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FACILITATION OF ENDOCANNABINOID EFFECTS IN THE VENTRAL HIPPOCAMPUS MODULATES ANXIETY-LIKE BEHAVIORS DEPENDING ON PREVIOUS STRESS EXPERIENCE

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Abstract—Although several pieces of evidence indicate that the endocannabinoid system modulates anxiety-like behaviors and stress adaptation, few studies have investigated the brain sites of these effects. The ventral hippocampus (VHC) has been related to anxiety behaviors and has a high expression of cannabinoid-1 (CB1) receptors. Moreover, endocannabinoid signaling in the hippocampus is proposed to regulate stress adaptation. In the present study we investigated the role of previous stressful experience on the effects of AM404, an anandamide uptake inhibitor, microinjected into the VHC of rats submitted to the elevated plus maze (EPM), a widely used animal model of anxiety. Stressed animals were forced restrained for two h 24 h before the test. AM404 (5–50 pmol) microinjection promoted an anxiogenic-like effect in non-stressed rats but decreased anxiety in stressed animals. AM251 (0.01 to 1000 pmol), a CB1 receptor antagonist, failed to change behavior in the EPM over a wide dose range but prevented the effects of AM404. Anxiolytic-like effects of AM404 (5 pmol) intra-VHC injection were also observed in the Vogel conflict test (VCT), another model of anxiety that involves previous exposure to stressful situations (48 h of water deprivation). These results suggest that facilitation of endocannabinoid system neurotransmission in the ventral hippocampus modulates anxiety-like behaviors and that this effect depends on previous stress experience.

Key words: anxiety, stress, ventral hippocampus, endocannabinoid system, CB1 receptors.

Endocannabinoids are retrograde messengers that belong to the class of eicosanoids and bind to cannabinoid receptors. Cannabinoid-1 (CB1), the first cloned cannabinoid receptor, is now recognized as the most prevalent G-protein coupled receptor in the CNS. They are pre-synaptically located and regulate both excitatory and inhibitory transmission in the brain (Freund et al., 2003; Matsuda et al., 1990). These receptors are highly expressed in brain areas related to the control of memory processing, anxiety and stress responses, such as the hippocampus, amygdala, prefrontal cortex and hypothalamus (Herkenham et al., 1991; Tsou et al., 1999). Anandamide (aracdonylethanolamide-AEA) is an endogenous cannabinoid that activate cannabinoid receptors and mimics the pharmacological actions of the major psychotomimetic compound of *Cannabis sativa* plant, Δ9-tetrahydrocannabinol (THC) (Devane et al., 1992). Depolarized neurons release AEA after a cleavage of a neural lipid membrane precursor through a calcium-dependent mechanism. Extracellular AEA is removed from the synaptic cleft by a high-affinity transport system present in neural and non-neural cells (Beltramo et al., 1997; Di Marzo et al., 1994; Hillard and Campbell, 1997) and is inactivated by a membrane-bound fatty acid amide hydrolases (FAAH). Drugs that induce a selective blockage of FAAH or AEA transporter could facilitate AEA signaling in specific brain structures by prolonging its effects. As a consequence, these drugs have been used as pharmacological tools to study the involvement of the endocannabinoid system on behavior (Beltramo et al., 1997; Kathuria et al., 2003).

Pharmacological or genetic blockage of FAAH promoted anxiolytic-like effect in ethologically-based models of anxiety such as the elevated plus-maze (EPM) and elevated zero-maze (Gaetani et al., 2003; Moreira et al., 2007, 2008). Moreover, AM404, an inhibitor of AEA transporter, significantly decreased restraint-induced serum corticosterone release in mice (Patel et al., 2004) and promoted anxiolytic-like effects in rats (Bortolato et al., 2006; Patel and Hillard, 2006).

These results corroborate those of several other studies indicating that the endocannabinoid system plays a complex role in the regulation of emotional states (for review, see Viveros et al., 2005) including acute or chronic stress responses (Hill and Gorzalka, 2004, 2006; Hill et al., 2005; Moreira et al., 2007, 2009). The brain structures involved in these effects, however, remain poorly understood and some studies employing intra-cerebral injections suggest that the effects of CB1 receptor activation on anxiety depend on the specific brain region (Rubino et al., 2008).

The hippocampus is a forebrain structure that shows a very high CB1 receptor expression (Tsou et al., 1999) and is proposed to play a central role in stress and anxiety processing (for review, see McEwen, 1999). Several studies, however, suggest that this structure presents a regional functional dissociation. While the ventral portion is proposed to modulate anxiety responses (Bannerman et al., 2004; