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Cannabinoid CB$_1$ receptors in the medial prefrontal cortex modulate the expression of contextual fear conditioning

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Abstract

The ventral portion of the medial prefrontal cortex (vMPFC) has been related to the expression of contextual fear conditioning. This study investigated the possible involvement of CB$_1$ receptors in this aversive response. Male Wistar rats were submitted to a contextual aversive conditioning session and 48 h later re-exposed to the aversive context in which freezing and cardiovascular responses (increase of arterial pressure and heart rate) were recorded. The expression of CB$_1$ receptor-mRNA in the vMPFC was also measured using real time-PCR. In the first experiment intra-vMPFC administration of the CB$_1$ receptor agonist anandamide (AEA, 5 pmol/200 nl) or the AEA transport inhibitor AM404 (50 pmol/200 nl) prior to re-exposure to the aversive context attenuated the fear-conditioned responses. These effects were prevented by local pretreatment with the CB$_1$ receptor antagonist AM251 (100 pmol/200 nl). Using the same conditioning protocol in another animal group, we observed that CB$_1$ receptor mRNA expression increased in the vMPFC 48 h after the conditioning session. Although AM251 did not cause any effect by itself in the first experiment, this drug facilitated freezing and cardiovascular responses when the conditioning session employed a lesser aversive condition. These results indicated that facilitation of cannabinoid-mediated neurotransmission in the vMPFC by local CB$_1$ receptor activation attenuates the expression of contextual fear responses. Together they suggest that local endocannabinoid-mediated neurotransmission in the vMPFC can modulate these responses.

Introduction

Contextual fear-conditioning responses, characterized by freezing immobility and mean arterial pressure (MAP) and heart rate (HR) increases, are elicited when rats are re-exposed to a chamber in which they have previously received electrical footshocks (Blanchard & Blanchard, 1969; Fanselow, 1980; LeDoux *et al.* 1988; Resstel *et al.* 2008*a*,*c*). These responses are associated with increased neuronal activity in the ventral portion of medial prefrontal cortex (vMPFC) (Beck & Fibiger,

1995). This region, composed by the infralimbic (IL) and ventral portion of prelimbic (PL) cortex (Resstel & Correa, 2006; Verberne & Owens, 1998), is involved with cardiovascular, neuroendocrine and behavioural defensive responses (Frysztak & Neafsey, 1994; Radley *et al.* 2009; Resstel *et al.* 2008*e*; Schulkin *et al.* 2005; Sierra-Mercado *et al.* 2006; Tavares *et al.* 2009). Associating electrical footshocks with cue auditory stimulus (CS), Frysztak & Neafsey (1994) described the first evidence relating the vMPFC with modulation of fear-conditioning responses. They showed that vMPFC lesions reduce the autonomic and behavioural responses during CS presentation. Corroborating these findings, recent data from our group showed that vMPFC is also important for the expression of contextual fear conditioning, modulating both cardio-vascular and behavioural responses associated with

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this aversive situation (Resstel *et al.* 2006*a*). Moreover, these effects seem to involve local neurotransmitter systems mediated by glutamate NMDA, nitric oxide (NO) and dopamine receptors (Pezze *et al.* 2003; Resstel *et al.* 2008*b*).

Several pieces of evidence indicate that the endocannabinoid system is critical for the processing of expression of emotional memory (Laviolette & Grace, 2006; Marsicano *et al.* 2002; Tan *et al.* 2010). In addition, systemic activation or blockade of cannabinoid CB_1 receptors (CB_1Rs) in the MPFC modulates emotional associative learning and memory formation (Laviolette & Grace, 2006).

Endocannabinoids (eCBs) can also modulate conditioned fear (Resstel *et al.* 2009). Cannabinoid CB_1R expression is considerably high in brain regions related to conditioned fear such as the vMPFC (Herkenham *et al.* 1991; Tsou *et al.* 1998). These receptors are predominantly localized on axon terminals where they modulate neurotransmitter release (Egertova *et al.* 2003; Howlett, 1995; Melvin *et al.* 1995; Wilson *et al.* 2001; Wilson & Nicoll, 2001), including those related to the regulation of fear conditioning (Beinfeld & Connolly, 2001; Davies *et al.* 2002; Herkenham *et al.* 1991). CB_1R agonists administered into the MPFC cause anxiolytic-like effects (Rubino *et al.* 2008*a,b*), reinforcing the proposal that eCBs in this brain area modulate emotional states. However, a possible involvement of CB_1Rs in the vMPFC in the expression of contextual fear-conditioning responses has not yet been investigated.

To address this problem we measured the expression of CB_1R-mRNA in vMPFC in fear-conditioned and non-conditioned rats. Moreover, we examined the effects of bilateral injections of the endocannabinoid anandamide (AEA) or the AEA re-uptake inhibitor AM404 into the vMPFC of rats submitted to a contextual fear-conditioning protocol. The possible involvement of local CB_1Rs in these effects was also investigated by combining these later drugs with the CB_1R antagonist AM251. In a last experiment, to verify if the eCB system in the vMPFC could tonically inhibit the expression of fear conditioning, we tested the effects of AM251 in a conditioning protocol that employed footshocks of lower intensities.

