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Research report

The endocannabinoid, endovanilloid and nitrergic systems could interact in the rat dorsolateral periaqueductal gray matter to control anxiety-like behaviors

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HIGHLIGHTS

- AEA induces biphasic effects in the modulation of anxiety-like behaviors in the dlPAG.
- AEA induces an anxiolytic-like effect in the dlPAG at lower doses.
- The lost of AEA anxiolytic-like effect at higher doses is influenced by NO formation.
- Concomitant NO formation and $TRPV_1$ activation facilitates defensive responses.

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ABSTRACT

Cannabinoid compounds usually produce biphasic effects in the modulation of emotional responses. Low doses of the endocannabinoid anandamide (AEA) injected into the dorsolateral periaqueductal gray matter (dlPAG) induce anxiolytic-like effects via CB_1 receptors activation. However, at higher doses the drug loses this effect, in part by activating Transient Receptor Potential Vanilloid Type 1 ($TRPV_1$). Activation of these latter receptors could induce the formation of nitric oxide (NO). Thus, the present study tested the hypothesis that at high doses AEA loses it anxiolytic-like effect by facilitating, probably via $TRPV_1$ receptor activation, the formation of NO. Male Wistar rats received combined injections into the dlPAG of vehicle, the $TRPV_1$ receptor antagonist 6-iodo-nordihydrocapsaicin or the NO scavenger carboxy-PTIO (c-PTIO), followed by vehicle or AEA, and were submitted to the elevated plus maze (EPM) or the Vogel conflict test (VCT). A low dose (5 pmol) of AEA produced an anxiolytic-like effect that disappeared at higher doses (50 and 200 pmol). The anxiolytic-like effects of these latter doses, however, were restored after pre-treatment with a low and ineffective dose of c-PTIO in both animal models. In addition, the combined administration of ineffective doses of 6-iodo-nordihydrocapsaicin (1 nmol) and c-PTIO (0.3 nmol) produced an anxiolytic-like response. Therefore, these results support the hypothesis that intra-dlPAG injections of high doses of AEA lose their anxiolytic effects by favoring $TRPV_1$ receptors activity and consequent NO formation, which in turn could facilitate defensive responses.

1. Introduction

Anxiety is an emotion controlled by several structures of the limbic system that include the periaqueductal gray matter (PAG). This midbrain region is involved in the control of defensive responses, defined as the behavioral and physiological reactions to potential (anxiety) or real (fear) threatening stimuli [1]. The PAG refers to the region around the cerebral aqueduct (Sylvius) and, in rodents, is subdivided radially into five distinct regions: dorsomedial, dorsolateral, lateral, ventral and ventrolateral [2]. Specifically, the dorsolateral (dlPAG) column has been proposed as one of the main neural substrate for the control of fear- and anxiety-related behaviors [3,4]. These responses are modulated by different brain systems and involve both typical (glutamate, GABA, serotonin, neuropeptides) and atypical neurotransmitters, such as nitric oxide (NO) and endocannabinoids [5].

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The endocannabinoid system includes the cannabinoid receptors Types 1 and 2 (CB_1 and CB_2, respectively), the endogenous agonists anandamide (AEA) and 2-arachidonoylglycerol (2-AG), both derived from arachidonic acid, and the proteins responsible for the synthesis and degradation of these substances [6,7]. When activated, CB_1 receptors induce a decrease in Ca^{2+} influx and activation of K^+ channels, resulting in hyperpolarization and inhibition of neurotransmitters release [8]. In the PAG, administration of CB_1 receptor agonists increases the expression of the neuronal activation marker cFos [9]. Moreover, in this region stressful stimuli induce 2-AG and AEA release [10]. Also, cannabinoid compounds administrated directly into this area are able to control anxiety-like behaviors. These pieces of evidence demonstrate that the endocannabinoid system plays a role in the PAG-induced modulation of defensive responses.

Besides being an agonist of CB_1 receptors, at higher doses AEA can also activate the Transient Receptor Potential Vanilloid Type 1 ($TRPV_1$) [11,12], a permeable-cation channel widely found in the periphery and also present in the central nervous system (CNS) [13]. Activation of these receptors, in contrast to the responses produced by CB_1 activity, results in increased Na^+ and Ca^{2+} conductance and consequent neuronal depolarization, which facilitates neurotransmitter release [13,14].

Thus, because of the ability of activating both CB_1 and $TRPV_1$ receptors, although with different affinities, AEA has been suggested to be, in addition to an endocannabinoid, also an endovanilloid [11]. This dual effect could help to explain its biphasic and complex responses in the brain [12]. In this sense, studies suggest that some behavioral effects mediated by CB_1 receptors are dose-dependently opposed to those regulated by $TRPV_1$ [15,16,17]. While activation of CB_1 receptors by agonists such as AEA produces anxiolytic-like effects in the PAG and other brain regions, higher doses are ineffective in changing anxiety-like behaviors or even become anxiogenic, due to concomitant activation of $TRPV_1$ receptors [15,18,19,20].

