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Opposing Roles for Cannabinoid Receptor Type-1 (CB_1) and Transient Receptor Potential Vanilloid Type-1 Channel (TRPV1) on the Modulation of Panic-Like Responses in Rats

Plínio C Casarotto[1], Ana Luisa B Terzian[2,3], Daniele C Aguiar[4], Hélio Zangrossi[1], Francisco S Guimarães[1], Carsten T Wotjak[2] and Fabrício A Moreira*,[4]

[1]Department of Pharmacology, School of Medicine of Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil; [2]Max Planck Institut für Psychiatrie, Research Group Neuronal Plasticity, Munich, Germany; [3]Graduate School of Neuroscience, Ludwig Maximilians Universität, Munich, Germany; [4]Department of Pharmacology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

The midbrain dorsal periaqueductal gray (dPAG) has an important role in orchestrating anxiety- and panic-related responses. Given the cellular and behavioral evidence suggesting opposite functions for cannabinoid type 1 receptor (CB_1) and transient receptor potential vanilloid type-1 channel (TRPV1), we hypothesized that they could differentially influence panic-like reactions induced by electrical stimulation of the dPAG. Drugs were injected locally and the expression of CB_1 and TRPV1 in this structure was assessed by immunofluorescence and confocal microscopy. The CB_1-selective agonist, ACEA (0.01, 0.05 and 0.5 pmol) increased the threshold for the induction of panic-like responses solely at the intermediary dose, an effect prevented by the CB_1-selective antagonist, AM251 (75 pmol). Panicolytic-like effects of ACEA at the higher dose were unmasked by pre-treatment with the TRPV1 antagonist capsazepine (0.1 nmol). Similarly to ACEA, capsazepine (1 and 10 nmol) raised the threshold for triggering panic-like reactions, an effect mimicked by another TRPV1 antagonist, SB366791 (1 nmol). Remarkably, the effects of both capsazepine and SB366791 were prevented by AM251 (75 pmol). These pharmacological data suggest that a common endogenous agonist may have opposite functions at a given synapse. Supporting this view, we observed that several neurons in the dPAG co-expressed CB_1 and TRPV1. Thus, the present work provides evidence that an endogenous substance, possibly anandamide, may exert both panicolytic and panicogenic effects via its actions at CB_1 receptors and TRPV1 channels, respectively. This tripartite set-point system might be exploited for the pharmacotherapy of panic attacks and anxiety-related disorders.
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INTRODUCTION

Panic disorder is a subtype of anxiety spectrum disorders characterized by recurrent episodes of panic attacks, which comprise intense feelings of fear and distress, accompanied by tachycardia, hyperventilation and increased blood pressure (Katon, 2006; Roy-Byrne *et al*, 2006). Although its precise neural basis has remained uncertain, evidence indicates that it might result from a malfunction in brain systems related to defense reactions (McNaughton and Gray, 2000). Accordingly, panic attacks exhibit isomorphism with flight responses regarding their autonomic, behavioral and psychological aspects (Bandler *et al*, 2000; McNaughton and Gray, 2000).

Among the brain regions that may participate in the elaboration of both panic and innate defense reactions is the midbrain dorsal periaqueductal gray (dPAG), comprising dorsomedial and dorsolateral columns (Del-Ben and Graeff, 2009; Schenberg *et al*, 2001). In humans, electrical stimulation of this structure induces an intensive feeling of aversion, fear and imminent death along with autonomic changes (Nashold *et al*, 1969). Moreover, neuroimaging studies detected increased activity in this region in patients suffering from panic (Del-Ben and Graeff, 2009) and in healthy volunteers exposed to a proximal threatening stimulus (Mobbs *et al*, 2007). In laboratory rats, local electrical or chemical dPAG stimulation induces autonomic changes along with escape responses (Beckett and Marsden, 1995; Krieger and Graeff, 1985; Schenberg *et al*, 2001). In addition, systemic or intradPAG injections of panicolytic drugs increase the threshold required to induce escape responses (Hogg *et al*, 2006; Jenck *et al*, 1995; Schenberg

*Correspondence: Dr FA Moreira, Department of Pharmacology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 Belo Horizonte, MG 31270-901, Brazil, Tel: +55 31 3409 2720, Fax: +55 31 3409 2645, E-mail: fabriciomoreira@icb.ufmg.br
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et al, 2001; Schütz et al, 1985). Due to its robust face, construct and predictive validity, the escape reaction resulting from electrical stimulation of the rat dPAG has been proposed as a model of panic attacks (Beckett and Marsden, 1995; Del-Ben and Graeff, 2009; Jenck et al, 1995; Schenberg et al, 2001).