Materials and methods

Animal preparation

Male Wistar rats weighing 230–270 g (aged 45–47 d) were used. Animals were kept in the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. Rats were housed individually in plastic cages under a 12-h light/dark cycle (lights on 06:30 hours) with food and water available *ad libitum*. The Institution's Animal Ethics Committee approved housing conditions and experimental procedures (process number: 215–2005).

Seven days before the test day rats were anaesthetized with tribromoethanol (Sigma-Aldrich, USA; 250 mg/kg i.p.). After scalp anaesthesia with 2% lidocaine the skull was surgically exposed and stainless-steel guide cannulae (26 G) were bilaterally implanted into the vMPFC using a stereotaxic apparatus (Stoelting, USA). Coordinates for cannulae implantation (AP = +2.2 mm, L = 2.8 mm from the medial suture, V = −3.3 mm from the skull with a lateral inclination of 23°) were selected from the rat brain atlas of Paxinos & Watson (1997). A control group of animals had stainless-steel guide cannulae implanted bilaterally into surrounding structures of the vMPFC such as cingulate cortex area 1 (AP = +1.2 mm, L = 1.5 mm from the medial suture, V = −2.3 mm from the skull) and the corpus callosum (AP = +1.2 mm, L = 2.8 mm from the medial suture, V = −2.3 mm from the skull). Cannulae were fixed to the skull with dental cement and one metal screw. After surgery, the animals received a poly-antibiotic injection (Pentabiotico®, Brazil) with streptomycins and penicillins to prevent infection and a nonsteroidal anti-inflammatory flunixine meglumine (Banamine®, Brazil) for post-operation analgesia.

One day prior to test day rats were anaesthetized with tribromoethanol and a catheter (a 4 cm PE-10 segment heat-bound to a 13 cm PE-50 segment, Clay Adams, USA) was inserted into the abdominal aorta through the femoral artery for cardiovascular recording. The catheter was tunnelled under the skin and exteriorized on the animal's dorsum.

Drugs

The endogenous cannabinoid AEA (Tocris, USA) and the AEA transporter inhibitor 4-hydroxyphenyl-arachidonylamide (AM404; Tocris) were 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. The CB_1R antagonist *N*-(piperidin-1 yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-H-pyrazole-3-carboxamide (AM251; Tocris) was dissolved in 10% DMSO 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, USA) and urethane (Sigma) were dissolved in distilled water and FG-7142 (Tocris) was suspended in poly-oxyethylenesorbitan monooleate (Tween 80, Sigma) 2% in saline.

Fear conditioning and testing

Habituation, conditioning and testing were performed in $25 \times 22 \times 22$ cm footshock chambers. The chambers had a grid floor composed of 18 stainless-steel rods (2 mm in diameter), spaced 1.5 cm apart and wired to a shock generator (Automatic Reflex Conditioner, model 8572; Ugo Basile, Italy). The chambers were cleaned with 70% ethanol between each animal. Preconditioning started 1 wk after stereotaxic surgery and consisted of one 10-min-long pre-exposure (habituation) to the footshock chamber. In the conditioning shock session, performed 24 h after the habituation session, animals were separated into two experimental groups: non-conditioned and conditioned. The non-conditioned group was exposed to the footshock chamber for 10 min but no shock was delivered. The conditioned group was submitted to a shock session consisting of six electric 1.5 mA/3 s footshocks (Resstel *et al.* 2008*a,b*) delivered at 20-s to 1-min intervals. Twenty-four hours after the conditioning session, a catheter was implanted into the femoral artery for cardiovascular recording.

An additional group of animals was submitted to a different conditioning protocol to obtain reduced conditioned fear responses as described previously (Baldi *et al.* 2004). Three electric footshocks of 0.85 mA/3 s were delivered at 20-s to 1-min intervals.

Cardiovascular and behavioural (freezing) responses evoked by conditioned emotional response to context were evaluated 2 d after the conditioning session. The test session consisted of a 10-min-long re-exposure to the footshock chamber without shock delivery. Animals were transferred from the animal room to the procedure room (a different room was used for conditioning) in their home box. MAP and heart rate HR were recorded using an HP-7754A amplifier (Hewlett Packard, USA) connected to a signal acquisition board (Biopac M-100, USA) and computer processed. Rats were tested one at a time. After 10 min adaptation, injections were performed into the vMPFC. Two 33 G needles (Small Parts, USA) 1 mm longer than the guide cannula and connected to a 10-μl syringe (7002-H, Hamilton Co., USA) through a PE-10 tubing were used. The needles were carefully inserted into the guide cannulae and the solutions were infused over a 30-s period with a rate of 400 nl/min. They

remained in place for an additional 20-s period to prevent reflux. The interval between the first and second microinjection was 5 min and the animals were tested 10 min after the last microinjection. Cardiovascular parameters recorded in the 10 min following the last drug injection were used as baseline measurements.

Freezing was evaluated during the test by an experimenter seated 50 cm from the footshock chamber who was blind to the treatment groups. Freezing was defined as the complete absence of movement while the animal assumed a characteristic tense posture (Fanselow, 1980; Resstel *et al.* 2006*b,c*). Since a pilot study indicated that no significant decrease of freezing responses occurs (data not shown) during the experimental session, the percentage of the total freezing time was used to evaluate the drug effects.

