densely expressed within the mesolimbic dopamine pathway (Tsou et al., 1998), and they are linked to the rewarding aspects of drugs of abuse (De Vries et al., 2001). In addition, CB1 receptors seem to mediate the expression of cocaine-induced locomotor sensitization (Kupferschmidt et al., 2012). The pharmacological blockade of cannabinoid CB1 receptors by rimonabant, a CB1 receptor antagonist, decreases psychostimulant-induced neurobiological effects, which is paralleled by the inhibition of their behavioral responses (Corbille et al., 2007; Filip et al., 2006; Mereu et al., 2013). However, little is known about the role of the endocannabinoid system and CB1 receptors on acute drug effects and on the development of addiction to other drugs of abuse, such as ethanol and opiates.

The present study aimed to investigate the dose-dependent effects of rimonabant on spontaneous locomotor activity of mice, on hyperlocomotion induced by acute drug administration and on the development of single injection-induced behavioral sensitization produced by three different drugs of abuse: ethanol, cocaine and morphine. Because clinical trials have revealed that rimonabant may induce symptoms of anxiety and depression (Moreira and Crippa, 2009), we also evaluated the possible depressive-like effect of an acute challenge with rimonabant at the highest dose as well as the central and peripheral locomotion frequencies of mice in the open-field under rimonabant effect as a measure of anxiety-like behavior in mice.

2. Materials and methods

2.1. Animals

Three-month-old Swiss EPM-M1 male mice (outbred, raised and maintained in the Center for Development of Experimental Models in Medicine and Biology of UNIFESP) were used. Animals weighing 30–35 g were housed under controlled temperature (22–23 °C) and light (12 h light, 12 h dark; lights on at 6 h 45 a.m.) conditions. Food and water were available *ad libitum* throughout the experiments. Animals were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023), revised in 2011, the EU Directive 2010/63/EU for animal experiments, and the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The Institutional Ethical Committee of UNIFESP approved the experimental procedures under protocol #470/07. The four different experiments were done with separate cohorts of naive animals.

2.2. Drugs

Absolute ethanol (Merck®), cocaine-HCl (Sigma®) and morphine (Sigma®) were diluted in 0.9% saline solution. Rimonabant (Sanofi-Aventis®) was dissolved in Tween 80 and propylene glycol and diluted to the correct concentrations with saline. A solution of saline + 1% Tween 80 + 3% propylene glycol was used as vehicle solution (Veh) for rimonabant. Drugs and vehicle solutions were administered intraperitoneally at 10 ml/kg of body weight. The selected dose range of rimonabant was based on previous literature (Gerdeman et al., 2008; Singh et al., 2004), and the doses of ethanol, cocaine and morphine used in the present study were based on previous studies conducted by our group (Fukushiro et al., 2008; Fukushiro et al., 2012a, 2012b; Procopio-Souza et al., 2011).

2.3. Open-field evaluation

Locomotor activity was measured in an open-field apparatus as described previously (Chinen and Frussa-Filho, 1999). The apparatus consisted of a circular wooden arena (40 cm in diameter and 50 cm high) with an open top and a floor divided into 19 squares. Hand-operated counters were used by an observer who was blind to the treatment to score total (total number of any squares entered), peripheral (number of entries into any floor unit contiguous to the apparatus walls) and central (number of entries into any floor unit not contiguous to the apparatus walls) locomotion frequencies during the 10-min sessions. To ensure inter- and intra-observer reliability, all researchers observed animals from all groups and the same observers were present during all behavioral evaluations of each experiment, observing the same animals on each day. Because cocaine- and amphetamine-induced behavioral sensitization shows a diurnal pattern (Akhisaroglu et al., 2004; Gaytan et al., 2000) and following the protocols previously established in our laboratory (Marinho et al., 2014; Procopio-Souza et al., 2011), all behavioral tests were conducted in the same period of the day, during the light phase of the cycle (2 h 00 p.m. to 5 h 00 p.m.).

2.4. Forced swim test

For the evaluation of a possible depressive-like effect of rimonabant at a high dose, mice were placed individually in a cylindrical glass container (30 cm height, 16 cm diameter, 11 cm of water depth, 23 °C) for 6 min. The duration of immobility was manually scored during the last 4 min by observers who were blind to the manipulation applied. A mouse was considered immobile when it floated in an upright position and made only small movements to keep its head above water.

2.5. Experimental procedure

2.5.1. Experiments I to III: effects of acute rimonabant administration on spontaneous locomotor activity, hyperlocomotion and behavioral sensitization induced by ethanol, morphine and cocaine

The experimental design was performed according to the model developed by our group (Marinho et al., 2014). For the first experiment, 70 mice were exposed to the open-field apparatus for 2 consecutive days

for habituation. All animals received saline (Sal) during the habituation sessions, and they were placed individually in the apparatus 30 min after injection. The total locomotion frequency was quantified during the 2nd exposure. On the 3rd day (24 h after the 2nd habituation session), mice were allocated to 5 experimental groups based on locomotion frequencies (*i.e.*, groups of mice with similar total locomotion frequencies). Before the session, mice received an i.p. injection of vehicle (Veh1, n = 10; Veh2, n = 10; Veh3, n = 10) or rimonabant (Rim, n = 10 per group/for each dose) at doses of 0.3, 1.0, 3.0 or 10 mg/kg. Animals were exposed to a 10-min open-field session 30 min after the injection, and their total, peripheral and central locomotion frequencies were evaluated. Immediately after behavioral evaluation, or 40 min after the rimonabant injection, 20 vehicle-treated animals received a Sal injection (Veh1–Sal, n = 10; Veh2–Sal, n = 10), and the other 10 vehicle-treated animals and all animals pretreated with Rim received 1.8 g/kg of ethanol (Veh3–Eth, Rim0.3–Eth, Rim1–Eth, Rim3–Eth and Rim10–Eth). Five minutes after Eth administration, mice were re-exposed to the open-field for locomotion quantification.

Seven days after the priming injection, mice were reallocated as follows: half of the saline-treated animals received another Sal injection (Veh1–Sal–Sal, n = 10), and the other half was treated acutely with 1.8 g/kg Eth (Veh2–Sal–Eth, n = 10). All other animals received a second 1.8 g/kg Eth injection. Rimonabant was not administered during the test for sensitization. Five minutes after Eth administration, mice were exposed to a 10-min open-field session and their total, peripheral and central locomotion frequencies were evaluated.

