Progress in Neuro-Psychopharmacology & Biological Psychiatry 35 (2011) 434–438

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Facilitation of CB1 receptor-mediated neurotransmission decreases marble burying behavior in mice

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ARTICLE INFO

Article history:
Received 25 May 2010
Received in revised form 2 November 2010
Accepted 18 November 2010
Available online 25 November 2010

Keywords:
Cannabinoids
Marble burying test
Obsessive–compulsive disorder
WIN55,212-2
AM404
URB597

ABSTRACT

Obsessive–compulsive disorder (OCD) is a common psychiatric disorder characterized by the occurrence of obsessions and compulsions. Glutamatergic abnormalities have been related to the pathophysiology of OCD. Cannabinoids inhibit glutamate release in the central nervous system, but the involvement of drugs targeting the endocannabinoid system has not yet been tested in animal models of repetitive behavior. Thus, the aim of the present study was to verify the effects of the CB1 receptor agonist WIN55,212-2, the inhibitor of anandamide uptake AM404 and the anandamide hydrolysis inhibitor URB597, on compulsive-associate behavior in male C57BL/6J mice submitted to the marble burying test (MBT), an animal model used for anti-compulsive drug screening. WIN55,212-2 (1 and 3 mg/kg), AM404 (1 and 3 mg/kg) and URB597 (0.1, 0.3 and 1 mg/kg) induced a significant decrease in the number of buried marbles compared to controls. Pretreatment with the CB1 receptor antagonist, AM251, prevented both WIN55,212-2 and URB597 effects. These results suggest a potential role for drugs acting on the cannabinoid system in modulating compulsive behavior.

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1. Introduction

The endocannabinoid system plays an important regulatory role in several brain functions (Ameri, 1999). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the two best characterized endocannabinoids, are synthesized "on demand" through cleavage of membrane phospholipids from post-synaptic neurons and act as retrograde messengers at central synapses. Cannabinoid receptor type-1 (CB1) is highly expressed throughout the central nervous system and modulates both excitatory and inhibitory neurotransmission (Herkenham et al., 1990; Schlicker and Kathmann, 2001). Activation of these receptors on axon terminals regulates ion channel activity inhibiting neurotransmitter release (Piomelli, 2003; Wilson and Nicoll, 2001).

The effects mediated by endocannabinoids are usually limited and short-lasting due to their fast removal from synaptic cleft by a two-step process that involves internalization and metabolism by the fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) enzymes for AEA and 2-AG, respectively (Elphick and Egertova, 2001; Giuffrida et al., 2001).

Obsessive–compulsive disorder (OCD) is a common psychiatric condition characterized by the occurrence of obsessions (persistent intrusive thoughts) and compulsions (defined as ritualistic repetitive behaviors) which are generally enacted in an effort to somehow alleviate intense anxiety caused by obsessions. Although OCD pathophysiology is not completely understood, the therapeutic effects of selective serotonin reuptake inhibitors suggest the involvement of serotonergic pathways [for review see Abramowitz et al., 2009]. However, preclinical and clinical data have also shown that attenuation of glutamate-mediated neurotransmission could be helpful in the treatment of OCD patients (Aboujaoude et al., 2009; Egashira et al., 2008; Grant et al., 2007) indicating that, in addition to serotonin, glutamatergic abnormalities could also be involved in the pathophysiology of OCD [for review see Pittenger et al., 2006; Ting and Feng, 2008].

Marble burying in mice was initially related to anxiety behavior (Njung'e and Handley, 1991). However, recent evidence suggests that it engages neural circuits implicated in compulsive-associated behavior (Thomas et al., 2009). As a consequence, the marble burying test (MBT) has been proposed as an animal model to investigate repetitive responses involved in OCD (Korff and Harvey, 2006; Thomas et al., 2009).

A recent study from our group demonstrated that cannabidiol, a major non-psychotomimetic component of *Cannabis sativa*, attenuates marble burying behavior. This effect was prevented by pretreatment with AM251, a CB1 receptor antagonist (Casarotto et al.,

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; CB1, cannabinoid receptor type 1; CSTC, cortico-striato-thalamo-cortical circuitry; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; MBT, marble burying test; OCD, obsessive–compulsive behavior; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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doi:10.1016/j.pnpbp.2010.11.027

2010). Although cannabidiol effects could involve several mechanisms (Izzo et al., 2009), it can enhance endocannabinoid-mediated actions by inhibiting the hydrolysis and reuptake of AEA (Bisogno et al., 2001). Therefore, the cannabidiol effect on marble burying behavior could have been mediated by facilitation of CB1 receptor-mediated neurotransmission, suggesting the involvement of the endocannabinoid system in this behavior.