Sample collection and RNA extraction

Independent groups of non-conditioned and conditioned animals were decapitated 48 h after the conditioning session (at the moment they should have been re-exposed to the aversive context) without chamber re-exposure between 09:00 and 10:00 hours. These animals were not submitted to any surgical intervention.

Immediately after decapitation, the vMPFC was collected by microdissection in RNAse-free conditions. Using a stainless-steel punch needle of 1.5 mm diameter, microdissections were obtained according to coordinates from the atlas of Paxinos & Watson (1997): vMPFC: 1000 μm; 3.2–2.2 from bregma. Tissue samples were transferred to a microtube with RNAlater reagent (Ambion, USA) and stored at -80 °C untILRNA isolation.

Total RNA was isolated using TRIzol reagent (Invitrogen®, New Zealand), according to the manufacturer's instructions. Briefly, TRIzol (750 μl/microtube) was added to the sample, shaken for 30 s and incubated on dry ice for 5 min. To each millilitre of the suspension, 200 μl chloroform (Sigma) and 10 μl glycogen (20 mg/ml) were added, vortexed, incubated at room temperature for 5 min and then centrifuged at 14 000 rpm for 25 min at 4 °C. The aqueous phase was transferred to a new microtube, to which 500 μl isopropanol (Sigma) was added. The sample was vortexed and incubated overnight at -80 °C. The next day, the samples were centrifuged at 14 000 rpm for 15 min at 4 °C. The pellet was washed in 70% ethanol, centrifuged at 14 000 rpm for 15 min at 4 °C and dried at room temperature. RNA samples were re-suspended in 15 μl diethylpyrocarbonate (DEPC)-treated water and stored at -80 °C. The concentration

of RNA was determined by UV spectrophotometer and then 500 ηg RNA was used for cDNA synthesis, using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA).

Real-time PCR reactions

Quantitative real-time PCR (qPCR) was performed using Applied Biosystems 7500 Real-Time PCR System. The following TaqMan® Gene Expression Assays (Applied Biosystems) were used in this study: Rn 00562880_m1 (CnR1). The PCR reaction was performed in triplicate. Water instead of cDNA was used as a negative control. Reference genes (β-actin and *GAPDH*) were run for each cDNA sample. Determination of gene transcript in each sample was obtained by the $\Delta\Delta C_q$ method. For each sample, the quantification cycle (Q_c) of mRNA was measured and normalized to the average of the reference genes ($\Delta C_q = C_q$ unknown $- C_q$ reference genes). The fold change of mRNA in the unknown sample relative to control group was determined by $2^{-\Delta\Delta C_q}$, where $\Delta\Delta C_q = \Delta C_q$ unknown $- \Delta C_q$ control. Data are shown as a relative percentage mRNA expression to the control group.

Experimental design

Each animal received two bilateral injections of 200 nl into the vMPFC of the drugs or their respective vehicles. The experimental groups were: vehicle + vehicle ($n=6$), vehicle + AEA (5 pmol, $n=6$), vehicle + AM404 (50 pmol, $n=6$), AM251 (100 pmol) + vehicle ($n=6$), AM251 (100 pmol) + AEA (5 pmol, $n=6$) or AM251 (100 pmol) + AM404 (50 pmol, $n=6$). The doses of AEA and AM404 were similar to those which had produced anxiolytic effects in the contextual fear conditioning and Vogel conflict tests after injection into the dorsolateral periaqueductal grey matter in previous studies (Lisboa *et al.* 2008; Resstel *et al.* 2008*d*). The dose of AM251 was the same that blocked eCB effects in the same studies. The interval between the first and second microinjection was 5 min and the animals were tested 10 min after the last microinjection.

In order to evaluate the effects of CB$_1$R antagonism in the vMPFC in a less aversive conditioning protocol, an additional animal group received a single bilateral microinjection of AM251 (100 pmol, $n=7$) 15 min before chamber re-exposure. As a positive control of the possible anxiogenic-like effect on fear-conditioning responses induced by AM251, the inverse benzodiazepine agonist FG-7142 (8 mg/kg, dose based on Hart *et al.* 1999, was administered systemically 20 min before chamber re-exposure in both aversive conditioning protocols ($n=5$ each intensity animal group).

Histological procedure

At the end of the experiments the rats were anaesthetized with urethane (1.25 g/kg i.p.) and 200 nl of 1% Evan's Blue dye was bilaterally injected into the vMPFC to mark the injection site. The chest was surgically opened; the descending aorta occluded; the right atrium severed and the brain perfused with 10% formalin through the left ventricle. Brains were post-fixed for 24 h at 4 °C, and 40-μm sections were cut using a cryostat (CM-1900, Germany). Serial brain sections were stained with 1% Neutral Red and injection sites determined using the rat brain atlas of Paxinos & Watson (1997) as reference.