Diverse types of receptors are expressed in the PAG, including the G-protein coupled cannabinoid type 1 receptor (CB_1) and the transient receptor potential type-1 (TRPV1) channel (Cavanaugh et al, 2011; Cristino et al, 2006; Herkenham et al, 1990, 1991; Mezey et al, 2000; Tóth et al, 2005; Tsou et al, 1998). These receptors may bind common endogenous ligands, which are thus termed endocannabinoids/endovanilloids, such as arachidonoyl ethanolamide (anandamide) (for reviews, see Di Marzo, 2008; Di Marzo et al, 2001 and Starowicz et al, 2007a). Despite this similarity, electrophysiological and neurochemical pieces of evidence indicate that they may have opposite functions. In the PAG, local CB_1 activation inhibits calcium channels and excitatory neuronal activity (Vaughan et al, 2000), whereas TRPV1 promotes calcium influx and glutamate release (Starowicz et al, 2007b; Xing and Li, 2007). At the behavioral level, CB_1 knockout mice show deficits in extinction and habituation of conditioned fear (Kamprath et al, 2006; Marsicano et al, 2002) and exacerbated anxiety-like behavior (Haller et al, 2002, 2004; Jacob et al, 2009; Martin et al, 2002). On the contrary, TRPV1 knockout mice show reduced fear- and anxiety-like responses (Marsch et al, 2007). Finally, either local activation of CB_1 or blockade of TRPV1 at level of the dorsolateral columns of the PAG-induced anxiolytic-like effects (Lisboa et al, 2008; Moreira et al, 2007, 2009; Terzian et al, 2009). The same is observed with either systemic or intraprefrontal cortex injections (Aguiar et al, 2009; Micale et al, 2009; Rubino et al, 2008).

So far, most studies have investigated the roles of CB_1 and TRPV1 separately. However, there is evidence that they may act in concert to modulate behavioral responses (Maione et al 2006; Micale et al, 2009; Rubino et al, 2008). They are co-expressed in several regions related to aversion, such as prefrontal cortex, amygdaloid complex and hippocampus, where they may modulate anxiety-related responses (Cristino et al, 2006; Micale et al, 2009), as well as in the ventrolateral PAG (vlPAG), where they control nociceptive reactions (Cristino et al, 2006; Maione et al, 2006).

Considering this background, the present study tested the hypothesis that CB_1 and TRPV1 exert opposite effects on panic-like responses at level of the dPAG and looked for interdependences of the two signaling systems.

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from the animal facilities at the University of São Paulo, Ribeirão Preto. The animals, weighing 300–330 g, were housed in groups of 4–6 per cage under a 12-h dark/12-h light cycle (lights on at 0700 h) at $22 \pm 1\,^{\circ}C$, and given free access to food and water. All experiments were carried in accordance with the Brazilian Society of Neuroscience and Behavior for the care and use of laboratory animals and were approved by the Experimental Animal Ethical Committee of University of São Paulo (protocol number: 114/2007). All efforts were made to minimize animal suffering.

Surgery

Rats were anesthetized with 2,2,2 tribromoethanol (250 mg/kg i.p.) combined with local anesthesia (2% lidocaine with a vasoconstrictor; Harvey, Brazil), and fixed in a stereotaxic frame (David Kopf) for implantation of a chemitrode in the dPAG (incisor bar 3.3 mm below the interaural line, 1.7 mm lateral to lambda at an angle of 22° with the sagittal plane, 5.0 mm below the surface of the skull; according to Paxinos and Watson, 1997). The chemitrode consisted of a stainless steel guide cannula (outer diameter: 0.6 mm, length: 13 mm) glued to a brain electrode made of stainless steel wire (diameter: 250 μm) that was insulated by enamel except for the cross-section of the tip. The electrode reached 1 mm below the end of the cannula. It was connected to a male pin parallel to the outer end of the cannula that could be plugged into an amphenol socket at the end of a flexible electrical cable. The chemitrode was fixed to the skull by means of acrylic resin and two stainless steel screws. A stylet was inserted into the guide cannula in order to prevent obstruction. At the end of the surgery, animals were treated with an antibiotic (Pentabiótico, Fort Dodge, Brazil; 1.0 ml/kg, i.m.) and fluxinin meglumine (Schering-Plough, Brazil; 2.5 mg/kg, s.c.), which has analgesic, antipyretic and anti-inflammatory properties. The animals were left undisturbed for 5–7 days after the surgery.

Drugs and Treatment

The CB_1 agonist, arachidonyl-2-chloro-ethylamide (ACEA; Tocris) was dissolved in Tocrisolve (vehicle). The CB_1 antagonist, *N*-(piperidin-1yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251; Tocris) and the TRPV1 antagonists, capsazepine (Tocris) and 4′-Chloro-3-methoxycinnamanilide (SB366791; Tocris), were dissolved in DMSO.

For local drug treatment, an injection needle (outer diameter: 0.3 mm) was inserted through the guide into the dPAG to a final depth of 6.0 mm below the surface of the skull. A volume of 0.2 μl was injected for 60 s using a 10-μl microsyringe (Hamilton) attached to a microinfusion pump (KD Scientific). The needle was left in place for another 60 s before removal.