The aim of the present study, therefore, was to test the hypothesis that facilitation of CB1 receptor-mediated neurotransmission would attenuate marble burying behavior.

2. Material and methods

2.1. Animals

The experiments were performed using male C57BL/6J mice weighing 25–30 g. The animals were housed in groups of 15 mice/cage under a 12 h light cycle (lights on at 7 am) with free access to food and water. Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and policies, and were approved by the local Ethical Committee (protocol number: 146/2009). All efforts were made to minimize animal suffering.

2.2. Drugs

The following drugs were used: WIN55,212-2 (a CB1 receptor agonist, Tocris, USA), AM404 (an inhibitor of AEA uptake, Tocris, USA), URB597 (a FAAH inhibitor, Calbiochem, USA) and AM251 (a CB1 receptor antagonist, Tocris, USA). All drugs were suspended in 2% Tween 80 in sterile saline (vehicle), except AM404 and AM251, which were suspended in 2% Tocrisolve™ 100 (Tocris, SA) in sterile saline and 10% DMSO in sterile saline, respectively. The drugs were injected intraperitoneally (ip) in a 10 mL/kg volume.

2.3. Apparatus and procedure

2.3.1. Marble burying test (MBT)

The test was performed using a squared box (38×32×28 cm) with a 5 cm sawdust layer covered floor. Twenty-five green clear glass marbles (1.5 cm diameter) were evenly spaced over the floor. One hour before testing the animals were left undisturbed in the experimental room and pre-exposed for 5 min to the sawdust box without marbles to avoid novelty seeking behavior during the test. Thirty minutes after the pre-exposition session the animals received the ip drug injections. In the test session the mice were placed in the center of marble containing box where they had been previously exposed to. Thirty minutes later the animals were taken from the box and the number of buried marbles was counted. The criteria for buried marbles included only those with at least two-thirds under sawdust (Njung'e and Handley, 1991).

2.3.2. Open field test

In order to control for a possible non-specific drug effect on locomotor activity that could interfere in the MBT, independent groups of animals were submitted to the open field test. It was performed in a Plexiglas circular arena (40 cm diameter), with 40 cm high walls. The animals were placed in the arena center and total distance traveled and the percent of time spent in central and peripheral zones were measured during 5 min using ANY-MAZE software (Stoelting, Illinois, USA).

2.4. Experimental design

2.4.1. Experiment 1: effects of WIN55,212-2, a CB1 receptor agonist, in the MBT

Naive mice were randomly divided into groups receiving ip injections of vehicle (n=6) or WIN55,212-2 (0.3, 1, 3 mg/kg; n=6/group). The animals were tested 30 min after drug injection. Independent groups receiving vehicle or WIN55,212-2 (1 or 3 mg/kg) were tested in the open field (n=5/group) 30 min after drug injection. The drug dose range was based on previous results from the literature (Haller et al., 2004; Rutkowska et al., 2006).

2.4.2. Experiment 2: effects of URB597, a selective FAAH inhibitor, in the MBT

Naive mice were randomly divided into groups receiving ip injections of vehicle (n=6) or URB597 (0.1, 0.3, 1 mg/kg; n=6–7/group). The animals were tested 30 min after drug injection. Independent groups of mice receiving vehicle or URB597 (0.1, 0.3, 1 mg/kg) were also tested in the open field (n=5/group) 30 min after drug injection. The drug dose range was based on previous results from the literature (Kathuria et al., 2003; Moreira et al., 2008).

2.4.3. Experiment 3: effects of AM404, an inhibitor of AEA uptake, in the MBT

Naive mice were randomly divided into groups receiving ip injections of vehicle (n=6) or AM404 (0.3, 1, 3 mg/kg; n=6/group). The animals were tested 30 min after drug injection. Independent groups receiving vehicle or AM404 (1 or 3 mg/kg) were tested in the open field (n=6/group) 30 min after drug injection. The drug dose range was based on previous results from the literature (Patel and Hillard, 2006).

2.4.4. Experiment 4: effects of AM251 on locomotor activity

Naive mice were randomly divided into groups receiving ip injections of vehicle or AM251 (1 or 3 mg/kg dose; n=6/group) and submitted to the open-field test 60 min after the drug injection. AM251 doses were based on the dose range that caused no significant effect in anxiety models (Umathe et al., 2009). Independent groups of animals also received vehicle or AM251 (3 mg/kg) and were submitted to the marble-burying test.