Data analysis

The possible differences in the intensity of CB$_1$R-mRNA expression in the vMPFC of conditioned and non-conditioned rats were compared using Student's *t* test.
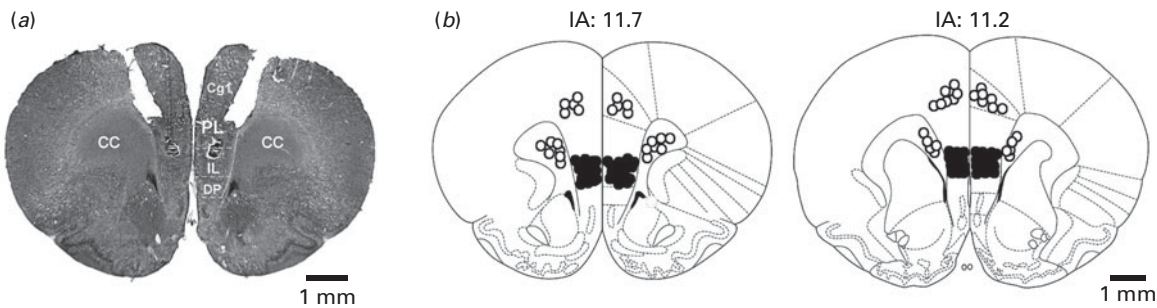
MAP and HR values were continuously recorded during the 5-min period before and the 10-min period after exposure to the footshock chamber. Data were expressed as means ± S.E.M. of MAP or HR changes (respectively ΔMAP and ΔHR) sampled at 60-s intervals. Points sampled during the 300 s before exposure were used as control baseline value. MAP and HR changes were analysed using three-way ANOVA with condition (conditioned or non-conditioned) and treatment (drug or vehicle) as main independent factors, and time as a repeated factor. When interaction between the factors was observed, groups were compared at specific times using Bonferroni's *post-hoc* test.

Freezing was expressed as percentage of the whole test period (600 s). Freezing, crossings and rearing were analysed using two-way ANOVA with condition (conditioned or non-conditioned) and treatment (drug or vehicle) as the two factors. When interaction between the factors was observed, specific one-way ANOVA followed by Bonferroni's *post-hoc* test was performed. $p < 0.05$ was assumed as statistically significant.

Results

Diagrammatic representation indicating the injection sites of vehicle and drugs and a photomicrograph showing a representative bilateral injection site in the vMPFC are presented in Fig. 1.

Fig. 1. (*a*) Photomicrograph of a coronal brain section showing bilateral microinjection sites in the vMPFC. (*b*) Diagrammatic representation based on the rat brain atlas of Paxinos & Watson (1997) indicating the injection sites in the vMPFC (●). Animals with drug injection sites outside the vMPFC were represented by open circles (○). Cg1, Cingulate cortex area 1; PL, prelimbic cortex; IL, infralimbic cortex; DP, dorsal peduncular cortex; CC, corpus callosum; IA, interaural.

CB$_1$-mRNA expression showed a significant increase (about 23%, $t = 9.17$, $p < 0.001$) in conditioned animals ($n = 6$) compared to control non-conditioned animals ($n = 8$) (Fig. 2).
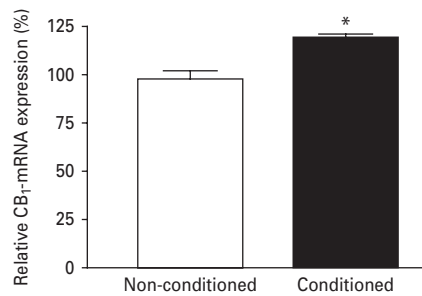
Behavioural responses to contextual fear conditioning

There were significant effects of condition ($F_{1,54} = 123$, $p < 0.001$), treatment ($F_{5,54} = 8.5$, $p < 0.001$) and condition × treatment interaction between the two factors ($F_{5,54} = 4.67$, $p < 0.001$) on time spent in freezing behaviour. Vehicle-treated rats which had received electrical footshocks (conditioned group) spent more time freezing during re-exposure to context than the animals that did not receive shocks (non-conditioned group) (Fig. 3). Bilateral injection into the vMPFC of AEA ($n = 6$, $p < 0.001$) or AM404 ($n = 6$, $p < 0.001$) reduced the time spent in freezing behaviour on conditioned animals when compared to the respective vehicle-treated group ($n = 6$, $F_{5,30} = 9.8$, $p < 0.001$, Fig. 3). Pretreatment with AM251 blocked the effects of AEA ($n = 6$) and AM404 ($n = 6$) (Fig. 3). Compared to vehicle-treated animals, AM251 ($n = 6$) did not change the time spent in freezing behaviour of conditioned animals ($p > 0.05$, Fig. 3).

In non-conditioned animals ($n = 5$ each group) no significant treatment effect was found ($F_{5,24} = 1$, $p > 0.05$, Fig. 3). No drug effect on motor activity was found in non-conditioned animals (crossings: $F_{5,24} = 0.9$, $p > 0.05$; rearings: $F_{5,24} = 0.7$, $p > 0.05$).

Cardiovascular responses to contextual fear conditioning

In both groups, conditioned and non-conditioned, bilateral injection of AEA, AM404 or AM251 into the
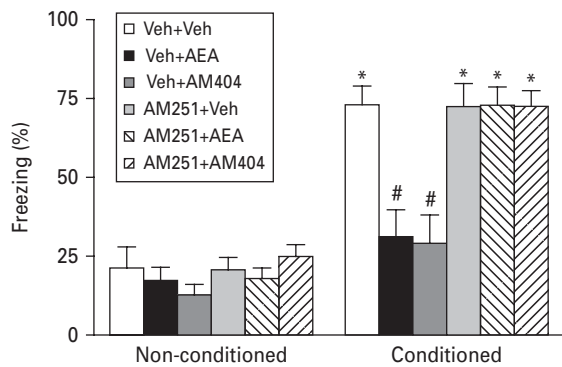

Fig. 2. CB$_1$-mRNA expression in the vMPFC of non-conditioned ($n = 8$) and conditioned ($n = 6$) animals observed 48 h after the fear-conditioning session. The columns represent the means and bars the S.E.M., * $p < 0.05$, Student's *t* test.

vMPFC had no effect on basal levels of both MAP and HR when compared to vehicle control group ($F_{7,36} = 0.97$, $p > 0.05$).

There were significant effects of condition, treatment and condition × time interaction on both HR ($F_{23,250} = 1.7$, $p < 0.05$) and MAP ($F_{23,250} = 2.5$, $p < 0.05$). In the conditioned group bilateral injection of either AEA or AM404 into the vMPFC significantly reduced the increase in MAP ($F_{5,450} = 80$, $p < 0.001$) and HR ($F_{5,450} = 31$, $p < 0.001$) (Fig. 4). In the non-conditioned group re-exposure to the context also induced an increase in HR and MAP, although smaller than that observed in the conditioned group (MAP: $F_{14,360} = 20$, $p < 0.01$; HR: $F_{14,360} = 6.9$, $p < 0.01$). However, no significant treatment effect was found on cardiovascular responses (MAP: $F_{5,360} = 0.7$, $p > 0.05$; HR: $F_{5,360} = 1$, $p > 0.05$) (Fig. 4).

Bilateral injection of AM251 had no effect on cardiovascular responses by itself on conditioned ($p > 0.05$)

Fig. 3. Effects of bilateral microinjections of 200 nl of vehicle + vehicle (Veh, $n = 6$), Veh + AEA (5 pmol, $n = 6$), Veh + AM404 (50 pmol, $n = 6$), AM251 (100 pmol) + Veh ($n = 6$), AM251 (100 pmol) + AEA ($n = 6$) or AM251 (100 pmol) + AM404 ($n = 6$) in the percentage of time spent in freezing behaviour in non-conditioned and fear-conditioned rats. Columns represent the means and bars the S.E.M., * $p < 0.05$ compared to the Veh + Veh non-conditioned group and # $p < 0.05$ compared to Veh + Veh conditioned group; Bonferroni's *post-hoc* test.

and non-conditioned ($p > 0.05$) groups. However, it antagonized the effects of AEA and AM404 on cardiovascular responses (MAP: $p > 0.05$; HR: $p > 0.05$) (Fig. 4).

Microinjection of AEA or AM404 into the cingulate cortex area 1 ($n = 6$ each drug) or the corpus callosum ($n = 6$ each drug) during the chamber re-exposure test in conditioned animals did not change behavioural ($F_{4,25} = 0.21$, $p > 0.05$) and cardiovascular (MAP: $F_{4,375} = 0.64$, $P > 0.05$; HR: $F_{4,375} = 0.51$, $p > 0.05$) responses compared to vehicle-treated animals ($n = 6$, data not shown).

Effects of bilateral vMPFC microinjections of AM251 or systemic administration of FG-7142 using a less aversive conditioning session

The group conditioned with a less aversive conditioning protocol ($n = 7$) spent reduced time in freezing behaviour ($41.6 \pm 5\%$ *vs.* $74 \pm 5\%$, $t = 4.3$, $p < 0.05$) and reduced cardiovascular responses (MAP: $F_{1,165} = 145$, $p < 0.001$; HR: $F_{1,165} = 183$, $p < 0.001$) during the re-exposure to the context in comparison to the group which received more aversive conditioning protocol ($n = 6$).

Using the more aversive conditioning protocol, FG-7142 ($n = 5$) had no effects on both freezing ($75.8 \pm 6.6\%$ *vs.* $79.5 \pm 7.1\%$, $t = 0.4$, $p > 0.05$) and cardiovascular (MAP: $F_{1,153} = 1.6$, $p > 0.05$; HR: $F_{1,153} = 0.04$, $p > 0.05$) responses when compared to the