In a previous study, we proposed that the loss of the anxiolytic-like effect induced by high AEA doses could be due to facilitation of glutamate release probably via activation of $TRPV_1$ receptors, since AEA effect was restored when combined to ineffective doses of $TRPV_1$ or *N*-methyl-D-aspartate (NMDA) antagonists [20]. It has been suggested that $TRPV_1$-induced glutamate release is mediated by the formation of NO [21,22,23], a very soluble gas that can act on adjacent cells without the involvement of a physical synapse [24]. NO is not stored in vesicles as other neurotransmitters, but is synthesized on demand, spreading rapidly to its site of action. Neuronal NO synthase (nNOS), the enzyme that catalyzes the reaction to produce NO in the CNS, is present in various structures that modulate responses to aversive events, including the dlPAG [25].

In the brain, NO has been shown to mediate synaptic plasticity and facilitate defensive responses [26,27,28]. In this sense, the present study evaluated the hypothesis that high and ineffective doses of AEA lose its anxiolytic-like effects by facilitating the release of NO in the dlPAG, probably via activation of $TRPV_1$ receptors. To further investigate this hypothesis, we also evaluated if the combined injections of ineffective doses of a $TRPV_1$ antagonist and a cell membrane impermeable NO scavenger would produce an anxiolytic-like effect.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 230–250 g were provided by the Central Animal Facility of the Medicinal School of Ribeirão Preto (FMRP-USP). The animals were housed in groups of four (cages size: 41 × 33 × 17 cm) in a temperature-controlled room (24 ± 2 °C) under standard laboratory conditions with free access to food and water and a 12-h light/12-h dark cycle (lights on: 6:30 a.m./lights off: 6:30 p.m.). The total number of rats used in this study was 151. Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and policies. All efforts were made to minimize animal suffering and the experimental protocols were approved by the local Ethical Committee.

2.2. Drugs

The following drugs were used: The CB_1/$TRPV_1$ receptors agonist, anandamide (AEA, Tocris, USA), at the doses of 5, 50 or 200 pmol [20,26], dissolved in Tocrissolve® (Tocris, USA). The $TRPV_1$ receptor antagonist 6-iodo-nordihydrocapsaicin (Tocris, USA) at the doses of 1 or 3 nmol [12], dissolved in DMSO 100%. The cell membrane impermeable NO scavenger Carboxy-PTIO (c-PTIO, Tocris, USA), at the dose of 0.3 nmol [25], dissolved in saline (NaCl 0.9%). Morphine (5 mg/kg, Merck, USA), dissolved in saline. The solutions were prepared immediately before use and kept on ice, protected from the light during the experimental sessions.

2.3. Surgery

Animals were submitted to a stereotaxic surgery to unilaterally implant cannulae (11 mm, 0.6 mm outside diameter, OD) into the dlPAG (coordinates: anteroposterior: 0 from lambda; lateral: −1.9 mm; depth: −4.3 mm; Angle: 16°). The cannulae were fixed to the skull with acrylic cement. The surgeries were performed under deep anesthesia with tribromoethanol 2.5% (10 mL/kg, intraperitoneally, i.p.) and immediately after the animals received a polyantibiotic (0.27 g/kg, intramuscular; Pentabiotico®, Fort Dodge, Brazil) to prevent infection and a non-steroidal anti-inflammatory (0.025 g/kg, s.c., Banamine®, Schering Plough, Brazil) for post-operative analgesia. After the surgery, animals underwent a recovery period of 5–7 days before the behavioral test.

2.4. Microinjection

Before being submitted to the behavioral tests, animals received unilateral microinjections of single or combined injections of drugs (AEA, c-PTIO and/or 6-iodo-nordihydrocapsaicin) and/or their respective vehicles (Tocrissolve®, DMSO 10% in saline and/or DMSO 100%) into the dlPAG. For this, microneedles (12 mm, 0.3 mm OD) were attached to a Hamilton microsyringe (10 μL) through a segment of polyethylene (P10) and inserted into the guide cannula. A 0.2 μL solution volume of each drug was injected over 30 s with the help of an infusion pump (KD Scientific, USA). In the case of combined drug injections, the final volume was 0.4 μL. After the injections, the needles remained inserted into the cannulae for additional 30 s to prevent drug reflux. In the experiments with two injections into the same animal there was a 5 min interval between them.

2.5. Apparatus

2.5.1. Elevated plus-maze (EPM)

The experiments were carried out in a wood-made elevated plus-maze (EPM) located in a sound attenuated, temperature controlled (23° ± 2 °C) room. The environment was illuminated by two fluorescent lights (40 W, 60 lx) located 1.3 m away from the EPM. The apparatus consisted of two opposing open arms (50 × 10 cm) without sidewalls, perpendicular to two enclosed arms (50 × 10 × 40 cm), with a central platform common to all arms