2.4.5. Experiment 5: effects of pretreatment with AM251, a CB1 receptor antagonist, on WIN55,212-2 effects in the MBT

Naive mice were divided into groups receiving a first ip injection of the CB1 receptor antagonist AM251 (AM; 1 mg/kg) or vehicle (VEH) followed, 30 min later, by a second ip injection of WIN55,212-2 (WIN; 1 mg/kg) or vehicle. The groups were: VEH–VEH, AM–VEH, VEH–WIN and AM–WIN (n=6/group). The animals were tested 30 min after the last drug injection.

2.4.6. Experiment 6: effects of pretreatment with AM251 on URB597 effects in the MBT

Naive mice were divided into groups receiving a first ip injection of the CB1 receptor antagonist AM251 (1 mg/kg) or vehicle followed, 30 min later, by a second ip injection of URB597 (URB; 0.3 mg/kg) or vehicle. The groups were: VEH–VEH, AM–VEH, VEH–URB, and AM–URB (n=7/group). As in experiment 3, the animals were tested 30 min after the last drug injection.

2.5. Statistical analysis

Experiments 1, 2, 3 and 4 were analyzed by one-way ANOVA, except MBT for experiment 4, which was submitted to Students' t test. A two-way ANOVA (factors being the first and second injections) was used to analyze experiments 5 and 6. Post-hoc analysis was

performed using the Newman–Keuls test. $P<0.05$ was considered significant.

3. Results

3.1. Experiment 1: WIN55,212-2 effects in the MBT

As seen in Fig. 1a, WIN55,212-2 (at 1 and 3 mg/kg dose) reduced the number of buried marbles in a dose-dependent manner compared to the control group [$F(3,20)=31.72$; Newman–Keuls, $P<0.05$]. However, the group treated with a 3 mg/kg dose exhibited a reduced locomotor activity in the open field [$F(2,12)=10.91$; $P<0.05$, Fig. 2a]. The drug did not change exploratory activity of the central or peripheral areas of the open field [percent time in the center: $F(2,12)=0.60$; percent time in the periphery $F(2,12)=0.84$; both $P>0.05$, Fig. 2b].

3.2. Experiment 2: URB597 effects in the MBT

URB597 (0.1, 0.3, 1 mg/kg) reduced the number of buried marbles in a dose-dependent manner compared to the control group [$F(3,21)=23.11$; Newman–Keuls, $P<0.05$, Fig. 1b] without affecting locomotor behavior [$F(3,16)=0.46$; $P>0.05$, Fig. 2c] or exploratory activity in the open field [percent time in the center: $F(3,16)=1.80$; percent time in the periphery: $F(3,16)=1.40$; both $P>0.05$, Fig. 2d].

3.3. Experiment 3: AM404 effects in the MBT

AM404 (1 and 3 mg/kg) reduced the number of buried marbles compared to the control group [$F(3,20)=3.72$; Newman–Keuls, $P<0.05$, Fig. 3a] without affecting locomotor behavior [$F(2,15)=0.19$; $P>0.05$, Fig. 3b] or exploratory activity in the open field [percent time in the center: $F(2,15)=0.86$; percent time in the periphery: $F(2,15)=0.68$; both $P>0.05$, Fig. 3c].

3.4. Experiment 4: effects of AM251 on locomotor activity

Although the higher dose of AM251 tended to decrease the total distance traveled in the arena, it failed to reach statistical significance [$F(2,15)=2.70$; $P=0.09$, Fig. 2e]. The drug did not modify the central and periphery explorations of the apparatus [percent time in the center: $F(2,15)=0.33$; percent time in the periphery: $F(2,15)=0.23$; both $P>0.05$, Fig. 2f]. No drug effect was observed in animals treated with the 3 mg/kg dose of AM251 in the MBT [$t(10)=0.38$, $P>0.05$; data not shown].

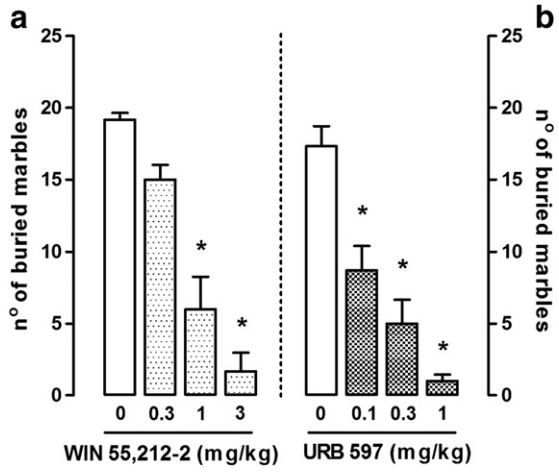

Fig. 1. (a) Effect of systemic (ip) injection of WIN55,212-2 (0, 0.3, 1 or 3 mg/kg, n = 6/group) on marble burying test (MBT). (b) Effect of ip injection of URB597 (0, 0.1, 0.3, 1 mg/kg; n = 6–7/group) on MBT. Data represent the mean ± SEM of buried marbles. * $P<0.05$ from respective control group.

3.5. Experiment 5: effects of pretreatment with AM251 on WIN55,212-2 effects in the MBT

Confirming results from experiment 1, WIN55,212-2 (1 mg/kg) decreased the number of buried marbles. AM251 was able to attenuate WIN55,212-2 effects on marble burying [first versus second drug injection interaction, $F(1,20)=5.79$, Newman–Keuls, $P<0.05$, Fig. 4a].

3.6. Experiment 6: effects of pretreatment with AM251 on URB597 effects in the MBT

As seen in experiment 1, URB597 (0.3 mg/kg) injection was able to decrease the number of buried marbles. This effect was prevented by pretreatment with AM251 [first versus second drug injection interaction, $F(1,24)=5.03$, $P<0.05$, Newman–Keuls, $P<0.05$, Fig. 4b].

4. Discussion

The present study showed that treatment with the CB1 receptor agonist WIN55,212-2 or the FAAH inhibitor URB597 inhibits marble burying behavior in a dose-dependent manner. These effects were prevented by a previous injection of the CB1 receptor antagonist AM251 at a dose that did not induce any significant effect by itself. Confirming the involvement of the cannabinoid system in this behavior, the endocannabinoid uptake inhibitor AM404 also decreased marble burying.

The MBT was first designed as an animal model aimed at detecting anxiolytic drug effects (Njung'e and Handley, 1991). However, several pieces of evidence have questioned this proposal. The observation that, contrary to most anxiety tests based on exploratory behavior, repeated exposure to marbles does not cause behavioral habituation, led to the proposal that the MBT, instead of measuring novelty-induced anxiety, would evaluate a natural, repetitive behavior, that can become compulsive (Njung'e and Handley, 1991; Thomas et al., 2009). Another argument favoring the proposal that the MBT reflects compulsive-related behavior is the development of tolerance after repeated treatment with classical anxiolytic compounds such as diazepam (Casarotto et al., 2010; Ichimaru et al., 1995).

Our laboratory has recently demonstrated an inhibitory effect of cannabidiol on marble burying behavior. This effect was probably mediated by facilitation of CB1 receptor-mediated neurotransmission, since it was prevented by previous administration of the CB1 receptor antagonist AM251 (Casarotto et al., 2010). The present study extends these previous findings showing that CB1 receptor agonist WIN55,212-2, AM404, a drug that is thought to exert its actions by increasing the availability of AEA for CB1 receptors by inhibiting their reuptake (Giuffrida et al., 2001), and URB597, a drug that produces dose-dependent rapid (<15 min) and persistent (>6 h) inhibition of brain FAAH activity and a significant increase in the brain content of AEA (Kathuria et al., 2003; Piomelli et al., 2006), are also able to inhibit marble burying behavior. Together, the results reinforce a possible involvement of the endocannabinoid system in the modulation of repetitive behaviors.

Impairment of motor function could be a confounding factor in the MBT and it has been described after treatment with drugs that act in an endocannabinoid system (Rodriguez de Fonseca et al., 1998; Sanudo-Pena et al., 2000; Varvel et al., 2005). Although the higher dose of WIN55,212-2 (3 mg/kg) reduced locomotor activity in the open field, no effect was found with the lower, but also effective dose (1 mg/kg) of the drug in the MBT. Moreover, confirming other studies (Piomelli et al., 2006), no effect on motor function was observed with drugs that facilitate endocannabinoid effects instead of producing a

