Progress in Neuro-Psychopharmacology & Biological Psychiatry 59 (2015) 76–83

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

Cannabinoid CB1 receptors in the dorsal hippocampus and prelimbic medial prefrontal cortex modulate anxiety-like behavior in rats: Additional evidence

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ARTICLE INFO

Article history:
Received 11 September 2014
Received in revised form 7 January 2015
Accepted 8 January 2015
Available online 14 January 2015

Keywords:
Anandamide
Anxiety
CB1 receptors
Dorsal hippocampus
Endocannabinoids
Prefrontal cortex

ABSTRACT

Endocannabinoids (ECBs) such as anandamide (AEA) act by activating cannabinoid type 1 (CB1) or 2 (CB2) receptors. The anxiolytic effect of drugs that facilitate ECB effects is associated with increase in AEA levels in several encephalic areas, including the prefrontal cortex (PFC). Activation of CB1 receptors by CB1 agonists injected directly into these areas is usually anxiolytic. However, depending on the encephalic region being investigated and on the stressful experiences, opposite effects were observed, as reported in the ventral HIP. In addition, contradictory results have been reported after CB1 activation in the dorsal HIP (dHIP). Therefore, in the present paper we have attempted to verify if directly interfering with ECB metabolism/reuptake in the prelimbic (PL) portion of the medial PFC (MPFC) and dHIP would produce different effects in two conceptually distinct animal models: the elevated plus maze (EPM) and the Vogel conflict test (VCT). We observed that drugs which interfere with ECB reuptake/metabolism in both the PL and in the dentate gyrus of the dHIP induced anxiolytic-like effect, in both the EPM and in the VCT via CB1 receptors, suggesting that CB1 signaling in these brain regions modulates defensive responses to both innate and learned threatening stimuli. This data further strengthens previous results indicating modulation of hippocampal and MPFC activity via CB1 by ECBs, which could be therapeutically targeted to treat anxiety disorders.

1. Introduction

Endocannabinoids (ECBs) are lipid compounds derived from arachidonic acid that act by activating cannabinoid type 1 (CB1) or 2 (CB2) receptors (Piomelli, 2003). In the central nervous system, ECBs are produced on demand and released from the postsynaptic neuronal membrane (Wilson and Nicoll, 2002). CB1 receptors are the most abundant metabotropic receptors in the mammalian brain (Herkenham et al., 1990). They are predominantly located in presynaptic terminals where they inhibit release of several classical neurotransmitters such as glutamate and GABA (Egertova et al., 1998). One of the main ECBs is arachidonoylethanolamide (Anandamide, AEA), a neurotransmitter that can activate, in addition to CB1 receptors, potential vanilloid type 1 receptors (TRPV1) (Aguiar et al., 2014). AEA actions terminate after an internalization process followed by enzymatic hydrolysis by fatty acid amide hydrolase (FAAH) in the postsynaptic neuron (Cravatt et al., 1996).

Extensive expression of CB1 receptors in encephalic regions such as the hippocampus (HIP), the medial prefrontal cortex (MPFC), the cerebellum, the periaqueductal gray matter (PAG) and the basal nuclei (Tsou et al., 1998) is probably related to the effects of Δ9-tetrahydrocannabinol (THC), the main psychoactive compound of the *Cannabis sativa* plant, on memory, cognition and behavior. Several studies have shown CB1 receptors are implicated in anxiety (Haller et al., 2004; Rey et al., 2012), mood (Steiner et al., 2008) and extinction of aversion-related memories processes (Marsicano et al., 2002; Metna-Laurent et al., 2012). For example, the anxiolytic and anxiogenic-like effects of drugs that facilitate ECB signaling are associated to increases in AEA levels in the PFC and the HIP (Bortolato et al., 2006; Draycott et al., 2014; Kathuria et al., 2003; Laviolette and Grace, 2006; Tan et al., 2011), suggesting these structures are involved in cannabinoid effects.

Activation of CB1 receptors by CB1 agonists injected directly into encephalic structures related to defensive responses such as the MPFC

Abbreviations: ECBS, Endocannabinoids; AEA, Anandamide; FAAH, fatty acid amide hydrolase; CB1, Cannabinoid type 1 receptor; CB2, Cannabinoid type 2 receptor; TRPV1, Transient potential vanilloid type 1 receptor; CBD, cannabidiol; PFC, Prefrontal cortex; MPFC, Medial prefrontal córtex; PL, Prelimbic; HIP, Hippocampus; dHIP, dorsal Hippocampus; PAG, Periaqueductal gray matter; THC, Δ9-tetrahydrocannabinol; EPM, Elevated plus maze; VCT, Vogel conflict test; AP, Anteroposterior; L, lateral; V, ventral

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http://dx.doi.org/10.1016/j.pnpbp.2015.01.005
0278-5846/