Experiments II and III were performed following the protocol for experiment I. Eth was replaced with morphine (Mor) at the dose of 20 mg/kg in the second experiment (in which the interval between Mor administration and behavioral evaluation was 20 min instead of 5 min) and with cocaine (Coc) at the dose of 10 mg/kg in the third experiment. The time-point between the administration of the drugs and the open-field exposure as well as the total amount of time of the locomotor evaluation sessions were established by previous studies conducted by our group (Berro et al., 2014; Fukushiro et al., 2012a, 2012b; Hollais et al., 2014; Marinho et al., 2014).

The three experiments were done with different animals using the same number mentioned above for each experiment. The experimental design of the three experiments is summarized in Fig. 1.

2.5.2. Experiment IV: effects of 10 mg/kg of rimonabant on depressive-like behavior evaluated by forced swim test in mice

Twenty mice were either treated with vehicle (n = 10) or Rim at a dose of 10 mg/kg (n = 10). Thirty minutes later, all of the animals were subjected to the forced swim test.

2.6. Statistical analysis

Before conducting the parametric tests, all variables were checked for normality (Shapiro–Wilk test) and homogeneity (Levene's test), which validated the use of the parametric test. For Experiments I, II and III, data obtained in each behavioral quantification (in response to the factors Rim treatment or drug treatment) were analyzed using one-way ANOVA. In Experiment IV, unpaired Student t-test was conducted for the analysis of the data obtained in response to the factor Rim treatment. Multiple comparisons were performed using Tukey's post hoc test when necessary. A p value less than 0.05 was considered a statistically significant difference.

3. Results

3.1. Experiment I: effects of acute rimonabant administration on spontaneous locomotor activity, acute ethanol-induced hyperlocomotion and ethanol-induced behavioral sensitization

Analysis of habituation using one-way ANOVA revealed no significant difference between groups (data not shown).

Rimonabant at all doses did not modify total (Fig. 2a), peripheral (Fig. 2b) or central (Fig. 2c) spontaneous locomotor activity compared with the vehicle group. ANOVA and Tukey's post hoc test revealed that acute Eth administration during the priming session increased total locomotion frequency (Fig. 2d, Veh3–Eth > Veh1–Sal), and this effect was abolished by the pre-administration of 10 mg/kg Rim [$F(6,63) = 9.54$; $p < 0.001$]. The peripheral locomotor activity showed a similar pattern of response to Eth and Rim, with Eth potentiating this parameter and pre-injected Rim at the dose of 10 mg/kg inhibiting acute Eth-induced peripheral hyperlocomotion [$F(6,63) = 8.72$; $p < 0.001$] (Fig. 2e). Acute Eth administration also potentiated the central locomotion frequency of mice, an effect that was abolished by Rim at all doses [$F(6,63) = 6.62$; $p < 0.001$] (Fig. 2f).

Mice were previously exposed/habituated to the open-field during the spontaneous locomotion evaluation for the subsequent within-day session on the first ethanol challenge and were re-exposed to the open-field on the test session only 7 days after the first ethanol injection. These different conditions could affect the locomotor activity of mice *per se*. Thus, to avoid an effect of this habituation factor between-sessions, the locomotor frequencies of mice were evaluated within-session, compared to the respective control groups. Mice acutely treated with Eth during the test session displayed increased total locomotion frequency (Fig. 2g, Veh2–Sal–Eth > Veh1–Sal–Sal), and this effect was potentiated in mice previously treated with this drug (Veh3–Eth–Eth > Veh2–Sal–Eth). These results demonstrate the development of behavioral sensitization to the stimulant effect of Eth. Pre-treatment with Rim at the doses of 3 and 10 mg/kg abolished Eth-induced behavioral sensitization [$F(6,63) = 8.54$; $p < 0.001$]. Previous treatment with Rim at the doses of 0.3 and 1 mg/kg also attenuated the development of behavioral sensitization to Eth, because both groups differed neither from the other Rim-treated groups nor from the Veh2–Sal–Eth group in the same experimental day (Fig. 2g). A second injection of Eth one week later also led to the development of peripheral locomotor sensitization (Veh1–Sal–Sal < Veh2–Sal–Eth < Veh3–Eth–Eth), which was abolished by Rim pre-treatment at all doses [$F(6,63) = 7.11$; $p < 0.001$] (Fig. 2h). The central locomotion of mice after a second Eth administration was attenuated by Rim pre-treatment at the doses of 3 and 10 mg/kg, in a pattern similar to that observed in the total locomotion frequency [$F(6,63) = 5.78$; $p < 0.001$] (Fig. 2i).

The results of the total, peripheral and central locomotion frequencies of Experiments I to III are summarized in Table 1 (Rimonabant effects on acute drug-induced hyperlocomotion) and in Table 2 (Rimonabant effects on drug-induced behavioral sensitization in a two-injection protocol).

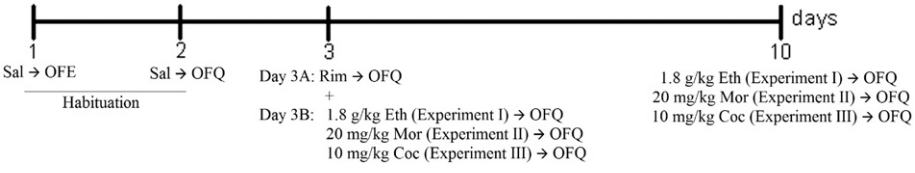

Fig. 1. Experimental design of the study. OFE: open-field exposure; OFQ: open-field quantification; Rim: rimonabant (0.3, 1, 3 or 10 mg/kg); Eth: ethanol; Coc: cocaine; Mor: morphine.

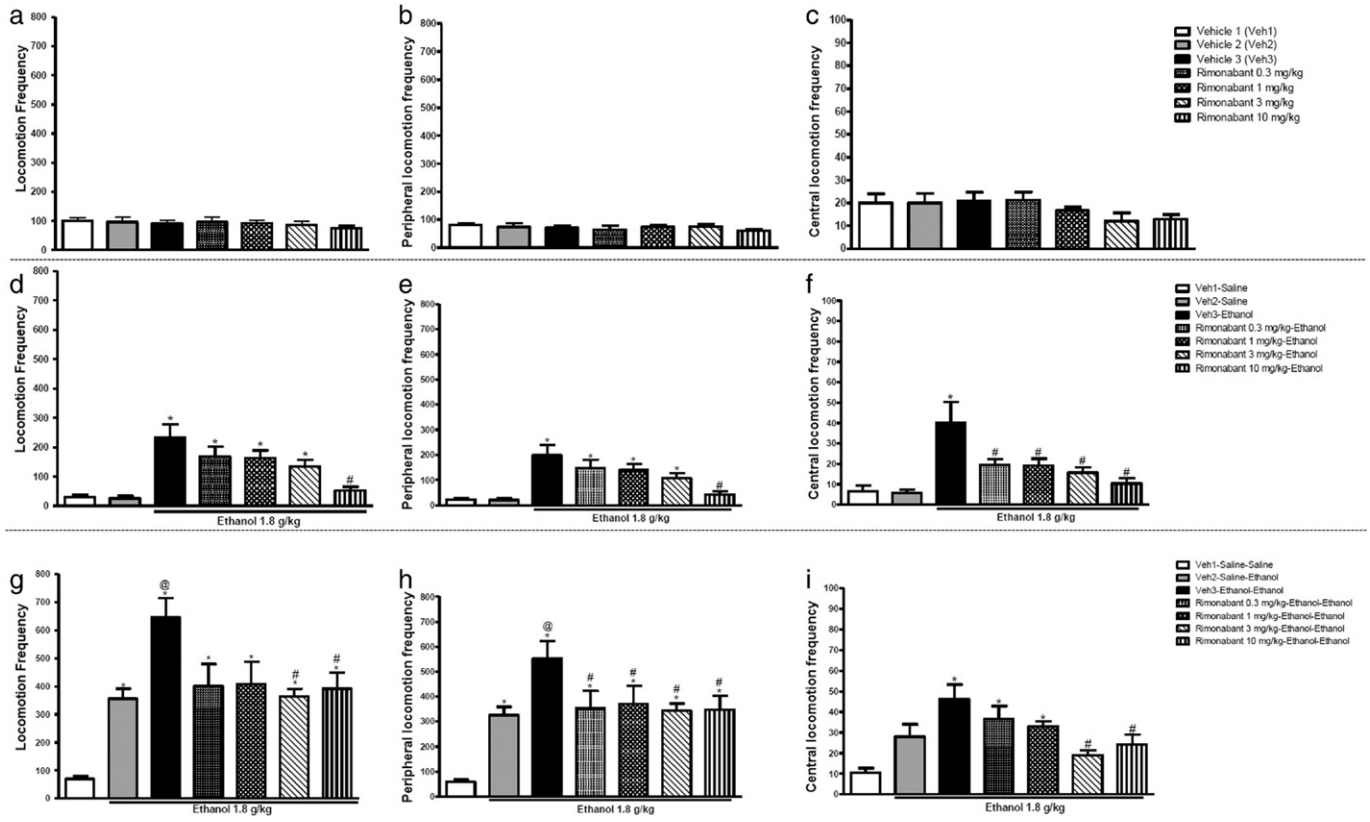

Fig. 2. Effects of i.p. treatment with either rimonabant (0.3, 1, 3 or 10 mg/kg) or vehicle on total (a) spontaneous locomotor activity of mice or in the (b) peripheral (floor units contiguous to the apparatus walls) and (c) central (floor units not contiguous to the apparatus walls) areas of the open-field and its subsequent effects on acute 1.8 g/kg ethanol-induced total (d), peripheral (e) and central (f) hyperlocomotion and total (g), peripheral (h) and central (i) locomotor sensitization after a 7-day interval. Data are reported as the means ± SEM. *$p < 0.05$ compared to Veh1–saline and Veh2–saline ((d), (e) and (f)) or Veh2–saline–saline ((g), (h) and (i)); #$p < 0.05$ compared to Veh3–ethanol ((d), (e) and (f)) or Veh3–ethanol–ethanol ((g), (h) and (i)); @$p < 0.05$ compared to Veh2–saline–ethanol ((g) and (h)). One-way ANOVA and Tukey's test.

3.2. Experiment II: effects of acute rimonabant administration on spontaneous locomotor activity, acute morphine-induced hyperlocomotion and morphine-induced behavioral sensitization

Analysis of the habituation test using one-way ANOVA revealed no significant differences between groups (data not shown).

Rim at all doses did not modify total (Fig. 3a), peripheral (Fig. 3b) or central (Fig. 3c) spontaneous locomotor activity compared with the vehicle group. ANOVA and Tukey's post hoc test revealed that acute Mor administration increased both total [$F(6,63) = 11.85$; $p < 0.001$] (Fig. 3d) and peripheral [$F(6,63) = 12.96$; $p < 0.0001$] (Fig. 3e) locomotion frequencies in all groups during the priming session, regardless of the previous treatment (Veh × Rim), compared to the control group (the Veh1–Sal group). Regarding the central locomotion frequency, pre-treatment with Rim at the lower doses (0.3 and 1 mg/kg) potentiated acute Mor-induced central hyperlocomotion, while both higher doses did not modify this parameter [$F(6,63) = 18.71$; $p < 0.0001$] (Fig. 3f).

Mice acutely treated with Mor displayed increased total locomotion frequency in the test session (Veh2–Sal–Mor > Veh1–Sal–Sal), which was potentiated in mice pretreated with this drug (Veh3–Mor–Mor > Veh2–Sal–Mor). These results demonstrate behavioral sensitization to

Table 1
Summary of the results: rimonabant effects on acute drug-induced hyperlocomotion.

Drug-induced hyperlocomotion				
Drug	Rim dose	Total LF	Peripheral LF	Central LF
Eth	Rim 0.3	–	–	X
	Rim 1	–	–	X
	Rim 3	–	–	X
	Rim 10	X	X	X
Mor	Rim 0.3	–	–	↑
	Rim 1	–	–	↑
	Rim 3	–	–	–
	Rim 10	–	–	–
Coc	Rim 0.3	↓	↓	X
	Rim 1	↓	↓	X
	Rim 3	↓	↓	X
	Rim 10	X	↓	X

LF — locomotion frequency; Rim — rimonabant; Eth — ethanol 1.8 g/kg; Mor — morphine 20 mg/kg; Coc — cocaine 10 mg/kg; X — abolishment; ↑ — potentiation; ↓ — attenuation.

Table 2
Summary of the results: rimonabant effects on drug-induced behavioral sensitization in a two-injection protocol.

Test session — drug-induced behavioral sensitization				
Drug	Rim dose	Total LF	Peripheral LF	Central LF
Eth	Rim 0.3	↓	X	–
	Rim 1	↓	X	–
	Rim 3	X	X	X
	Rim 10	X	X	X
Mor	Rim 0.3	↓	↓	–
	Rim 1	↓	↓	–
	Rim 3	↓	–	X
	Rim 10	↓	–	X
Coc	Rim 0.3	X	X	X
	Rim 1	X	X	X
	Rim 3	X	X	X
	Rim 10	X	X	X

LF — locomotion frequency; Rim — rimonabant; Eth — ethanol 1.8 g/kg; Mor — morphine 20 mg/kg; Coc — cocaine 10 mg/kg; X — abolishment; ↑ — potentiation; ↓ — attenuation.

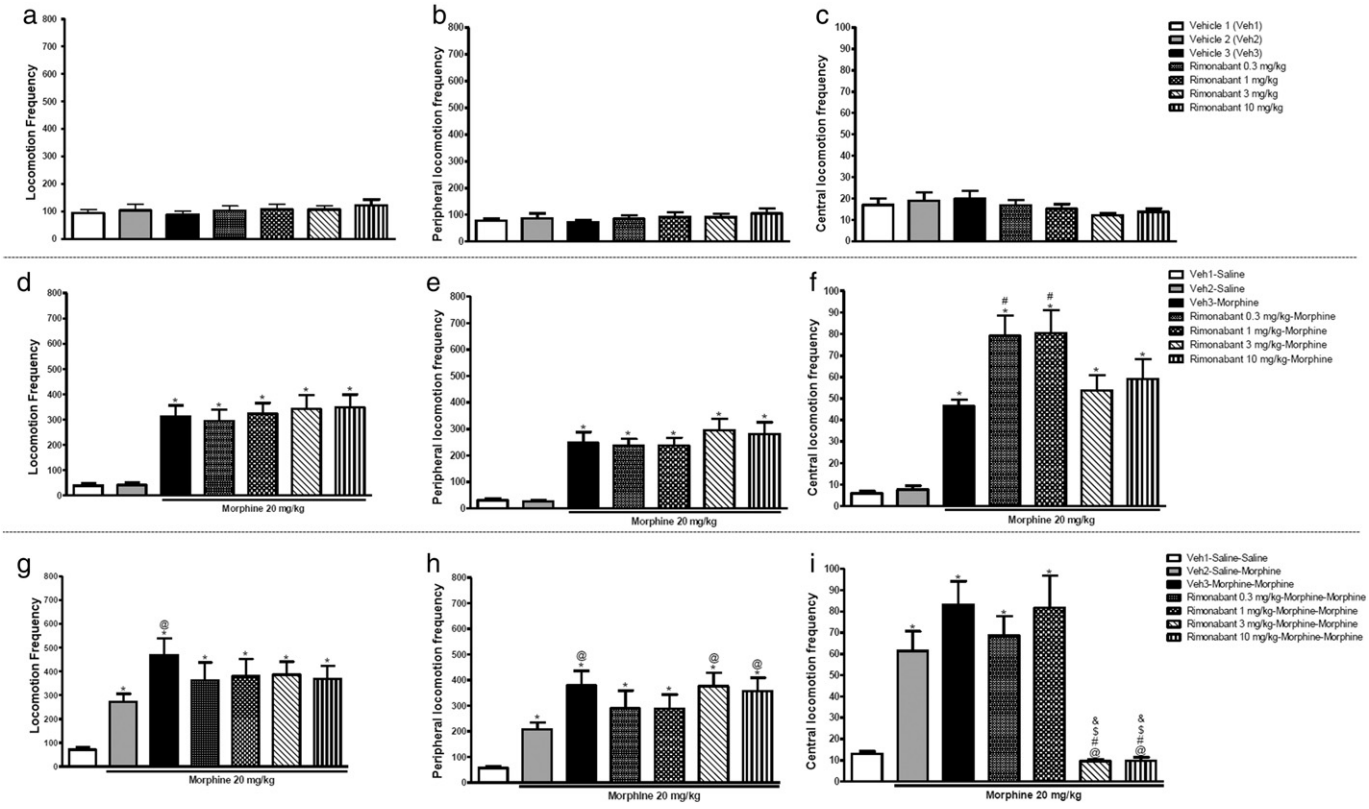

Fig. 3. Effects of i.p. treatment with either rimonabant (0.3, 1, 3 or 10 mg/kg) or vehicle on total (a) spontaneous locomotor activity of mice or in the (b) peripheral (floor units contiguous to the apparatus walls) and (c) central (floor units not contiguous to the apparatus walls) areas of the open-field and its subsequent effects on acute 20 mg/kg morphine-induced total (d), peripheral (e) and central (f) hyperlocomotion and total (g), peripheral (h) and central (i) locomotor sensitization after a 7-day interval. Data are reported as the means ± SEM. *$p < 0.05$ compared to Veh1–saline and Veh2–saline ((d), (e) and (f)) or Veh2–saline–saline ((g), (h) and (i)); $^{\#}p < 0.05$ compared to Veh3–morphine (f) or Veh3–morphine–morphine (i); $^{@}p < 0.05$ compared to Veh2–saline–morphine ((g) and (h)); $^{\$}p < 0.05$ compared to rimonabant 0.3 mg/kg–morphine–morphine (i); $^{\&}p < 0.05$ compared to rimonabant 1 mg/kg–morphine–morphine (i). One-way ANOVA and Tukey's test.

the stimulant effect of Mor. Previous treatment with Rim at all doses attenuated the development of behavioral sensitization to Mor, because the Rim-treated groups differed neither from the Veh3–Mor–Mor group nor from the Veh2–Sal–Mor group in the same experimental day [$F(6,63) = 4,98$; $p = 0.001$] (Fig. 3g). The peripheral locomotor activity showed a similar pattern of response to Mor and Rim at the doses of 0.3 and 1 mg/kg. A second injection of Mor one week later also led to the development of peripheral locomotor sensitization (Veh1–Sal–Sal < Veh2–Sal–Mor < Veh3–Mor–Mor), which was abolished by Rim pre-treatment at the lower doses (0.3 and 1 mg/kg), while both higher doses did not modify this parameter [$F(6,63) = 5.31$; $p < 0.0001$] (Fig. 3h). The central locomotion of mice after a second Mor administration was attenuated by Rim pre-treatment at the doses of 3 and 10 mg/kg. In fact, the central locomotor activity of groups pre-treated with Rim at the higher doses differed from that of all other groups on the Mor test day, with the exception of the saline control group (Veh1–Sal–Sal) [$F(6,63) = 15.88$; $p < 0.0001$] (Fig. 3i).

3.3. Experiment III: effects of acute rimonabant administration on spontaneous locomotor activity, acute cocaine-induced hyperlocomotion and cocaine-induced behavioral sensitization

Analysis of the habituation data using one-way ANOVA revealed no significant differences between groups (data not shown).