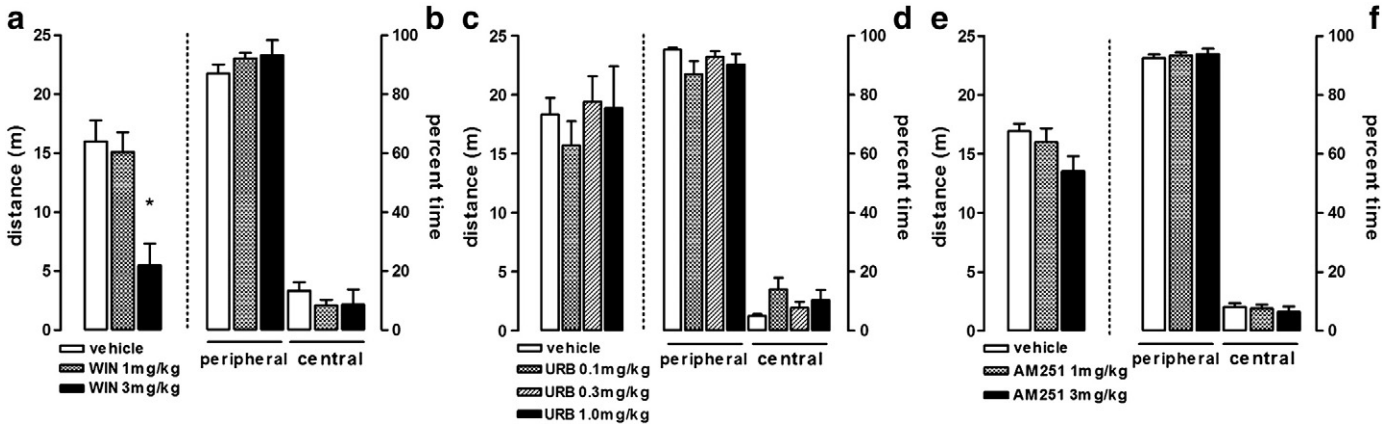

Fig. 2. (a) Effect of ip injection of WIN55,212-2 (0, 1 or 3 mg/kg) on total distance traveled and (b) percent time spent in peripheral and central zones in the open-field (n = 5/group). (c) Effect of ip injection of URB597 (0, 0.1, 0.3, or 1 mg/kg) on total distance traveled and (d) percent time spent in peripheral and central zones in the open-field (n = 5/group). (e) Effect of ip injection of AM251 (0, 1 or 3 mg/kg) on total distance traveled and (f) percent time spent in peripheral and central zones in the open-field (n = 6/group). Data expressed as mean ± SEM. * $P<0.05$ from respective control group.

general activation of CB1 receptors. Together, these findings suggest that our results in the MBT cannot be attributed to impairment of motor function.

Anxiolytic effects have been described after treatment with CB1 receptor agonists and FAAH inhibitors (Kathuria et al., 2003; Patel and Hillard, 2006). Although these effects could have influenced the present results, at the doses used WIN55,212-2, AM404 and URB597 did not change the percentage of time spent in the center or periphery of the open field. Since anxiolytic drugs usually increase exploratory activity of the former area (Prut and Belzung, 2003), acute anxiolytic effects seem to not be responsible for our results in the MBT.

So far there has been little evidence pointing to an involvement of cannabinoids in OCD related behaviors. Results obtained from a pilot study indicated that a single-dose treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC) improved compulsive behavior in patients with Tourette's Syndrome (Muller-Vahl et al., 2002), a neuropsychiatric disorder characterized by the presence of multiple physical (motor) tics commonly associated with OCD (Hounie et al., 2006; Jankovic, 2001). Additionally, an "add-on" effect of dronabinol, a synthetic form of Δ^9-THC, improving OCD treatment has been observed (Schindler et al., 2008).

The neurotransmitter systems related to marble burying behavior and OCD are still poorly understood. The inhibitory effects of SSRIs in this test and its therapeutic effect in OCD patients suggest the involvement of serotonergic mechanisms (Casarotto et al., 2010). In addition, recent evidence suggests that glutamatergic neurotransmission could also be involved (Egashira et al., 2008; Iijima et al., 2010). Glutamate is the major neurotransmitter in the cortico-striato-thalamo-cortical (CSTC) circuitry that has been implicated in the pathophysiology of OCD [for review see Carlsson, 2000; Pittenger et al., 2006]. Brain imaging studies of OCD patients have demonstrated a dysfunction of glutamatergic neurotransmission in CSTC circuitry and patients with OCD show increased glutamate levels in cerebrospinal fluid compared to healthy subjects (Chakrabarty et al., 2005). Moreover, both preclinical and clinical studies suggest that drugs that attenuate glutamate neurotransmission such as riluzole and memantine are helpful in the treatment of OCD patients (Aboujaoude et al., 2009; Grant et al., 2007) and are effective in the MBT (Egashira et al., 2008; Iijima et al., 2010). However, there are also contradictory results regarding the possible role of glutamate in this disorder. For example, a decrease in anterior cingulate cortex glutamate concentration was found in OCD patients (Arnold et al., 2009) and in transgenic models an anti-glutamatergic drug exacerbated OCD-associated behaviors (McGrath et al., 2000). Taken together, these data suggest that OCD could involve, rather than just a general increase, disregulation of glutamatergic neurotransmission in specific