(Fogaca et al., 2012; Rubino et al., 2008) and the dorsolateral PAG (Moreira et al., 2007) is usually anxiolytic. There are, however, several contradictory results. In addition to producing an inverted U-shaped dose–response curve, these drugs can also be anxiogenic (Campos et al., 2010; Hakimizadeh et al., 2012; Moreira et al., 2012; Roohbakhsh et al., 2007), indicating anxiety modulation by CB1 receptors is probably more complex than initially thought. These opposite effects could depend, in addition to the encephalic region being investigated, also on the stress experience of the subjects before or at the time of behavioral tests (Campos et al., 2010; Lisboa et al., 2008, 2010). In line with this proposal, we observed that intra-ventral HIP injection of AEA reuptake inhibitor produces anxiogenic and anxiolytic effects in naïve rats tested in the elevated plus maze (EPM) and the Vogel conflict tests, respectively. The anxiogenic effect observed in the EPM turned into an anxiolytic effect when rats were previously stressed by forced restraint (2 h) 24 h before the EPM test (Campos et al., 2010). Similar model- and stress-dependent effects were found after intra-PL injection of cannabidiol (CBD) (Fogaca et al., 2014; Lemos et al., 2010), a phytocannabinoid that can, among other effects, inhibit the FAAH enzyme (for review, see Campos et al., 2012). The involvement of the ECB system in these effects, however, is not clear, since CBD can act by several other mechanisms, including facilitation of 5HT1A-mediated neurotransmission (Resstel et al., 2009). Therefore, in the present paper we will directly verify if interference with AEA metabolism/reuptake in the PL and the dorsal HIP would produce different effects in two conceptually distinct animal models of anxiety, the EPM and the VCT.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 230–270 g were used. Animals were maintained at the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. Rats were housed in groups of 4 in plastic cages with free access to food and water and under a 12 h light/dark cycle (lights on at 06:30 h). Independent groups of animals were used in all experiments. The Institution's Animal Ethics Committee approved housing conditions and experimental procedures (protocol n° 143/2007). All efforts were made to minimize animal suffering, to reduce the number of animals used.

2.2. Stereotaxic surgery

Rats were anesthetized with tribromoethanol (Sigma-Aldrich; 250 mg/kg i.p.). After scalp anesthesia with lidocaine (2% epinephrine as vasoconstrictor; subcutaneous), the skull was surgically exposed and stainless steel guide cannula (26G) were bilaterally implanted into the PL or the dentate gyrus of the dHIP using a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA). The Bregma was used as a reference point. Coordinates for cannula implantation into the PL (incisor: −3.3 mm; AP = −3.3 mm; L = 1.9 mm from the medial suture, V = −2.6 mm from the skull with a lateral inclination of 22°) or dHIP (incisor: −2.5 mm; AP = −4.0 mm from bregma; L = +2.8 mm from the medial suture, V: −2.1 mm from the skull) were based on the rat brain atlas of Paxinos and Watson (2006). Cannulae were fixed to the skull with dental cement and a metal wire was inserted into the cannula to prevent obstruction. After surgery, the animals received an intramuscular injection of a poly-antibiotic (Pentabiotico®, Fort Dodge, Brazil; 0.2 ml) and a subcutaneous injection of the nonsteroidal anti-inflammatory flunixine meglumine (Banamine®, Schering Plough, Brazil) for analgesia.

2.3. Drugs

The AEA transporter inhibitor 4-hydroxyphenylarachidonylamide (AM404; Tocris, Westwoods Business Park Ellisville, MO, USA) 50 pmol was dissolved in Tocrisolve TM 100 (a solvent that contains a 1:4 ratio of soya oil/water, emulsified with the block co-polymer Pluronic F68) as recommended by the manufacturer. Cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl Ester (URB597, inhibitor of FAAH enzyme; Calbiochem) 0.01 nmol was dissolved in DMSO 10% in saline (0.9% NaCl). The CB1 receptor antagonist N-(piperidin-1yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-Hpyrazole-3-carboxamide (AM251; Tocris, Westwoods Business Park Ellisville, MO, USA) 100 pmol was dissolved in DMSO 10% in saline (0.9% NaCl). The solutions were prepared immediately before use and were kept on ice and protected from the light during the experimental sessions. Tribromoethanol (Sigma, St. Louis, Missouri, USA) and Urethane (Sigma, St. Louis, Missouri, USA) were dissolved in distillated water. Morphine hydrochloride (5 mg/kg, Merck) was dissolved in saline (0.9% NaCl). AM404 and URB597 doses were chosen based on previous studies from our group showing these doses modulate anxiety-related behavior (Lisboa et al., 2008, 2010; Moreira et al., 2007) and from pilot studies showing these same doses modified anxiety-like behavior when injected into the dHIP or the MPFC. From these studies, we also choose a dose of AM251 that did not modify anxiety-like behaviors by itself.

3. Experimental procedures

In the test day, five to seven days after surgery, independent group of animals received two bilateral injections into the dHIP or PL. The first microinjection of vehicle (500 nl into the dHIP or 200 nl into the PL) or AM251 (100 pmol) was followed, 5 min later, by a second injection of vehicle, AM404 (50 pmol) or URB597 (0.01 or 0.1 nmol). Ten minutes later, the animals were submitted to the test session (Vogel or EPM).

In the experiments measuring tail withdrawal latency and water consumption, in which rats received only AM404 or URB, the animals were submitted to the tests 10 min after the drugs. Morphine hydrochloride 5 mg/kg (1 ml/kg) was injected systemically as a positive control in the tail flick test 30 min before evaluation.

3.1. Vogel conflict test

This test was performed in a Plexiglas box (42 × 50 × 25 cm) with a stainless grid floor. The metallic spout of a drinking bottle containing water projected into the box and the contact of the animal with the spout and the grid floor closed an electrical circuit controlled by a sensor (Anxio-Meter model 102, Columbus, USA). This sensor produced 7 pulses/s whenever the animal was in contact with both components. Each pulse was considered as a lick and after every 20 licks, a 0.5 mA/2 s shock was delivered in the metallic drinking spout. The sensor recorded the total number of licks and shocks delivered during the test period. The whole apparatus was located inside a sound-attenuated cage (Lisboa et al., 2008).

Animals were water deprived for 48 h before the test. After the first 24 h, the animals were allowed to drink freely for 3 min in the test box in order to find the bottle spout. The animals that did not find the spout were excluded from the experiment. After an additional 24 h period of water deprivation the drugs were injected into the dHIP or PL and 10 min later the animals were placed into the test box for the 3 min test session. The number of licks and shocks delivered were registered. Although the number of shocks delivered by the system was proportional to the number of licks performed by the rat (one shock at every 20 licks), sometimes at the end of the test the animal was still licking but had not yet received the next shock. Therefore, the number of licks is usually slightly higher than one would expect considering the number of shocks.