Escape Threshold Determination

Escape behavior induced by dPAG electrical stimulation was performed in accordance with previous work (Casarotto et al, 2010). The animals were evaluated in a circular arena (40 cm in diameter) with 40 cm high walls made of transparent Plexiglas. Brain stimuli were generated by a sine-wave stimulator. The stimulation current (peak-to-peak) was monitored on the screen of an oscilloscope (Minipa, Brazil). The brain electrode was connected to the stimulator by means of an electromechanical swivel and a flexible cable, allowing ample movement of the animal inside the experimental cage. The escape threshold was determined in response to an electrical stimulus (AC, 60 Hz, 10 s)

presented through the chemitrode. The interstimulus interval was 10 s. The current intensity started at 20 μA and was increased by steps of 4 μA until the rat started running around the circular arena (escape behavior). In most cases, this vigorous reaction was accompanied by vertical jumps. When these behaviors were observed, electrical stimulation to the dPAG was interrupted by the experimenter that was blind to the treatment groups. The basal escape threshold was defined as the lowest current intensity that evoked escape in three successive trials of electrical stimulation. Animals with basal thresholds >150 μA were excluded from the study. Following basal escape threshold determination, independent groups of animals were injected with the drugs or vehicle into the dPAG, and the escape threshold was reanalyzed 10 min later. The variation in escape threshold (Δ) was then calculated for each animal and refers to the difference between escape threshold values obtained post- and pre-treatment.

Experiments

Independent batches of animals were used in each experiment. In experiment 1, the rats were treated with ACEA (0.01, 0.05 and 0.5 pmol) or vehicle ($n = 6$–7). In experiment 2, new groups of animals received AM251 (75 pmol) or its vehicle followed by ACEA (0.05 pmol) or its respective vehicle 5 min later ($n = 5$–6). In experiment 3, they were injected with capsazepine (0.1, 1 and 10 nmol) or vehicle ($n = 6$–9). In experiment 4, capsazepine (0.1 nmol) or vehicle injections were followed by ACEA (0.5 pmol) 5 min later ($n = 5$ per group). In experiment 5, the rats were treated with AM251 (75 pmol) or vehicle followed by capsazepine (10 nmol) or vehicle 5 min later ($n = 5$–7). Finally, in experiment 6, they were treated with AM251 (75 pmol) or vehicle followed by SB366791 (10 nmol) or vehicle 5 min later ($n = 5$–7). The doses of ACEA, AM 251 and capsazepine were selected based on previous works performing injections into the dPAG (Moreira *et al*, 2007; Terzian *et al*, 2009). The dose of SB366791 was selected based on the affinity of this substance for TRPV1 as compared with other antagonists (Gawa *et al*, 2005; Gunthorpe *et al*, 2004).

Histology

After the experiments, animals were deeply anesthetized with chloral hydrate. The brain was perfused through the heart with saline solution followed by 10% formalin solution, before being removed and fixed in 10% formalin. Frozen sections of 55 μm were cut using a microtome to localize the positions of the electrode tips according to the atlas by Paxinos and Watson (1997). Only data from rats having chemitrode tips inside the dPAG were included in the statistical analysis.

Immunofluorescence

Male Wistar rats were anaesthetized with urethane and transcardially perfused with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS, pH 7.4). The brain was removed and post-fixed over 2 h in PFA 4% and stored for at least 30 h in 30% sucrose for cryoprotection. Coronal sections (25 μm) were obtained in a cryostat and were washed in PBS 0.01 M. After this, the sections were incubated in glycine 0.1 M, washed again and incubated for 1 h in 5% bovine serum (BSA, sigma) in PBS 0.01 M, pH 7.4 containing 0.3% Triton X-100. Thereafter, the sections were incubated for 2 days at 4 °C in a mixture of anti-CB1 receptor N-terminus (1 : 250; Abcam raised in rabbit) to anti-TRPV1 receptor N-terminus (1 : 100; Santa Cruz raised in goat) diluted in PBS 0.01 M containing BSA 5% and 0.3% Triton X-100. After incubation in the primary antiserum, the tissue sections were washed in PBS and sequentially incubated in a mixture of Alexa 594 donkey anti-rabbit IgGs (1 : 1000; Molecular Probes, Eugene, OR) and Alexa 488 donkey anti-goat IgGs (1 : 1000; Molecular Probes) for 1 h. Slides were rinsed in PBS and coverslipped with Fluormount-G (Electron Microscopy Sciences, Hatfield, PA). Images were first obtained using an Olympus BX50 microscope through computerized image system (Image Pro-Plus 4.0, Media Cybernetics). For a more appropriate analysis of double-stained cells, the slides were re-examined using a modified Olympus BX61WI confocal microscope in an upright configuration, using an Argonium laser at 488 nm. Immunostained slides (Alexa Fluor 488-coupled anti-CB_1 receptors; Alexa Fluor 546-coupled anti-TRPV1 receptors) were exposed for 2.71 s and green and red channels were merged digitally by Fluoview software. Images were further exported in TIFF format. The specificity of the primary antibodies has been demonstrated before by means of CB_1 and TRPV1 knockout mice (Maione *et al*, 2006). Nevertheless, we additionally performed negative controls for non-specific labeling. The protocol was the same as described above, except for the fact that the primary antibodies for CB_1 and TRPV1 were switched, so that each protocol would include a primary antibody for one receptor and a secondary for the other.

Statistical Analysis

The effects of intradPAG drug injections were analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test, when appropriate. Data were expressed as mean ± SEM. Statistical significance was accepted if $p < 0.05$.