vehicle-treated group ($n = 6$). However, in the less aversive conditioning protocol ($n = 5$), FG-7142 increased freezing ($44.7 \pm 4.9\%$ *vs.* 73.6 ± 3.2, $t = 4.9$, $p < 0.01$) and cardiovascular (MAP: $F_{1,120} = 51.5$, $p < 0.001$; HR $F_{1,120} = 35.5$, $p < 0.001$) responses when compared to the vehicle-treated group ($n = 5$; data not shown). Similarly, bilateral administration of AM251 into the vMPFC of conditioned animals submitted to the less aversive conditioning protocol ($n = 6$) increased freezing ($44.9 \pm 6.2\%$, *vs.* $71.58 \pm 6.2\%$, $t = 3.2$, $p < 0.01$) and cardiovascular (MAP: $F_{1,165} = 69.3$, $p < 0.001$; HR: $F_{1,165} = 79.8$, $p < 0.001$) responses ($n = 7$) (Fig. 5). A diagrammatic representation indicating the vMPFC injection sites of vehicle or AM251 in this latter group of animals is presented in Fig. 5.
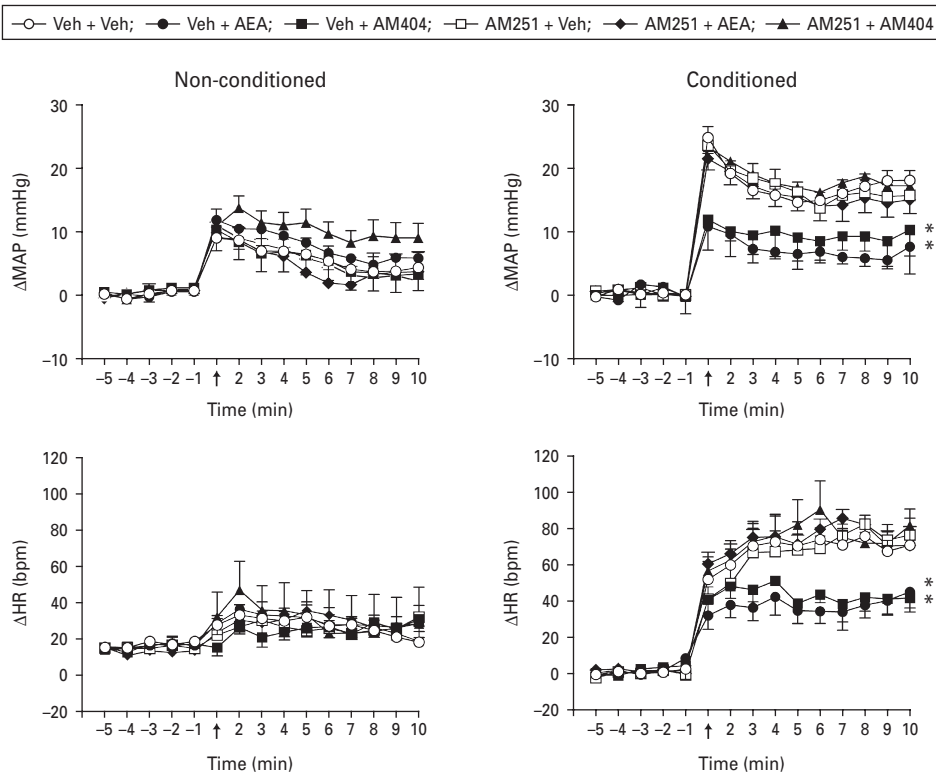
Discussion

The main result of the present study is that CB_1Rs located in the vMPFC could play a key role in fear-conditioning responses associated with aversive context. Moreover, this study provides the first indication that a tonic CB_1-dependent mechanism in the vMPFC modulates the expression of contextual fear conditioning.

Several studies have indicated the participation of eCBs in defensive responses. For example, Bortolato *et al.* (2006) using different rat models of anxiety showed that systemic administration of AM404 exerted dose-dependent anxiolytic-like effects associated with increased levels of AEA in vMPFC, which were prevented by pre-administration of the CB_1R antagonist rimonabant (SR141716). Similar results were also observed after systemic administration of the CB_1R agonists CP 55,940 and WIN 55212-2, whereas the antagonists SR141716 and AM251 produced opposite responses (Patel & Hillard, 2006).

Similar to our results, Pamplona *et al.* (2006) described systemic treatment with WIN 55212-2 that decreased the expression of contextual fear conditioning, an effect prevented by pretreatment with the CB_1R antagonist SR141716. We now extend these findings by implicating the vMPFC as a possible site of these effects, since similar results were observed after local microinjection of AEA, a CB_1R agonist, or AM404, an AEA transport inhibitor.