3.2. Water consumption evaluation

Apparatus was the same used in the test above; however, the electric shock delivering system was rendered inoperative.

3.3. Tail-flick test

Apparatus consisted of an acrylic platform with a nichrome wire coil (Insight Instruments. Brazil) maintained at room temperature (24–26 °C). Rats were gently handled and their tails were laid across the coil. Coil temperature was then raised at a 9 °C/s rate by the passage of electric current. System had a cut-off time of 6 s to prevent tissue damage when the coil temperature approached 80 °C. The time to withdraw the tail was recorded as tail-flick latency. Electric current was calibrated to provoke this reflex within 2.5–3.5 s in non-treated animals (Lisboa et al., 2008; Resstel et al., 2008b).

3.4. Elevated plus maze (EPM)

Experiments were carried out in a wood apparatus located in a sound attenuated and temperature controlled (24 °C) room, which was illuminated by a 40-W incandescent light placed 3 m away from the EPM. Apparatus consisted of two opposite open arms (50X10 cm), crossed at a right angle by two arms of the same dimensions enclosed by 40-cm high walls with no roof. The maze was located 50 cm above the floor. The AnyMaze™ software (version 4.7, Stoelting) was employed for behavioral analyzes. It detects the position of the animal in the maze and calculates the percentage of entries and time spent in open and the number of enclosed arms entries.

3.5. Histology

At the end of the experiments, rats were anesthetized with chloral hydrate (5%, 1 ml/kg, i.p.), their chests were surgically opened, the descending aortas occluded, the right atrium severed and the brains perfused with 10% formalin through the left ventricle. The brains were post fixed in 10% formalin for 24 h at 4 °C and 40 μm sections were cut with the help of a cryostat (CM 1900, Leica, Germany). The placement of the injection needles was identified with the help of the rat brain atlas of Paxinos and Watson (2006). A representative photomicrography and the injection sites can be seen in Fig. 1. Rats that received injections outside the aimed area were excluded from the analyzes.

3.6. Statistical analysis

All data are expressed as the means ± SEM. Results from the Vogel conflict test and elevated plus-maze were analyzed by one-way analyzes of variance (ANOVA). Student–Newman–Keuls (S-N-K) post-hoc test was used when significant differences were observed. The data from water consumption and tail-flick tests were analyzed by repeated measures ANOVA, with time as the repeated measure and treatment as independent factor. Dunnett post-hoc test was used when significant difference was observed. In case of non-homogeneity of variances a logarithm transformation was performed. Statistical differences were considered significant when $p \leq 0.05$.

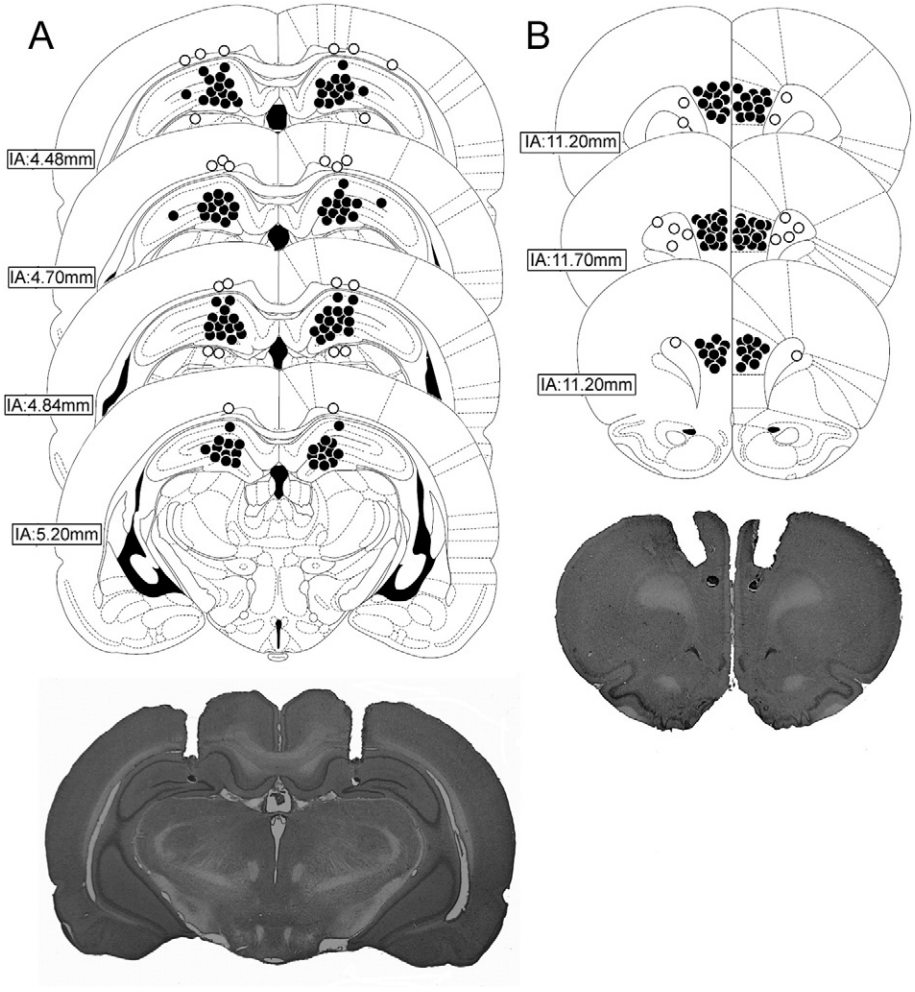

Fig. 1. Histological localization of injection sites located in the dHIP (left panel; black circles) or in the PL MPFC (right panel; black circles) in diagrams based on the atlas of Paxinos and Watson (2006). Due to overlap, the number of points represented is fewer than the real number of rats used in the experiments. The open circles represent the injections outside the dHIP and PL.