RESULTS

The sites of electrical stimulation and drug injection can be seen in Figure 1. Electrical stimulation of the dPAG (black dots), but not of surrounding regions (gray dots), caused an explosive panic response, which was characterized by running and jumping. Activation of CB_1 receptors by local administration of ACEA modified the effects of electrical stimulation in a bell-shaped manner. It increased the threshold for inducing escape behavior at 0.05 pmol, though not at 0.01 or 0.5 pmol ($F(3,23) = 4.06$; $p < 0.05$; Figure 2). The panicolytic-like effect of 0.05 nmol ACEA was mediated by CB_1 receptors, since it could be blocked by pre-treatment with the selective antagonist AM251 (75 pmol) ($F(3,17) = 13.86$; $p < 0.01$; Figure 3). Local blockade of TRPV1 channels by capsazepine also raised the escape threshold at 1 and 10 nmol ($F(3,25) = 16.58$; $p < 0.05$; Figure 4).

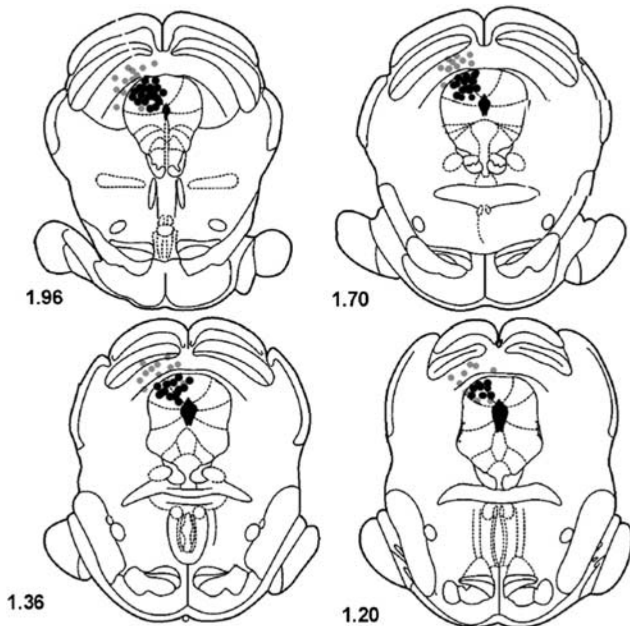

Figure 1 Schematic representation of coronal sections from interaural line of the rat brain (Paxions and Watson, 1997) showing the injection/electrical stimulation sites inside (dark circles) of the dPAG. Sites where electrical stimulation failed to induce escape responses are also presented (gray circles). Due to overlaps, the number of points represented is fewer than the number of rats actually employed in the experiments.

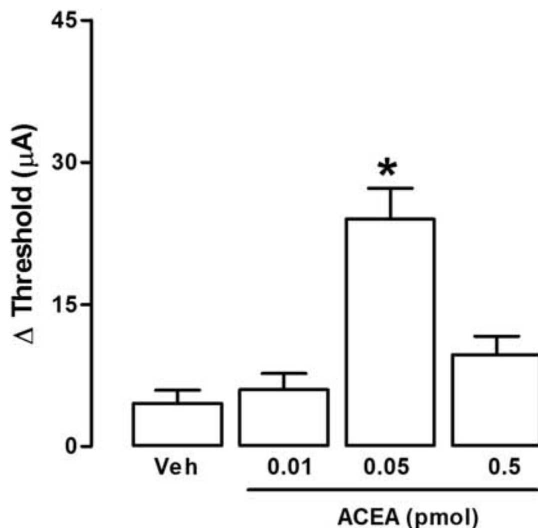

Figure 2 Local injection of the selective CB_1-receptor agonist, ACEA, into the dPAG increases the threshold of electric current necessary for inducing panic-like response ($n = 7, 6, 7, 7$). Following basal escape threshold determination, independent groups of animals were injected with vehicle or ACEA (0.01–0.5 pmol), and the escape threshold was re-analyzed 10 min later. The variation in escape threshold (Δ) was then calculated for each animal and refers to the difference between escape threshold values obtained post- and pre-treatment. *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test).

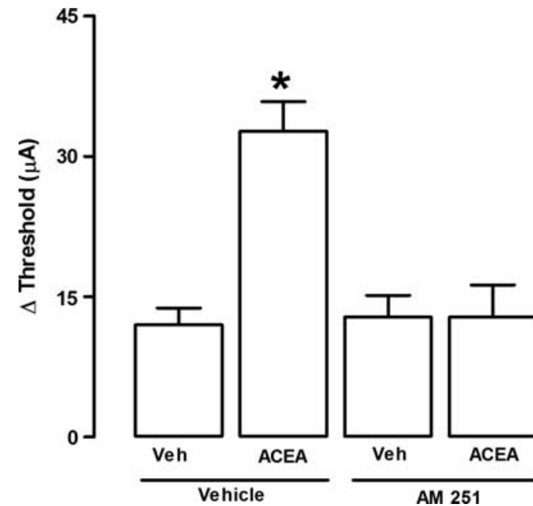

Figure 3 Local pre-treatment (5 min) with the CB_1 antagonist, AM251 (75 pmol), prevents ACEA (0.05 pmol)-induced panicolytic-like effect ($n = 5, 6, 5, 5$). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

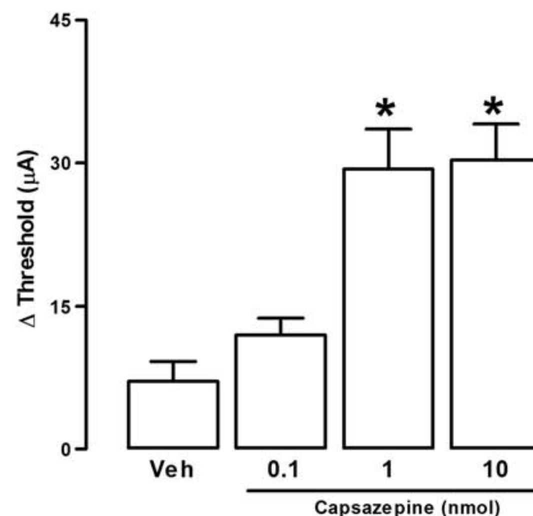

Figure 4 Local injection of the TRPV1 antagonist, capsazepine (CPZ; 0.1–10 nmol), into the dPAG increases the threshold of electric current required for inducing panic-like response ($n = 9, 7, 6, 7, 7$). *$p < 0.05$ Compared with vehicle-treated rats (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

These results suggest that TRPV1 has diametrically opposite functions as compared with CB_1. Considering that certain cannabinoids may also bind TRPV1 at higher concentrations (Huang *et al*, 2002; Price *et al*, 2004; Silveira *et al*, 2010; Smart *et al*, 2000; Zygmunt *et al*, 1999), simultaneous CB_1 and TRPV1 activation could explain the bell-shaped effects observed with ACEA in the first experiment (Figure 2). High-dose ACEA could activate both CB_1 and TRPV1, occluding the CB_1-mediated effects. Indeed, an ineffective dose of capsazepine (0.1 nmol) unmasked the panicolytic-like effect of ACEA (0.5 pmol) ($t(8) = 3.48$; $p < 0.01$; Figure 5).

Finally, we tested the hypothesis that the panicolytic-like effect of TRPV1 blockade would occur because it would shift the action of a common endogenous agonist toward CB_1 activation. Supporting this hypothesis, the panicolytic-like effect of capsazepine at higher doses (10 nmol) could be

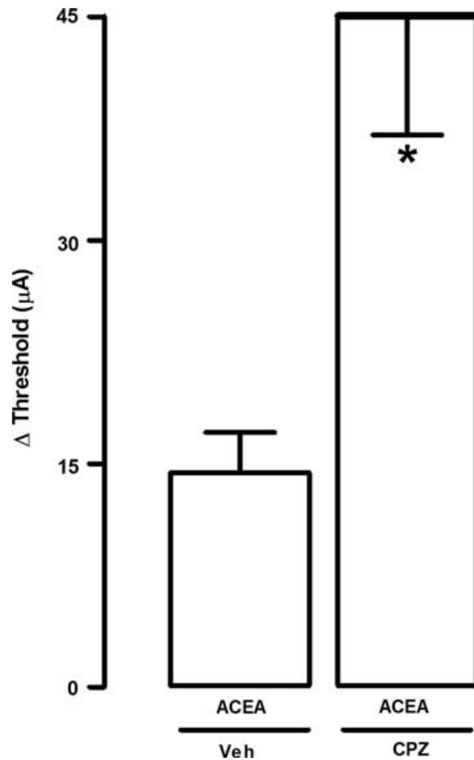

Figure 5 Local pre-treatment (5 min) with the TRPV1 antagonist, capsazepine (CPZ; 0.1 nmol), unmasks the panicolytic-like effect of high-dose ACEA (0.5 pmol) ($n = 5$ per group). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

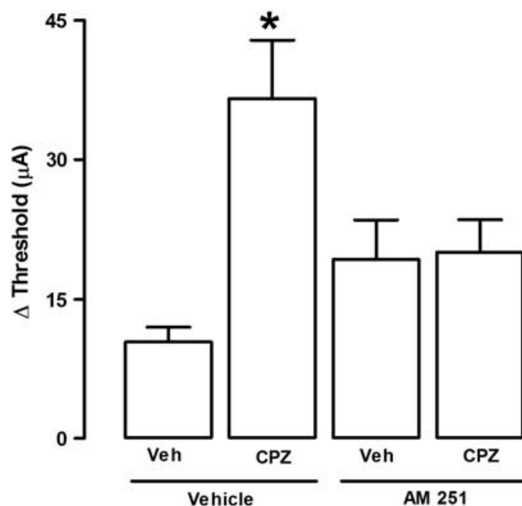

Figure 6 Local pre-treatment (5 min) with the CB$_1$ antagonist, AM251 (75 pmol), prevents the panicolytic-like effect of capsazepine (CPZ; 10 nmol)-induced ($n = 5$, 7, 5, 7). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

prevented by pre-treatment with AM251 (75 pmol), demonstrating its dependency on CB$_1$ signaling ($F(3,20) = 5.74$; $p < 0.01$; Figure 6). Remarkably, this data could be reproduced with a more selective TRPV1 antagonist, SB366791 (10 nmol), which increased the threshold for

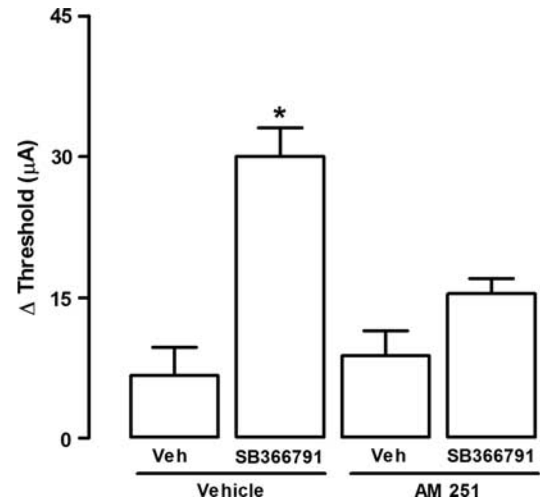

Figure 7 Local pre-treatment (5 min) with the CB$_1$ antagonist, AM251 (75 pmol), prevents the panicolytic-like effect of SB366791 (10 nmol) ($n = 6$, 6, 5, 7). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

panic-like responses to the same extent as capsazepine, again in a CB$_1$-dependent manner (75 nmol), as presented in Figure 7 ($F(3,20) = 16.3$; $p < 0.01$; Figure 7).

At the cellular level, both CB$_1$ and TRPV1 are expressed in neurons within the dPAG (Figure 8). Immunofluorescent labeling revealed neurons that were exclusively CB$_1$ or TRPV1 positive. However, many neurons co-expressed both receptors in their cell bodies. This could be certified through confocal microscopy examination, which allowed us to distinguish double-stained neurons from pairs of mono-stained neurons located in adjacent plans. There was a medium degree of colocalization throughout the dorsolateral and dorsomedial aspects of the PAG. This pattern of co-expression supports the notion that they could be simultaneously activated at a given synapse. Importantly, no staining was observed in protocols in which the antibodies were switched, so that the primary for one receptor was combined with the secondary for the other (Figure 9). This re-assures the specificity of the antibodies used in the present protocols.

DISCUSSION

The present work provides evidence for opposite functions of CB$_1$ and TRPV1 in an animal model of panic attacks, the electrical stimulation of the dPAG. In this model, local application of antipanic drugs increases the current threshold necessary for the induction of explosive escape behaviors. We demonstrated that the selective CB$_1$ agonist, ACEA, increased the threshold for inducing panic-like reaction in a bell-shaped manner. This panicolytic-like effect was completely prevented by the CB$_1$ receptor antagonist, AM251. Although this effect was lost in a higher dose, it could be unmasked by previous treatment with an inactive dose of capsazepine, a TRPV1 antagonist. In addition, pharmacological blockade of TRPV1 *per se* also exerted panicolytic-like effects, suggesting the existence of

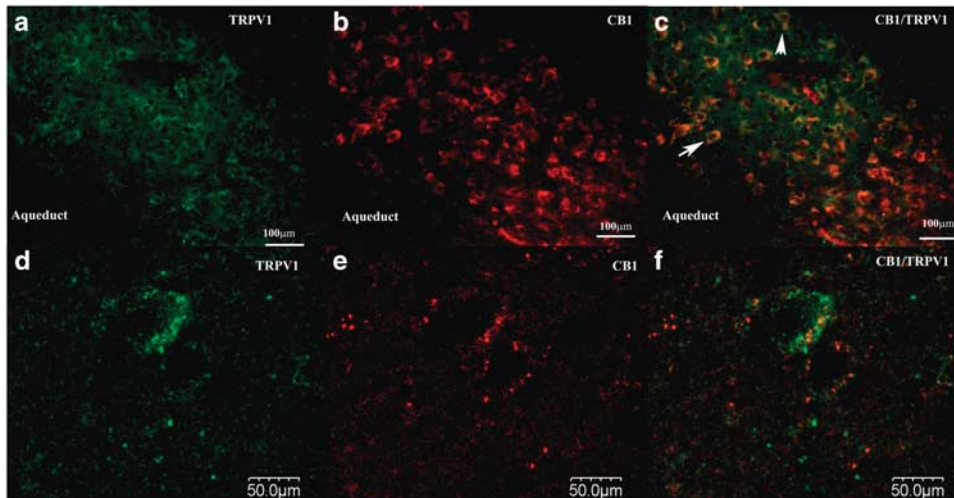

Figure 8 CB_1 receptors and TRPV1channels are co-expressed in neurons in the dPAG. The photomicrographs (25 μm thick section) show image of double-label immunofluorescence of CB_1 (red, Alexa Fluor 594; a, d) and TRPV1 receptors (green, Alexa Fluor 488; b, e), as revealed by fluorescence (a–c) and confocal (d–f) microscopy. Panel (c) (the composite images of (a, b)) shows neurons expressing both receptors. One representative double-stained neuron is shown in higher magnification in f (arrow). Scale bars: 100 μm (a–c) and 50 μm (d–f).