Rimonabant at all doses did not modify total (Fig. 4a), peripheral (Fig. 4b) or central (Fig. 4c) spontaneous locomotor activity compared with the vehicle group. ANOVA and Tukey's post hoc test revealed that Coc administration increased total locomotion frequency (Veh3–Coc > Veh1–Sal) during the priming session. This increase was attenuated by pre-administration of the lower doses of Rim (0.3, 1 or 3 mg/kg) and abolished by the highest dose of this drug (10 mg/kg) (Veh3–Coc > Rim10–Coc = Veh1–Sal) [$F(6,63) = 13.32$; $p < 0.001$] (Fig. 4d). The peripheral locomotor activity showed a similar pattern of response to Coc and Rim, with Coc potentiating this parameter and pre-injected Rim at the doses of 0.3, 1 and 3 mg/kg attenuating it. However, the highest dose of Rim (10 mg/kg) was not effective in abolishing acute Coc-induced peripheral hyperlocomotion, only attenuating it (Veh3–Coc > Rim10–Coc > Veh1–Sal) [$F(6,63) = 12.18$; $p < 0.0001$] (Fig. 4e). Acute Coc administration also potentiated the central locomotion frequency of mice, an effect that was abolished by Rim at all doses [$F(6,63) = 19.39$; $p < 0.0001$] (Fig. 4f).

Mice that received an acute Coc injection presented increased total locomotion frequency compared to the control group during the test session (Veh2–Sal–Coc > Veh1–Sal–Sal). Mice previously treated with Coc exhibited greater hyperlocomotion (Veh3–Coc–Coc > Veh2–Sal–Coc), which demonstrates the development of behavioral sensitization to the stimulant effect of this drug. Pre-treatment with Rim at all doses blocked Coc-induced behavioral sensitization (0.3–10 Rim–Coc–Coc groups < Veh3–Coc–Coc) [$F(6,63) = 6.06$; $p < 0.001$] (Fig. 4g). Regarding the peripheral locomotor activity, this parameter showed a similar pattern of response in the Coc test session, with a pre-treatment with Rim at all doses blocking Coc-induced peripheral locomotor sensitization [$F(6,63) = 4.72$; $p < 0.0001$] (Fig. 4h). The central locomotion of mice after a second Coc administration was attenuated by Rim pre-treatment at all doses [$F(6,63) = 9.38$; $p < 0.0001$] (Fig. 4i).

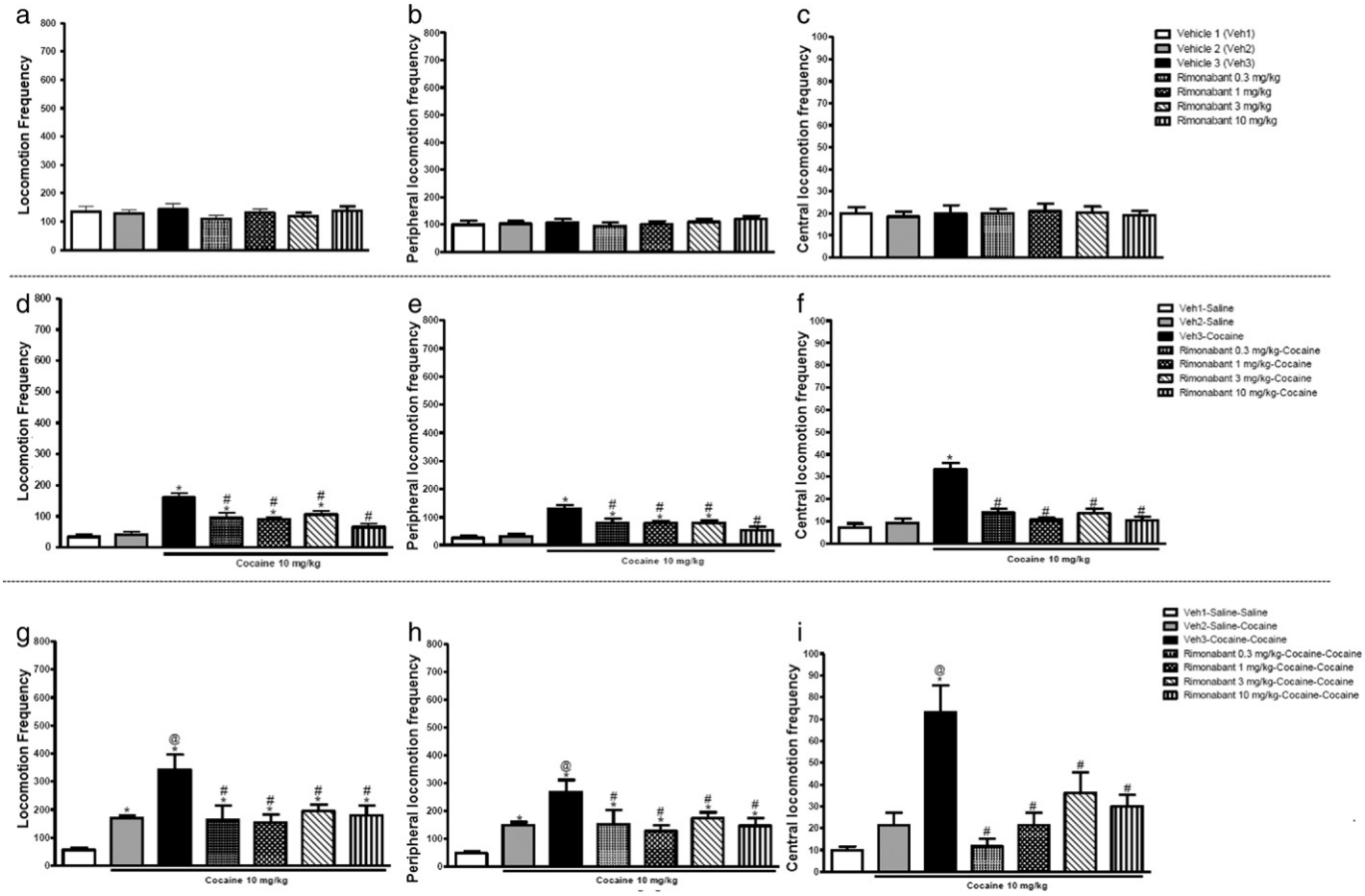

Fig. 4. Effects of i.p. treatment with either rimonabant (0.3, 1, 3 or 10 mg/kg) or vehicle on total (a) spontaneous locomotor activity of mice or in the (b) peripheral (floor units contiguous to the apparatus walls) and (c) central (floor units not contiguous to the apparatus walls) areas of the open-field and its subsequent effects on acute 10 mg/kg cocaine-induced total (d), peripheral (e) and central (f) hyperlocomotion and total (g), peripheral (h) and central (i) locomotor sensitization after a 7-day interval. Data are reported as the means ± SEM. $^{*}p < 0.05$ compared to Veh1–saline and Veh2–saline ((c), (e) and (f)) or Veh1–saline–saline ((g), (h) and (i)); $^{\#}p < 0.05$ compared to Veh3–cocaine ((d), (e) and (f)) or Veh3–cocaine–cocaine ((g), (h) and (i)); $^{@}p < 0.05$ compared to Veh2–saline–cocaine ((g) and (h)). One-way ANOVA and Tukey's test.