The involvement of eCB-mediated neurotransmission in the vMPFC in aversive responses has been suggested by previous studies. Rubino *et al.* (2008 a, b) showed that microinjection of Δ^9-tetrahydrocannabinol (THC) or the AEA analogue methanandamide into the vMPFC produced anxiolytic-like effects in rats. These effects were prevented by pretreatment with
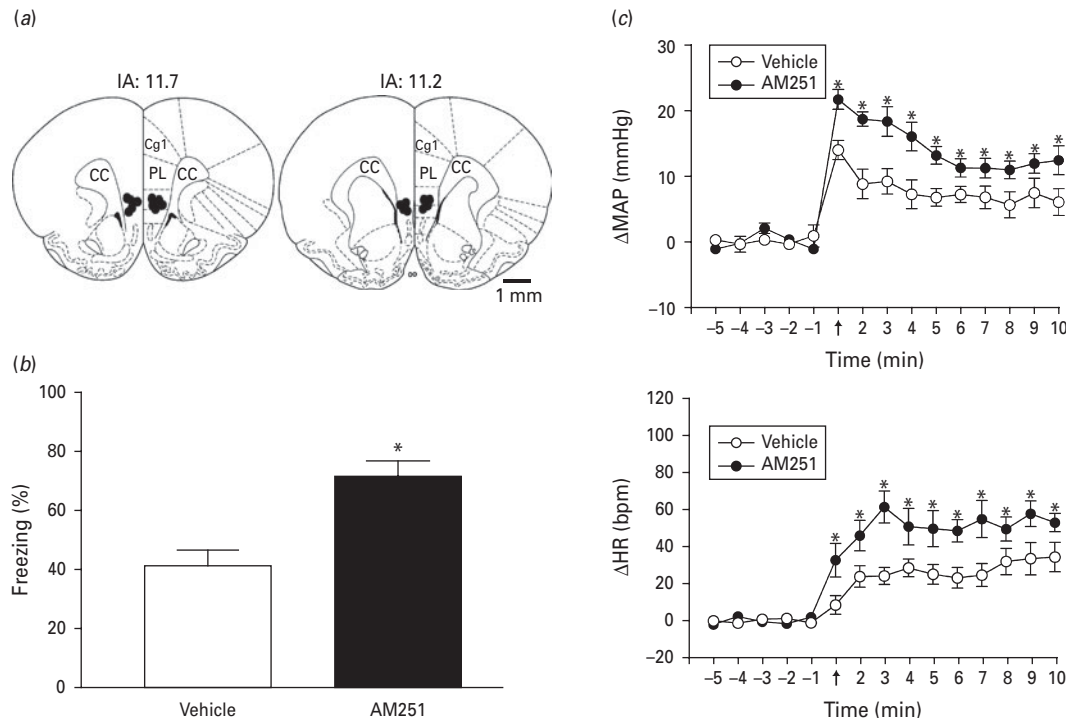
Fig. 4. Time-course of the effects of bilateral microinjection of 200 nl of vehicle + vehicle ($n = 6$), vehicle + AEA (5 pmol, $n = 6$), vehicle + AM404 (50 pmol, $n = 6$), AM251 (100 pmol) + vehicle ($n = 6$), AM251 (100 pmol) + AEA ($n = 6$) or AM251 (100 pmol) + AM404 ($n = 6$) in the mean arterial pressure (ΔMAP) and heart rate (ΔHR) recorded in non-conditioned and fear-conditioned rats. Arrows indicate the start of the chamber re-exposure. Symbols represent the means and bars the S.E.M. The asterisk indicates significant treatment difference ($p < 0.05$, Bonferroni's *post-hoc* test) over the whole footshock chamber exposure period compared to vehicle-treated animals.

the CB₁R antagonist AM251 (Rubino *et al*. 2008*a*, *b*). Moreover, infusion of AM251 into the vMPFC blocked the extinction of fear memory in a fear-potentiated startle model whereas WIN 55212-2 facilitated extinction (Lin *et al*. 2009). Together, these studies suggest that activation of CB₁Rs in the vMPFC could modulate conditioned fear responses, reinforcing the proposed role of this brain area in this process (Resstel *et al*. 2006*a*, *b*). However, in our first experiment AM251 had no effect on the fear-conditioning response by itself, which might indicate that endogenous eCB-mediated neurotransmission is not related to this process. Nevertheless, fear-conditioning responses to context are known to increase as footshock intensity is increased (Baldi *et al*. 2004) and rats exposed to 1.2 mA footshocks expend about 80% of the session in freezing behaviour. To test if a ceiling effect was interfering with our results we performed an additional experiment decreasing footshock intensity in the conditioning session. Similar to the results of Baldi *et al*. (2004), this lower intensity (0.85 mA) caused a significant decrease in freezing time. Using a higher

electrical footshock intensity (1.5 mA) we have previously failed to detect a potentiation of contextual fear-conditioned responses after systemic injection of the inverse benzodiazepine receptor agonist FG-7142 (Berntson *et al*. 1996), although this anxiogenic compound has been shown to increase defensive-like cardiovascular reactivity to a moderate-intensity auditory stimulus (Quigley *et al*. 1994).

Corroborating this later study, FG-7142 evoked an increase in fear-conditioning responses when compared to the vehicle-treated group in the less aversive paradigm (data not shown). These results suggest that a ceiling effect caused by a more aversive conditioning session could have prevented the detection of AM251 effects. In agreement with this suggestion, intra-vMPFC administration of AM251 increased the freezing and cardiovascular contextual fear responses when a reduced electrical footshock intensity was used in the conditioning session.

This ceiling effect could help to explain contradictory results regarding the role of eCBs on fear conditioning. For example, whereas systemic

(a)

(b)

(c)

Fig. 5. (a) Diagrammatic representation based on the rat brain atlas of Paxinos & Watson (1997) indicating injections sites of vehicle or AM251 into the vMPFC of rats conditioned with reduced electrical footshock intensity. Cg1, Cingulate cortex area 1; PL, prelimbic cortex; CC, corpus callosum; IA, interaural. (b) Effects of bilateral microinjections of 200 nl vehicle ($n=7$) or 100 pmol AM251 ($n=6$) in the percentage of time spent in freezing behaviour in rats conditioned with reduced electrical footshock intensity. Columns represent the means and bars the S.E.M., * $p<0.05$, Student's t test. (c) Time-course of the effects in the mean arterial pressure (ΔMAP) and heart rate (ΔHR) of bilateral microinjections of 200 nl vehicle ($n=7$) or 100 pmol AM251 ($n=6$) in rats conditioned with reduced electrical footshock intensity. Arrows indicate the start of the chamber re-exposure. Symbols represent the mean and bar the S.E.M. * $p<0.05$, Bonferroni's *post-hoc* test.

administration of CB_1R antagonists increased the expression of fear conditioning to tone (Arenos *et al.* 2006; Kamprath *et al.* 2006; Niyuhire *et al.* 2007; Reich *et al.* 2008) and CB_1 knockout mice showed increased freezing to an auditory cue (Kamprath *et al.* 2006), the systemic administration of CB_1R antagonists failed to affect contextual freezing expression (Arenos *et al.* 2006; Pamplona *et al.* 2006; Suzuki *et al.* 2004), except when the contextual fear-conditioning protocol also included tone presentation. On the other hand, genetic disruption of CB_1Rs or systemic administration of the CB_1R antagonist AM251 abolished contextual conditioned fear responses in mice (Mikics *et al.* 2006). Different protocols and species used (rats or mice) could also be involved in these contradictory results (for review see Resstel *et al.* 2009).