4. Results

4.1. AM404 and URB597 in the dHIP induced an anti-conflict effect in the Vogel test by activation of CB1 receptors

AM404 and URB597 injected into the dHIP significantly increased the number of punished licks ($F_{5,54} = 24.8$, $p < 0.0001$). Pre-treatment with AM251 blocked this effect ($p > 0.05$, n = 6–10/group; Fig. 2).

To control for possible analgesic or thirsty confounding effects, independent groups of animals were also submitted to the tail-flick withdrawal and water consumption tests.

In the water consumption test, multivariate ANOVA showed that there was a significant effect of day ($F_{1,52} = 5.8$, $p < 0.05$), but not of treatment ($p > 0.05$) or interaction between them ($p > 0.05$, n = 9–11/group), indicating that the treatments did not increase water consumption (Fig. 3).

In the tail-flick test (Fig. 3) there were significant effects of time ($F_{4,13} = 13.0$, $p < 0.0001$), treatment ($F_{3,16} = 96.2$, $p < 0.0001$) and interaction between them ($F_{12,35} = 12.7$, $p < 0.0001$). Morphine significantly increased the latency to tail withdrawal, an antinociceptive effect, 10 ($F_{3,16} = 26.0$, $p < 0.0001$, Dunnett), 20 ($F_{3,16} = 153,4$, $p < 0.0001$, Dunnett), 30 ($F_{3,16} = 57.0$, $p < 0.0001$, Dunnett) and 40 min ($F_{3,16} = 62.7$, $p < 0.0001$, Dunnett) from its administration. Neither AM404 nor URB597 interfere with tail withdrawal latency, indicating that these drugs did not cause analgesic effects ($p > 0.05$, n = 5/group).

4.2. AM404 and URB597 in the dHIP induced anxiolytic-like effect in the EPM by activation of CB1 receptors

Both AM404 and URB597 increased open arm exploration in the elevated plus-maze (percentage of open arms entries, $F_{5,59} = 5.6$, $p < 0.0001$, Percentage of time spent in open arms, $F_{5,59} = 4.0$, n = 9–13/group; $p < 0.005$, S-N-K). Pre-treatment with AM251 attenuated this effect. There was no effect on the number of entries into the enclosed arms ($p > 0.05$) (Fig. 4).

4.3. AM404 and URB597 in the PL induced an anti-conflict effect in the Vogel test by activation of CB1 receptors

AM404 and URB597 (Fig. 5) significantly increased the number of punished licks ($F_{5,53} = 5.2$, $p < 0.0005$; $p < 0.05$, S-N-K). Pretreatment with AM251 blocked these effects ($p > 0.05$; n = 8–11/group).

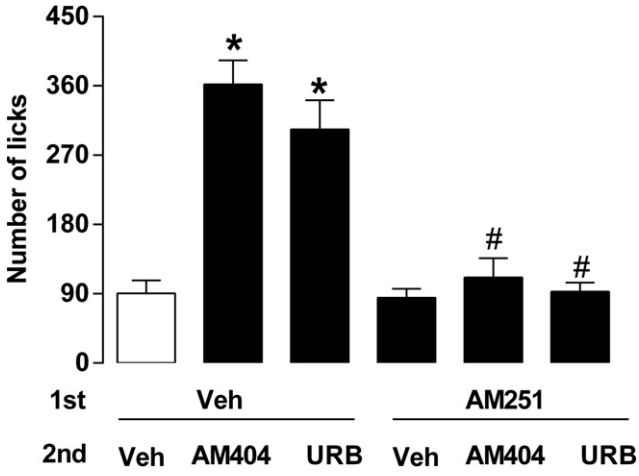

Fig. 2. Effect of anandamide (AEA) modulation in the dHIP in animals submitted to the Vogel conflict test. Vehicle (Veh; 500 nl), AM404 (50 pmol), URB (URB597; 0.01 nmol), AM251 (100 pmol). Results are expressed as means ± SEM of 6–10 animals/group. *$p < 0.05$ compared to other groups, S-N-K, #$p < 0.05$ compared to AM251 + AM404 or AM251 + URB597, S-N-K.

In the water consumption test there was a significant effect of day ($F_{1,32} = 14.0$, $p < 0.001$), but not of treatment ($p > 0.05$) or interaction between them ($p > 0.05$; n = 6–7/group; Fig. 6).

In the tail-flick test (Fig. 6) there were significant effects of time ($F_{4,17} = 8.9$, $p < 0.0001$), treatment ($F_{3,20} = 27.4$, $p < 0.0001$) and interaction between them ($F_{12,47} = 4.3$, $p < 0.005$). Morphine significantly increased the latency to tail withdrawal, an antinociceptive effect, after 10 ($F_{3,20} = 12.4$, $p < 0.0001$, Dunnett), 20 ($F_{3,20} = 14.1$, $p < 0.0001$, Dunnett), 30 ($F_{3,20} = 8.8$, $p < 0.005$, Dunnett) and 40 min ($F_{3,20} = 5.4$, $p < 0.01$, Dunnett) of its administration. Thirty min after its administration, URB597 decreased the latency to tail withdrawal. Neither AM404 nor URB597 had any other effect ($p > 0.05$, n = 5–7/group).

4.4. URB597 in the PL induced anxiolytic-like effects in the EPM by activation of CB1 receptors

URB597 significantly increased the percentage of entries in the open arms (n = 7; $F_{3,20} = 4.6$, $p < 0.02$). There was also a tendency for the same effect in the percentage of time ($F_{3,20} = 2.5$, $p = 0.09$) spent in these arms. There was no effect in the number of enclosed arm entries ($p > 0.05$). Pretreatment with AM251 blocked the anxiolytic-like effect of URB597 in the percentage of entries in the open arms (n = 6, $p > 0.05$; Fig. 7).

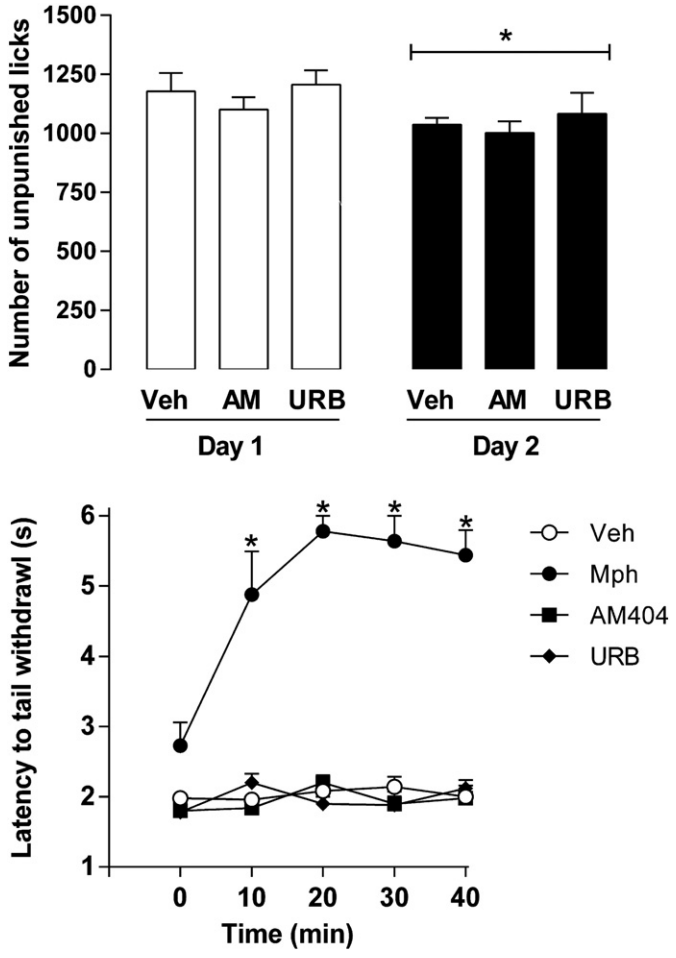

Fig. 3. Effect of anandamide (AEA) modulation in the dHIP in animals submitted to the water consumption test and tail-flick test. Upper panel: Vehicle (Veh, 500 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol). Results are expressed as means ± SEM of 9–11 animals/group. *$p < 0.05$ compared to Day 1, Repeated measures ANOVA. Lower panel: Veh (200 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol), Morphine (Mph; 5 mg/kg). Results are expressed as means ± SEM of 5 animals/group. *$p < 0.05$ compared to other groups, Dunnett.

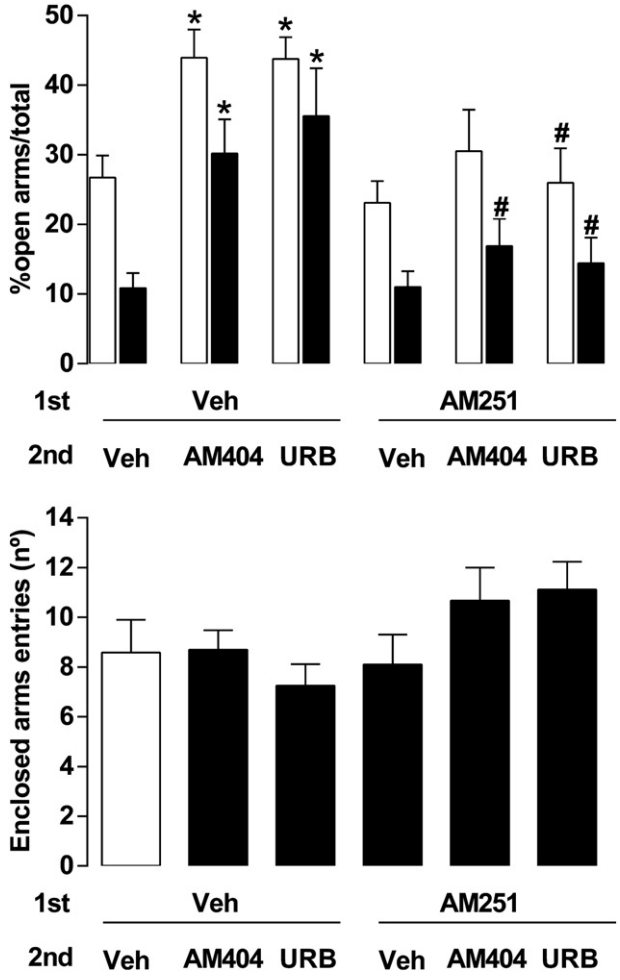

Fig. 4. Effect of anandamide (AEA) modulation in the dHIP in animals submitted to the elevated plus maze. Upper panel: percentage of open arms related to total arm entries (% open arms/total); Lower panel: number of enclosed arm entries. Vehicle (Veh, 500 nl). Results are expressed as means ± SEM of 9–13 animals/group. White bars: % entries; Black bars: % time. *$p < 0.05$ compared to veh–veh group, S-N-K, #$p < 0.05$ compared to veh + AM404 or veh + URB597 groups, S-N-K.