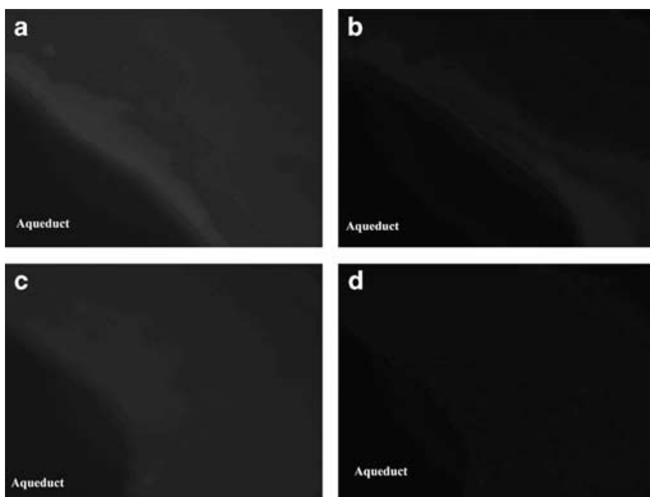

Figure 9 Negative controls for the double-labeled immunofluorescence. The photomicrographs (25 μm thick section) show images of the dPAG from protocols combining TRPV1 secondary (Alexa Fluor 488) with CB_1 primary antibodies (a) or CB_1 secondary (Alexa Fluor 594) with TRPV1 primary antibodies (b). Images resulting from protocol in which the primary antibodies were omitted as seen in the microscopy though filters for Alexa Fluor 488 (c) and Alexa Fluor 594 (d) are also shown.

tonic modulation by TRPV1, whose function opposed that of CB_1. Remarkably, capsazepine effect could be prevented by AM251, revealing that the effect of TRPV1 blockade was completely dependent on CB_1 signaling. Noteworthy, capsazepine effect could be mimicked by another TRPV1 antagonist, SB366791, whose action was also prevented by AM251. Together with the co-expression of CB_1 and TRPV1 observed at level of the dPAG, these data suggest the existence of a set-point system, whereby activation of TRPV1 *vs* CB_1 balances the induction of panic-like responses.

The dPAG model of panic has some limitations in terms of a broader interpretation in relationship to human psychopathology. It mimics only the panic attacks, which actually occur with several other symptoms of panic disorder, such as behavioral and physiological signs of intense anxiety, fear and distress (Katon, 2006; Roy-Byrne *et al*, 2006). Moreover, it is based on the measure of an all-or-none response to electrical stimulation of a specific brain structure, which is only part of a more complex neural circuitry generating fear, anxiety and panic responses (Del-Ben and Graeff, 2009). Despite of these drawbacks, this model presents face, construct and predictive validities (Jenck *et al*, 1995; Beckett and Marsden, 1995; Del-Ben and Graeff, 2009; Schenberg *et al*, 2001). The face validity is supported by the fact that the behavioral and autonomic responses observed in this model resemble those seen in panic attacks (Schenberg *et al*, 2001). As for the theoretical construct, the PAG is a midbrain structure activated during escape responses induced by natural aversive stimulus either in laboratory animals or in healthy volunteers (Dielenberg *et al*, 2001; Mobbs *et al*, 2007). Neurosurgery patients, in whom this structure has been electrically stimulated, reported feelings of fear and despair similar to those described in panic patients (Nashold *et al*, 1969). Finally, and more relevant for the present study, the pharmacological predictability of the model is supported by the observations that drugs used to treat panic disorder increase the threshold of escape responses induced by electrical stimulation of the dPAG (Jenck *et al*, 1995; Schenberg *et al*, 2001; Schütz *et al*, 1985).

In this model, ACEA induced a panicolytic-like effect in an intermediary dose, which could be prevented by AM251, in line with previous experiments in anxiety-related reactions (Moreira *et al*, 2007). These results support the concept of general anti-aversive effects of CB_1 signaling at level of the PAG. Accordingly, local injections of other cannabinoids inhibit the escape responses induced by local injection of an excitatory amino acid (Finn *et al*, 2003) and

promote anxiolytic-like effects in several animal models (for a review, see Moreira *et al*, 2009).

Contrary to CB_1, TRPV1 seems to mediate pro-aversive effects, since we detected a clear panicolytic-like effect after its blockade with capsazepine or SB366791. Moreover, an ineffective dose of capsazepine was able to unmask the panicolytic-like effects of higher dose of ACEA. Therefore, the bell-shaped dose–response curve of ACEA seems to result from an activation of CB_1 at lower concentrations (panicolytic), whereas higher concentration of ACEA also activates the less affine TRPV1 channels (panicogenic), counteracting CB_1-mediated actions, resulting in a net null effect. This is in line with ACEA affinities profile, binding CB_1 in lower dose (Gawa *et al*, 2005) and both CB_1 and TRPV1 in higher dose (Huang *et al*, 2002; Price *et al*, 2004; Silveira *et al*, 2010; Smart *et al*, 2000; Zygmunt *et al*, 1999).