3.4. Experiment IV: effects of 10 mg/kg rimonabant on depressive-like behavior evaluated by forced swim test in mice

Student t-test revealed no significant differences between groups, demonstrating that Rim at the dose of 10 mg/kg did not exert a depressive-like behavior in mice (Fig. 5).

4. Discussion

The experimental design for the present study was performed according to a model developed by our group (Marinho et al., 2014). This is a skillful and reliable model that allows a simultaneous evaluation (*e.g.*, in the same group of animals) of the effects of pharmacological agents on the spontaneous locomotor activity of rodents and on the acute effect of drugs of abuse, as well as on the subsequent development of behavioral sensitization in a two-injection protocol. The behavioral sensitization phenomenon has been suggested to be useful for studying the mechanisms underlying dopaminergic mesoaccumbens plasticity (Henry and White, 1991; Kalivas and Stewart, 1991; Wolf et al., 1994), which appears to share neuronal mechanisms with drug craving in humans (Robinson and Berridge, 1993). In fact, the neurocircuitry that underlies behavioral sensitization and relapse to drug seeking behavior is similar in both neurochemistry and neuropharmacology (for a review see Steketee and Kalivas, 2011). Of note, all drugs of abuse employed in the present study exert their stimulant effects by increasing mesolimbic dopaminergic transmission *via* distinct mechanisms (Cheer et al., 2007; Gessa et al., 1985; Lupica and Riegel, 2005), which increases locomotor activity in rodents (Einhorn et al., 1988; Ellinwood et al., 2000).

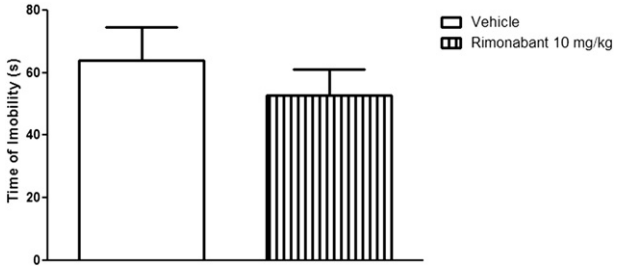

Fig. 5. Effects of i.p. treatment with either 10 mg/kg of rimonabant or vehicle on the immobilization of mice submitted to the forced swim test. Data are reported as the means ± SEM. Student t-test.

The ventral tegmental area (VTA), which contains dopaminergic cell bodies, and its projections to the nucleus accumbens (NAc) are critical elements of the circuits that mediate drug-related behaviors, including the development of drug-induced behavioral sensitization (Carr and Sesack, 2000; Fields et al., 2007; Humphries and Prescott, 2010). Notably, the endocannabinoid system also modulates drug-seeking motivation *via* mechanisms dependent on dopamine release in the NAc (Oleson and Cheer, 2012). The acute administration of ethanol (Basavarajappa et al., 2008), morphine (Viganò et al., 2004) or cocaine (Palomino et al., 2014) enhances the levels of endocannabinoids in rodents. Although the mechanisms underlying these effects are still unclear, all these drugs of abuse increase the levels of anandamide (Ceccarini et al., 2013; Centonze et al., 2004; Viganò et al., 2004),

which preferably binds to CB1 endocannabinoid receptors (Gonsiorek et al., 2000; Di Marzo, 2008). CB1 cannabinoid receptors are present in different regions of the brain reward circuitry, including the VTA and NAc (Gardner, 2005). Acting as retrograde messengers on CB1 receptors, endocannabinoids modulate the glutamatergic excitatory and the gamma-aminobutyric acid (GABAergic) inhibitory inputs into the VTA (Maldonado et al., 2006). The activation of CB1 receptors present in axon terminals of GABAergic neurons in the VTA inhibits GABA transmission, removing the inhibitory input on dopaminergic neurons (Lupica and Riegel, 2005; Riegel and Lupica, 2004) and thereby contributing to the addictive properties induced by different drugs of abuse that increase dopaminergic neuron firing rates, such as opiates, nicotine and alcohol (Maldonado et al., 2006).

In fact, synthetic CB1 receptor agonists inhibit both excitatory postsynaptic currents mediated by glutamate, and inhibitory postsynaptic current mediated by GABA through the inhibition of synaptic transmission in the VTA (Melis et al., 2004a; Pan et al., 2008; Riegel and Lupica, 2004; Szabo et al., 2002), which in dopaminergic neurons is blocked by the antagonism of CB1 receptors (Melis et al., 2004b). Furthermore, it has been demonstrated that the increased release of dopamine in the NAc induced by drugs of abuse is partly dependent upon endocannabinoid activity *in vivo* (Cheer et al., 2007), which implicates that endocannabinoids might regulate drug-related behaviors by modulating dopamine signaling. In fact, disrupting CB1 receptor activation in the VTA dramatically reduce, whereas augmenting levels of endocannabinoids increase, cue-evoked dopamine concentrations in the NAc and reward seeking (Oleson et al., 2012). In this regard, Lupica and Riegel hypothesized that the activation of CB1 receptors by endocannabinoids modulates afferents impinging upon these cells to further sculpt neuronal activity in the VTA (Lupica and Riegel, 2005). Thus, the modulation of the synaptic activity by endocannabinoids is thought to influence the firing activity of VTA dopaminergic neurons and ultimately impact behavioral outcomes (for a review see Wang and Lupica, 2014).

4.1. Ethanol results

In the above detailed scenario, by exerting an antagonistic property at CB1 receptors, Rim increases GABAergic activity in the mesoaccumbens and regulates the firing of these neurons (Cheer et al., 2007). This effect would be expected to inhibit the primary effects of Eth and Mor. In fact, the administration of the highest dose (10 mg/kg) of Rim inhibited acute Eth-induced hyperlocomotion by blocking both peripheral (Fig. 2e) and central (Fig. 2f) locomotor activities. In addition, Rim at the doses of 0.3, 1 and 3 mg/kg selectively inhibited the central hyperlocomotion induced by an acute Eth injection without affecting total locomotion. These results suggest a selective effect of 10 mg/kg Rim on the peripheral hyperlocomotion induced by Eth and a broad effect of Rim on the classic Eth-induced anxiolytic effect (Sanday et al., 2013). In this respect, a growing body of evidence suggests that the endocannabinoid system plays an important role in regulating anxiety- and alcohol-related behaviors, commonly comorbid situations (Kessler et al., 1997; Kushner et al., 1990).