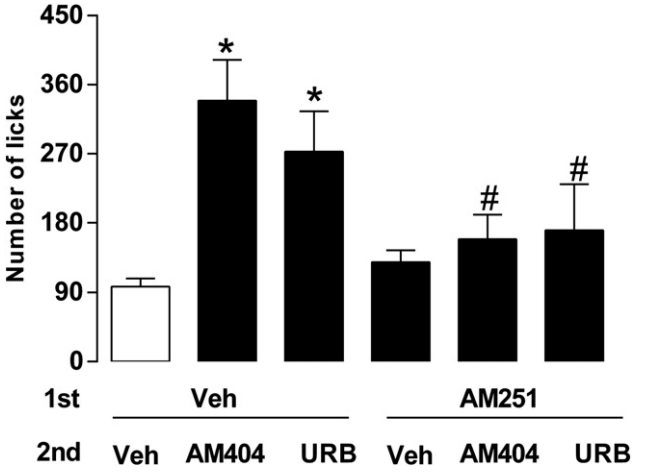

Fig. 5. Effect of anandamide (AEA) modulation in the PL MPFC in animals submitted to the Vogel conflict test. Vehicle (Veh, 200 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol), AM251 (100 pmol). Results are expressed as means ± SEM of 8–11 animals/group. *$p < 0.05$ compared to other groups, S-N-K; #$p < 0.05$ compared to AM251 + AM404 or AM251 + URB597, S-N-K.

5. Discussion

The present study shows that administration of the FAAH enzyme (URB597) or the ECBs transporter (AM404) inhibitors into the dHIP or the PL attenuated both innate and learned-anxiety in rats, effects prevented by the CB1 receptor antagonist AM251.

Since AM404 has been reported to increase levels of both AEA and 2-AG (Beltramo et al., 1997; Bisogno et al., 2001; Di et al., 2005; Hajos et al., 2004), we cannot exclude involvement of 2-AG in the observed effects of this drug. However, URB597 is an inhibitor of the FAAH enzyme, selectively increasing AEA, but not 2-AG, levels in the mouse brain after systemic injection (Fowler, 2012, 2013). This latter evidence suggests that AEA is related to our present findings.

Behavioral animal models used to assess drug effects on anxiety can be separated into two groups: those based on associative (conditioned) learning and on innate (unconditioned) fear (Millan and Brocco, 2003). Whereas the VCT is based on associative learning, the elevated plus maze (EPM) is associated with innate fear. In the VCT, water deprived rats were allowed to freely drink for 3 min. However, this behavior was also punished by a mild but aversive shock delivered on the spout of the drinking bottle, which is thought to generate a conflict situation inhibiting drinking behavior (Lisboa et al., 2008). The present results showed CB1 activation, probably due to increased ECB levels in the dHIP and the PL, reduced this conflict. Considering the VCT is a paradigm based on the conflict between water appetite and punishment, drugs influencing nociceptive thresholds or drinking motivation might yield confounding results (Millan and Brocco, 2003). Therefore, we conducted experiments to control for these possibilities. Although the test was sensitive to the positive control (morphine), dHIP or PL injection of AM404 or URB597 failed to modify withdrawal latencies in the tail-flick model 10 min after its administration, suggesting a gross change in nociceptive threshold was not responsible for the anti-punishment effects observed (Millan and Brocco, 2003). URB597 decreased the latency to tail-withdrawal 30 min after its administration into the PL. However, this effect could not explain the anti-punishment action of the drug because the VCT was performed 10 min after injection whereas the pro-nociceptive effect occurred 30 min later. Treatments also failed to influence water intake in a non-punished situation. The present results are similar to previous reports showing activation of CB1 receptors in different encephalic structures such as the dlPAG (Lisboa et al., 2008) and ventral hippocampus (Campos et al., 2010) induced anti-conflict effect in the VCT.

Independent groups of animals were also tested is the EPM, which is based on innate aversion rodents present to open and illuminated places (File, 1990). Results suggested that, similar to the VCT, activation of CB1 receptors in the dHIP and in the PL decreases nxiolytic-like behaviors. Together, our data indicate CB1 signaling in these brain structures could similarly modulate associative and innate fear.

Identification and better characterization of the ECB system led to the development of pharmacological agents that increase cannabinoid signaling, potentially being future new tools to treat anxiety-related disorders (Hill and Patel, 2013). Even if the effects of cannabinoid drugs on emotional related behaviors appear to be highly influenced by the experimental context (Haller et al., 2009; Manduca et al., 2014; Naidu et al., 2007), several studies showed anxiolytic effects (Haller et al., 2004; Rey et al., 2012) and facilitated extinction of aversion-related memories (Marsicano et al., 2002; Metna-Laurent et al., 2012) by CB1 receptors activation. The anxiolytic effects of AM404 or FAAH inhibitors systemically administrated are paralleled by increases in AEA concentrations in brain regions that include the PFC (Bortolato et al., 2006; Kathuria et al., 2003), indicating this structure could be involved in these effects. Supporting this proposal, local activation of CB1 in encephalic structures related to anxiety, such as the PAG, the MPFC and the ventral HIP decreases anxiety-related behaviors (Campos et al., 2010; Fogaca et al., 2012; Lisboa et al., 2008, 2010; Moreira et al., 2007; Resstel et al., 2008c; Rubino et al., 2008).