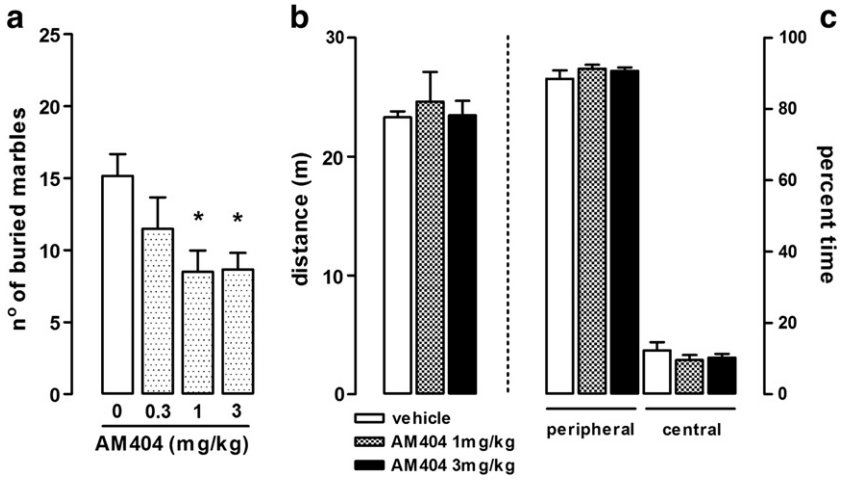

Fig. 3. (a) Effect of systemic (ip) injection of AM404 (0, 0.3, 1 or 3 mg/kg, n = 6/group) on marble burying test (MBT). (b) Effect of ip injection of AM404 (1 or 3 mg/kg) on total distance traveled and (c) percent time spent in peripheral and central zones in the open-field (n = 6/group). Data represent the mean ± SEM of buried marbles. * $P<0.05$ from respective control group.

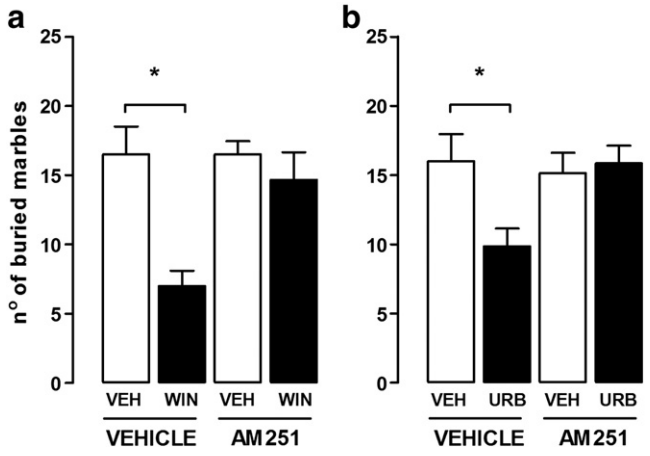

Fig. 4. Effect of a first ip injection of vehicle (VEH) or AM251 (1 mg/kg) followed by a second ip injection of VEH, (a) WIN55,212-2 (1 mg/kg) or (b) URB597 (0.3 mg/kg) on MBT (n = 6–7/group). Data represent the mean ± SEM of buried marbles. * $P<0.05$ from respective control group.

brain areas. Therefore, although cannabinoid agonists and pharmacological agents that enhance endogenous cannabinoid signaling could be interfering in marble burying behavior by facilitating a CB1 receptor-mediated decrease of glutamate release in neural pathways involved in OCD, further studies using intra-cerebral drug injections are needed to elucidate the mechanisms of this effect.

In conclusion, the present results indicated that facilitation of CB1 receptor-mediated neurotransmission inhibits marble burying behavior, suggesting the involvement of the endocannabinoid system in the pathophysiology of OCD. They also suggest that drugs targeting this system could be effective in the control of compulsive associated behavior.

Acknowledgements

The authors thank JC de Aguiar for technical assistance. This research was supported by grants from FAPESP and CNPq.

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