The panicolytic consequences of TRPV1 antagonists speak for a tonic activation of TRPV1 under basal conditions. This is in accordance with previous studies, which reported decreased anxiety upon pharmacological or genetic interruption of TRPV1 signaling. For instance, systemic injection of capsazepine reduced anxiety-like behavior in rats (Kasckow *et al*, 2004). Moreover, a detailed investigation on the behavior of TRPV1 knockout mice unveiled a phenotype of reduced anxiety-like behavior in the elevated plus maze and in the light-dark box, as well as reduced fear conditioning (Marsch *et al*, 2007). In addition, the effect of systemically administered capsazepine is mimicked by local injection into specific structures, namely the ventral hippocampus (Santos *et al*, 2008), the dorsolateral PAG (Terzian *et al*, 2009) and the prefrontal cortex (Aguiar *et al*, 2009; Rubino *et al*, 2008). This is consonant with neurochemical and electrophysiological data showing that this ion channel stimulates calcium influx and glutamate release in the PAG, increasing local neuronal activity (Starowicz *et al*, 2007b; Xing and Li, 2007). In addition, it has been demonstrated in other brain regions related to anxiety and emotional processing, such as the hippocampus and the nucleus accumbens, that anandamide acts upon TRPV1 modulates long-termed depression of synaptic activity (Chavez *et al*, 2010; Grueter *et al*, 2010). Although it remains unclear how these electrophysiological events would reflect behavioral responses, these studies suggest that anandamide could be the endogenous substance activating this ion channel (Di Marzo, 2010; Chavez *et al*, 2010; Grueter *et al*, 2010). Other lipids, including *N*-arachidonoyl dopamine, N-oleoyldopamine and some lypooxigenase products have also been among the main candidates proposed as endovanilloids (Di Marzo *et al*, 2001; Starowicz *et al*, 2007a).

Remarkably, the effects of TRPV1 blockade could be fully prevented by pre-treatment with the selective CB_1 antagonist, AM251. Thus, we have shown that the effect of TRPV1 antagonists is ultimately mediated by CB_1 receptor, possibly because the actions of an endogenous agonist would be entirely shifted to CB_1, after TRPV1 blockade. This finding adds to previous observations about opposite roles for CB_1 and TRPV1 in various behavioral responses (Maione *et al*, 2006; Micale *et al*, 2009; Rubino *et al*, 2008). We suggest that CB_1 and TRPV1 might be simultaneously activated at a given synapse, since we observed co-expression of CB_1 and TRPV1 at level of the dPAG. Similar observations were obtained before in the prefrontal cortex, amygdala complex, hippocampus and vlPAG (Cristino *et al*, 2006; Maione *et al*, 2006; Micale *et al*, 2009). In accordance with previous studies, we also detected dPAG neurons that were either exclusively CB_1 positive (Herkenham *et al*, 1990, 1991; Tsou *et al*, 1998) or TRPV1 positive (McGaraughty *et al*, 2003; Mezey *et al*, 2000; Tóth *et al*, 2005).

Our pharmacological and histological experiments support a scenario in which TRPV1 and CB_1 would counteract each other to balance the threshold for panic-like responses in multiple ways. For instance, the opposite effects of CB_1 and TRPV1 could be mediated by different neurons that converge on the same output system. Alternatively, they could result from divergent influences on intracellular signaling, neural firing rates and neurotransmitter release within the same neurons (Kawahara *et al*, 2010; Starowicz *et al*, 2007b; Vaughan *et al*, 2000; Xing and Li, 2007). Regarding the physiological modulation of these mechanisms, there might be an endogenous agonist, with affinity for both, which would be part of a tripartite system to establish set points for behavioral responses. One candidate would be anandamide, which has been proposed as an endocannabinoid/endovanilloid (Zygmunt *et al*, 2000), with higher affinity for CB_1 as compare with TRPV1 (Di Marzo *et al*, 2001; Starowicz *et al*, 2007a).

Despite of the facts that anandamide has lower affinity for TRPV1 and has its actions limited by the enzyme fatty acid amide hydrolase (FAAH; Piomelli, 2003), the local levels of this substance are significantly increased by electrical stimulation of the dPAG (Walker *et al*, 1999). Therefore, it is conceivable that, in the present model, anandamide levels would increase and act upon both receptors, whereby TRPV1-mediated actions may prevail over CB_1 (Tognetto *et al*, 2001), thus facilitating panic-like behavior. Blockade of this ion channel would increase the threshold by shifting anandamide action entirely to CB_1 receptors, whose selective activation is panicolytic. This hypothesis is reinforced by recent electrophysiological and behavioral experiments. In PAG slices, anandamide could either facilitate or inhibit excitatory transmission through TRPV1 and CB_1, respectively, in the presence of the hydrolysis inhibitor URB597 (Kawahara *et al*, 2010). Accordingly, FAAH knockout mice have a phenotype of lower anxiety-like behavior due to higher CB_1 activation by anandamide. However, CB_1-antagonist treatment induced anxiogenic-like effects in these animals, but not in wild-type controls, possibly by shifting anandamide activity toward TRPV1 (Cassano *et al*, 2010).

In conclusion, we have shown opposite functions for TRPV1 and CB_1 receptors in the modulation of panic-like responses at the level of the dPAG. Anandamide actions via CB_1 *vs* TRPV1 may constitute a set-point system that represents a promising target for the pharmacotherapy of panic and other anxiety spectrum disorders.

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DISCLOSURE

The authors declare no conflict of interest.

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