Powers et al. (2010) demonstrated that the endocannabinoid system modulation influences both anxiety-like and conditioned alcohol reward behaviors. However, an endocannabinoid uptake inhibitor was not effective in blocking alcohol drinking behavior in their study. Thus, the selective effect of Rim on the central hyperlocomotion induced by Eth seems to be related to the inhibition of the Eth-induced anxiolysis. Of note, Rim also did not affect Eth-induced hyperlocomotion at low doses, being effective in the abolishment of total and peripheral locomotion only at the dose of 10 mg/kg. The low sensitivity of Rim in the inhibition of the acute Eth stimulant effect may be due to the inhibition of GABAergic interneurons by Eth at low doses (Lupica et al., 2004), which increases dopaminergic activity in the VTA. Rimonabant disinhibits these same neurons; therefore, this antagonist would only abolish Eth-induced hyperlocomotion at concentrations that prevent GABAergic interneuron inhibition, which may have occurred at the highest dose.

Interestingly, while only the highest dose (10 mg/kg) abolished the acute stimulant effect of Eth, previously administered Rim at the doses of 3 and 10 mg/kg hindered Eth-induced behavioral sensitization due to the inhibition of both central and peripheral locomotor frequencies. Thus, high doses of Rim would still inhibit Eth-induced anxiolysis even one-week later after its administration (Fig. 2i). In addition, the lower doses of Rim selectively inhibited the peripheral hyperlocomotion induced by a second Eth injection (Fig. 2h). In this scenario, all doses of Rim would be equally effective in abolishing Eth-induced long-lasting behavioral effects. Because the mesolimbic dopamine system is involved in the mediation of Eth-induced hyperlocomotion and reinforcement (Cheer et al., 2007; Di Chiara and Imperato, 1988; Gessa et al., 1985; Lupica and Riegel, 2005) one may suppose that the cannabinoid CB1 receptor antagonist is capable of removing dopamine-mediated appetitive attributes of Eth, thereby preventing Eth-induced behavioral sensitization. In fact, studies have been demonstrating that Rim is effective in inhibiting the development of Eth self-administration behavior (Dyr et al., 2008; Economidou et al., 2006) in rodents, which seems to be linked to a reduction of reward-related responding instead of drug-induced motor deficits (Economidou et al., 2006).

4.2. Morphine results

In contrast, Rim did not reduce Mor-induced acute stimulant effects at any dose (Fig. 3d, e and f). These results suggest a distinct effect of Rim, which is likely due to the different mechanisms of action of Eth and Mor. Both drugs increase dopaminergic neuron firing rates from the VTA to the NAc, with Eth enhancing dopamine release in the NAc through its action on $GABA_A$ receptors in the VTA (Hyman et al., 2006). On the other hand, opiates increase dopaminergic activity in the limbic system *via* the activation of μ-opiate receptors on the cell bodies of the GABAergic interneurons in the VTA, and VTA dopaminergic neurons are tonically inhibited by these GABAergic interneurons (Matsui et al., 2014). Acute treatment with opiates inhibits them, thus disinhibiting the dopaminergic projection neurons, which then release dopamine in the NAc and other terminal fields (Hyman et al., 2006). Thus, despite the effects of Rim on the GABAergic transmission in the VTA, this drug would not counteract the acute stimulant effects of Mor, regardless of the dose administered.

At the doses of 0.3 and 1 mg/kg Rim actually potentiated Mor-induced acute central hyperlocomotion (Fig. 3f). Mor also exerts its effects by potentiating glutamatergic neurotransmission in the VTA (Harris et al., 2004). The first explicit demonstration of glutamatergic involvement in the development of Mor-induced addictive behaviors was the blockade of the development of behavioral sensitization by systemic administration of antagonists of the NMDA subtype of the ionotropic glutamate receptors (Karler et al., 1989). As previously mentioned, endocannabinoids also modulate the glutamatergic excitatory inputs into the VTA (Maldonado et al., 2006). Thus, the final effect on the modulation of VTA dopaminergic activity by endocannabinoids depends on the functional balance between the inhibitory GABAergic and excitatory glutamatergic inputs. In this scenario, while the GABAergic neurons are inhibited by an acute Mor injection, the glutamatergic neurotransmission would be potentiated by both Mor and Rim, thereby leading to an enhanced central locomotion frequency. At higher doses, Rim would counterbalance this effect by potentiating the GABAergic inhibition.

Although Rim did not modify acute total and peripheral Mor-induced hyperlocomotion, it was effective in attenuating Mor-induced behavioral sensitization at all doses. These data are in line with a previous study by Viganò et al. (2004) demonstrating that Rim modified the signs of Mor sensitization when administered in its expression phase, whereas co-administration of Rim and Mor in the induction phase only slightly affected the behavioral responses. The lower doses of Rim

(0.3 and 1 mg/kg) exerted this inhibitory effect mainly due to a decrease in the peripheral locomotion frequency (Fig. 3h), which might be explained by the absence of Rim in the test session. In the first Mor challenge, mice experienced Mor effects in the presence of Rim 0.3 or 1 mg/kg potentiating effects, which could lead to the development of a state dependency. This phenomenon determines that the retrieval of learned information requires the animal to be in a state similar to that in which the memory for this information was acquired (Izquierdo et al., 1981). In the present study, we used a context-dependent behavioral sensitization protocol, in which the presentation of the drug is paired to a specific context (open-field apparatus) other than the mice's home-cage. Thus, the applied protocol involves the creation of associations between the drug effect and the exteroceptive (environmental) and interoceptive (drug-related) cues (Bloise et al., 2007). In the sensitization test, the same test environment cues were present as those during acquisition. However, mice would not be under the effect of Rim to retrieve the interoceptive conditioning information, failing to respond with a higher peripheral locomotion frequency in the absence of this drug.

On the other hand, the attenuation of behavioral sensitization to Mor induced by pre-treatment with Rim at the doses of 3 and 10 mg/kg was a result of the abolishment of the central locomotion sensitization (Fig. 3i). Of note, it has been shown that there is an interaction between the cannabinoid and the opioid systems in the modulation of anxiety (Zarrindast et al., 2008), which could explain the long-term effects of high doses of rimonabant in the anxiety-like behavior of mice under Mor condition. However, further studies are needed in order to better understand this interaction and the mechanisms underlying these effects.