Studies employing electrophysiological recording, neuronal inactivation and local drug microinjection have related NMDA receptor-mediated glutamatergic neurotransmission in the vMPFC to the expression of contextual fear-conditioning responses (Beck & Fibiger, 1995; Corcoran & Quirk, 2007; Resstel *et al.* 2006a). In the prefrontal cortex, CB_1Rs are localized pre-synaptically in glutamatergic neuron terminations (Auclair *et al.* 2000) and the CB_1R agonists WIN 55212-2 and CP 55,940 (Devane *et al.* 1988) decrease excitatory post-synaptic currents (EPSCs) whereas the CB_1R antagonist SR141716A increased them (Auclair *et al.* 2000). In agreement with these observations, systemic administration of a CB_1R antagonist increased neuronal activation in the vMPFC (Alonso *et al.* 1999). These results suggest that glutamatergic EPSCs evoked in vMPFC cells are tonically inhibited by endogenous cannabinoids through CB_1Rs. Recent evidence has also shown that CB_1Rs mediate fear adaptation, an effect that depends on eCBs interference with glutamatergic transmission in cortical brain structures (Kamprath *et al.* 2009). These results and our present findings suggest that eCBs could play a major role in the expression of contextual fear

conditioning by controlling glutamatergic synaptic transmission in the vMPFC.

However, cannabinoids could also interfere with fear conditioning in the vMPFC by modulating other neurotransmission systems. For example, local infusion of CB_1 agonist WIN 55212-2 into this brain region has been proposed to facilitate extinction of fear-potentiated startle by decreasing GABA release (Lin *et al.* 2008). Moreover, opposite consequences of local application of cannabidiol or electrical stimulation of either the IL or the PL cortices on the expression of conditioned fear have been reported (Lemos *et al.* 2009; Vidal-Gonzalez *et al.* 2006). Although in the present studies these two regions have not been separated, most of our injections were performed into the ILand ventral portion of PL cortex. Previous studies showed that neurotransmission inhibition of this area inhibits the expression of contextual fear conditioning (Corcoran & Quirk, 2007; Resstel *et al.* 2006*a*). Therefore, more studies are needed to fully understand how eCB modulation of local neurotransmission in subregions of the vMPFC interferes with fear-conditioning responses.

CB_1R-mRNA expression was increased in the vMPFC 48 h after the aversive conditioning session. Although further studies investigating if this effect is reflected by CB_1 protein expression changes, this result agrees with several reports showing changes in eCB-mediated neurotransmission after stress exposure (for review see Lutz, 2009) and that CB_1R density increases in the prefrontal cortex of rodents exposed to chronic unpredictable stress for 21 d (Hill *et al.* 2008).

Previous studies have shown that acquisition and retention of conditioned fear extinction, elicited by discrete cues are reduced in CB_1 knockout mice or by systemic administration of a CB_1R antagonist (Kamprath *et al.* 2006, 2009; Marsicano *et al.* 2002; Niyuhire *et al.* 2007). Similar, but not the same, results have also been reported for contextual fear conditioning. Suzuki *et al.* (2004), for example, failed to find any effect of CB_1R antagonism on extinction acquisition in mice, but did find that this treatment was able to block retention of extinction. In rats, however, systemic administration of a CB_1R agonist facilitated the acquisition of extinction of contextual fear conditioning whereas CB_1R antagonism disrupted it (Pamplona *et al.* 2006). Despite the contradictory results, these results indicate that either genetic ablation or pharmacological CB_1R antagonism modulates the acquisition and retention of fear memories extinction. The vMPFC has also been related to this effect. For example, irreversible vMPFC electrolytic lesions or acute and reversible vMPFC inhibition by tetrodotoxin prior to the acquisition session were able to impair recall of extinction (Quirk *et al.* 2000; Sierra-Mercado *et al.* 2006). In addition, microinjections of the protein synthesis inhibitor anisomycin microinjected into vMPFC impaired fear extinction (Santini *et al.* 2004). The involvement of vMPFC eCBs on fear extinction, however, has not been investigated and needs further consideration.

Despite the use of single doses being a limitation of the present work, the results agree with previous studies employing intracerebral microinjection where these doses have been effective (Lisboa *et al.* 2008; Resstel *et al.* 2008*d*).

In conclusion, the present results suggest that eCB-mediated neurotransmission in the vMPFC plays an important role on the expression of fear conditioning. They also agree with the proposal that the eCB system acts as a 'stress buffer', attenuating aversive responses (Lutz, 2009), and suggest that the vMPFC could be a brain site for this effect.

Acknowledgements

Statement of Interest

None.

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