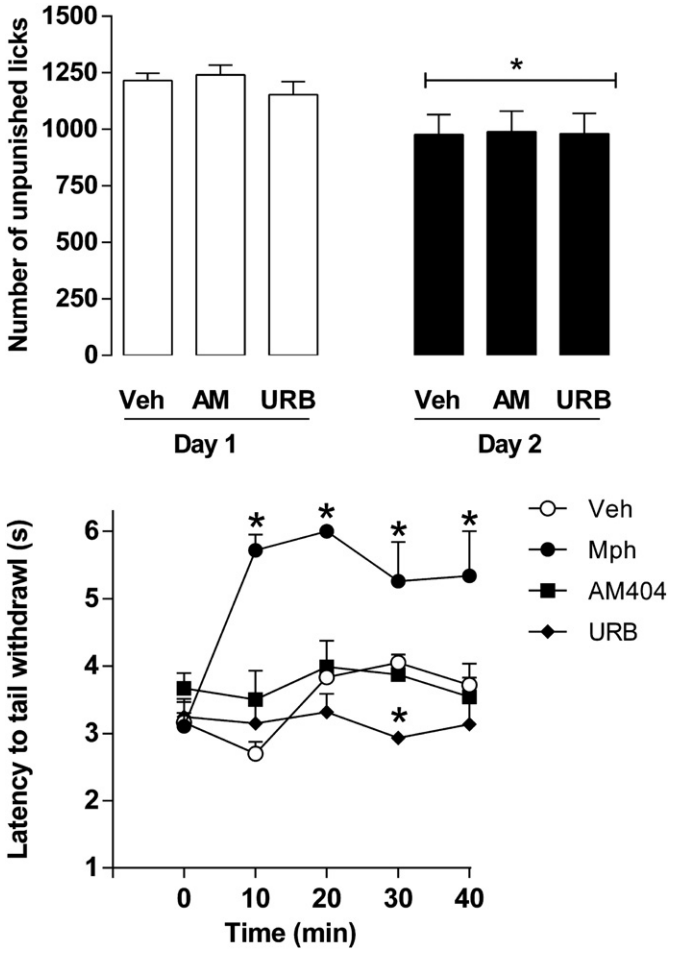

Fig. 6. Effect of anandamide (AEA) modulation in the PL MPFC in animals submitted to the water consumption test and tail-flick test. Upper panel: Vehicle (Veh, 200 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol). Results are expressed as means ± SEM of 9–11 animals/group. *p < 0.05 compared to Day 1, Repeated measures ANOVA. Lower panel: Veh (200 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol), Morphine (Mph; 5 mg/kg). Results are expressed as means ± SEM of 5 animals/group. *p < 0.05 compared to other groups, Dunnett.

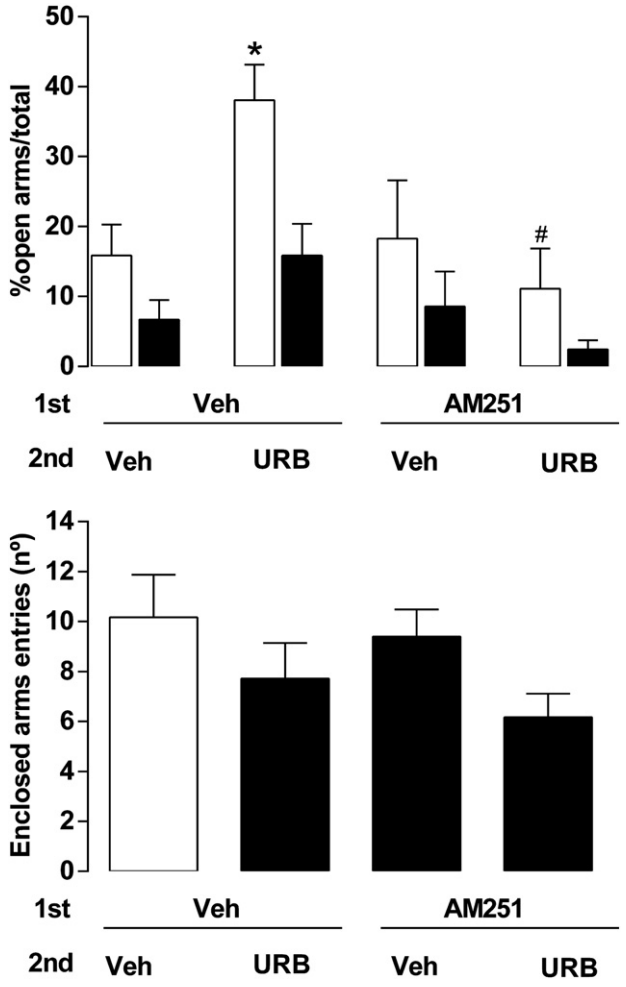

Fig. 7. Effect of anandamide (AEA) modulation in the PL MPFC in animals submitted to the elevated plus maze. Upper panel: percentage of open arms related to total arm entries (% open arms/total); Lower panel: number of enclosed arm entries. Vehicle (Veh, 200 nl), AM (AM404; 50 pmol), URB (URB597; 0.01 nmol), AM251 (100 pmol). Results are expressed as means ± SEM of 5–7 animals/group. White bars: % entries; Black bars: % time. *p < 0.05 compared to veh–veh group, S-N-K.

The MPFC and the dHIP are involved in stress- and anxiety-related disorders such as PTSD and depression (Francati et al., 2007). Stress exposure increases glutamate release in brain areas related to defensive responses (Riaza Bermudo-Soriano et al., 2012). Several pieces of evidence indicate cannabinoids modulate glutamate release in these areas (Auclair et al., 2000; Domenici et al., 2006; Kamprath et al., 2009). Glutamate not only exerts its effects through direct activation of glutamate receptors, but also modulates release of several neurotransmitters involved in stress-response, including AEA and other ECBs. Inhibition of glutamatergic neurotransmission in the MPFC induces anxiolytic-like effect in the VCT and in the CFC (Lisboa et al., 2011; Resstel et al., 2008a).