4.3. Cocaine results

At the highest dose (10 mg/kg), Rim also inhibited Coc acute stimulant effect, attenuating it at the other doses (0.3, 1 and 3 mg/kg) (Fig. 4d), which was due to a decrease in both peripheral (Fig. 4e) and central (Fig. 4f) locomotion frequencies at all doses. As far as we know, this is among the first studies demonstrating a potential role for the endocannabinoid system on primary locomotor stimulant effects of a psychostimulant, in contrast with several other studies demonstrating that CB1 receptors are not required to obtain the activation of the mesolimbic circuitry by drugs of abuse (for a review see Maldonado et al., 2006). Unlike Eth and Mor, psychostimulants enhance dopamine levels in the NAc by directly acting on dopaminergic axon terminals (Maldonado et al., 2006). Cocaine blocks the reuptake transporters on presynaptic dopaminergic terminals from the VTA, increasing dopamine availability in the NAc, which potentiates the firing of GABAergic neurons (Bocklisch et al., 2013). Rimonabant by exerting an antagonistic property at CB1 receptors would further potentiate this GABAergic activity in the mesoaccumbens, decreasing the VTA dopaminergic neurotransmission and consequently the acute Coc-induced hyperlocomotion.

Importantly, all doses of Rim prevented the development of behavioral sensitization induced by Coc also due to the abolishment of both peripheral (Fig. 4h) and central (Fig. 4i) locomotor activities of mice. In this scenario, Rim seems to block the development of Coc-induced behavioral sensitization regardless of the specific locomotor activity in distinct segments of the open-field. Conflicting results have been reported by several research groups about the involvement of cannabinoid receptors in the development of Coc-induced sensitization. Our data are in line a with previous study by Mereu et al. (2013) showing that a single Coc injection in mice produced behavioral sensitization paralleled by a large stimulation of extracellular dopamine levels in the NAc core, and that both behavioral and neurochemical effects were reversed by CB1 receptor blockade produced by Rim (Mereu et al., 2013). Cocaine has been suggested to release endocannabinoids (Cheer et al., 2007). As showed in our third experiment, blockade of cannabinoid receptors before the sensitizing injection of Coc significantly attenuated the occurrence of behavioral sensitization. Thus, our results support the hypothesis raised by Mereu et al. (2013) that neuroadaptations induced by a single injection of Coc require the release of endocannabinoids. In addition, blockade of the cannabinoid CB1 receptors during the maintenance (Gerdeman et al., 2008) or expression (Kupferschmidt et al., 2012; Ramiro-Fuentes and Fernandez-Espejo, 2011) of a previously established behavioral sensitization to Coc inhibits the manifestation of this phenomenon.

4.4. Ethanol vs morphine vs cocaine

In summary, the main results of the present study where that the highest dose of Rim abolished ethanol- and cocaine-induced hyperlocomotion and behavioral sensitization without modifying spontaneous and central locomotor activity or inducing depressive-like behavior on the forced swim test in mice. Lower doses of Rim also selectively blocked acute ethanol-induced central hyperlocomotion. Although rimonabant at 0.3 and 1 mg/kg potentiated the central hyperlocomotion induced by acute morphine injection, it was effective in attenuating morphine-induced behavioral sensitization at all doses. Of note, Rim was effective in either attenuating or blocking the development of behavioral sensitization to all drugs of abuse at all doses, providing evidence that CB1 receptor antagonism is capable of blocking neural adaptations resulting from a single drug exposure.

4.5. Final considerations

Regarding the chosen target drug, Rim has been approved in several countries mainly for the treatment of obesity and associated metabolic dysregulation. However, some clinical studies showed specific psychiatric side-effects – mainly depression- and anxiety-like states – with high doses of Rim (Moreira and Crippa, 2009), which appear to be reversible after cessation of the drug (Moreira et al., 2009). Thus, one could assume that our data, demonstrating an immediate effect of Rim on acute Coc-, Mor- and especially Eth-induced hyperlocomotion and its subsequent sensitization could be due to a depressive effect of Rim. Importantly, in the present study Rim, at the dose that showed high specificity in preventing both acute and sensitized drug effects, did not induce depressive-like behavior in mice (Fig. 5). Furthermore, the chosen dose range of Rim did not exert anxiety-like states *per se*, which is demonstrated by the lack of difference between the vehicle- and Rim-treated groups in the central spontaneous locomotion of mice in all three experiments (Figs. 2c, 3c and 4c). These results discard a possible bias and strengthen the relevance of our results and their implications.

Thus, our data indicate that Rim selectively modulates, inhibiting or at least attenuating, the neural changes responsible for the initiation of Eth, Mor and Coc locomotor sensitization at doses that do not induce adverse effects in mice (*e.g.* doses that do not modify spontaneous behavior or induce a depressive-like state). Because locomotor sensitization in rodents seems to share plastic mechanisms with drug addiction in humans, corresponding to some aspects of drug abuse, such as compulsive drug-seeking behavior (for a review see Steketee and Kalivas, 2011), our findings are in accordance with other studies demonstrating a strong evidence that the endocannabinoid system is involved in drug-seeking behavior (Justinova et al., 2009). Although one must always be wary of extrapolating clinical relevance from animal data, the above-discussed considerations suggest that cannabinoid CB1 receptor antagonists would be a good target for the development of clinical preventive addiction therapies.

5. Conclusions

In conclusion, our data indicate that Rim selectively modulates, inhibiting or at least attenuating, behavioral sensitization to Eth, Mor and Coc. Because the neural basis of this phenomenon has been

proposed to correspond to some components of addiction, our findings demonstrate a strong evidence that the endocannabinoid system might be involved in ethanol, cocaine and morphine abuse, including in compulsive drug-seeking behavior.

Conflict of interest

The authors declare that except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. We are entirely responsible for the scientific content of the paper.

Authors' contributions

EAVM, AJO-L and RF-F were responsible for the study concept and design. EAVM, AJO-L, RW-S, RS, MAB, TSY, AWH and LFB contributed to the acquisition of animal data. EAVM, AJO-L, RW-S, RS, MAB, AWH, LFB and RF-F assisted with data analysis and interpretation of the findings. EAVM, AJO-L, KAZ, CLP, BML, LFB and RF-F drafted the manuscript. RF-F (in memoriam) although helped to draft the manuscript, to interpret the results and critically revised the paper, unfortunately passed away prior to submission. All authors critically reviewed the content and approved the final version for publication.

Role of funding source

The funding sources had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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