Glutamatergic pyramidal cells in the MPFC send afferents to several limbic regions, particularly to the HIP and the amygdaloid complex (Myers et al., 2011) and control HPA axis activity (Herman et al., 2004). Interference with glucocorticoids and/or glutamate transmission in these structures has been suggested as a possible mechanism of cannabinoid effects on stress-related changes. HPA axis activation in stress situations, by increasing glucocorticoid levels, could recruit MPFC ECB signaling, dampening further HPA activation by CB1 receptors and controlling stress effects (McLaughlin et al., 2014).

Therefore, PL MPFC activation of CB1 receptors could have induced anxiolytic-like effects in our study by preventing HPA axis activation and corticosterone secretion. Although this hypothesis seems unlikely in the case of the EPM since this exposure only lasts for 5 min, it could be considered for VCT results. In this test, animals received electrical shocks and the test was performed 24 h later, when increasing ECB signaling could have attenuated corticosterone release and decreased the conflict behavior. Supporting this proposition, a recent report showed that URB597 administration prior to restraint stress attenuated corticosterone release during stress (McLaughlin et al., 2014).

In the MPFC CB1 receptors are present on pre-synaptic glutamatergic neurons (Auclair et al., 2000). CB1 receptor agonists and antagonists decrease and increase, respectively, excitatory postsynaptic currents (EPSCs) in this area (Auclair et al., 2000; Devane et al., 1988). Corroborating these observations, systemic administration of a CB1 receptor antagonist increased neuronal activation in the MPFC (Alonso et al., 1999). Overall, these data suggest that glutamatergic EPSCs evoked in MPFC cells are tonically inhibited by ECBs through CB1 receptors. Therefore, in our study local activation of CB1 receptors could have attenuated anxiety-like behavior by decreasing glutamate levels.

The septum–hippocampal system performs context analyzes of threatening situations and generates anxiety in response to conflict by interrupting ongoing behavior and increasing level of arousal and attention (Gray and McNaughton, 2000). CB1 receptors are widely expressed in presynaptic terminals in the HIP (Herkenham et al., 1990). Although they are prominently present in GABAergic terminals (Marsicano and Lutz, 1999), CB1 receptors are also found in other hippocampal

neuronal subpopulations, including glutamatergic, serotonergic and cholinergic (for review see Katona and Freund, 2008).

Hippocampal CB1 receptors located on glutamatergic axonal terminals control glutamatergic synaptic neurotransmission (Domenici et al., 2006). Administration of a cannabinoid agonist to mice hippocampal slices containing axonal terminals decreased glutamatergic excitatory transmission only when CB1 receptors were absent in the GABAergic neurons, but not when they were absent in all forebrain neurons, indicating that excitatory synaptic transmission in forebrain areas is directly modulated by CB1 receptors expressed on glutamatergic presynaptic axon terminals (Domenici et al., 2006). Furthermore, long-term potentiation (LTP) deficit observed in the HIP after treatment with CB1 agonists is attributed to diminished glutamate release to levels lower than those necessary to release Mg^{+2} from NMDA receptors (Misner and Sullivan, 1999). Corroborating possible involvement of hippocampal glutamatergic CB1 receptors in neurotransmission and behavior control, depolarization-induced suppression of inhibition (DSI) was completely absent whereas depolarization-induced suppression of excitation (DSE) was rescued in genetically modified knockout CB1 mice after a rescued expression of these receptors only in telencephalic glutamatergic neurons (Glu-CB1-RS) (Ruehle et al., 2013). This indicates that expression of CB1 receptors in these glutamatergic neurons is sufficient for restoration of hippocampal DSE. Therefore, by activation of CB1 receptors in the dHIP, URB597 or AM404 probably attenuated anxiety-like behavior by decreasing glutamate levels.

Conflicting results have been reported concerning the effects on anxiety of drugs that interfere with ECBs in the dHIP. URB597 induced anxiolytic effect in the EPM when injected unilaterally into the CA1 region of the dHIP (Hakimizadeh et al., 2012). However, anxiogenic effect was observed in this brain region it the same model after injection of the CB1 receptor agonist WIN55212-2 (Roohbakhsh et al., 2007). The present results confirmed, in two different models, the anxiolytic effects of URB597. In addition to methodological (unilateral CA1 versus bilateral dentate gyrus injections) and dosage differences, the anxiogenic effect of WIN55212-2 could be reflecting a non-selective activation of CB1 receptors in this brain area, influencing, for example, both glutamatergic and GABAergic synapses. URB597 and AM404, on the other hand, would have a more selective effect, facilitating AEA only where it is being formed on demand.

In conclusion, the present results suggest that AEA in the PL and in the dHIP modulates, via CB1 receptors, aversive stimulus-induced responses both in innate and learned fear animal models. This data further strengthens previous results indicating modulation of hippocampal and MPFC activity via CB1 by ECBs, which could be therapeutically targeted to treat anxiety disorders.

Authors' disclosure

Sabrina F. Lisboa, Francisco S. Guimarães and Leonardo B. M. Resstel designed the study and wrote the protocol. Sabrina F. Lisboa, Priscila Nejo, Anna A. Borges and Aline Fassini managed the literature searches and performed the experiments. Sabrina F. Lisboa undertook the statistical analyzes and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Fundings

The research was supported by grants from FAPESP (2007/06999-9, 2012/17626-7) and CNPq. These agencies had no further role in 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.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank José C. de Aguiar, Eleni T. Gomes and Laura H. A. Camargo (University of São Paulo) for the excellent technical assistance.

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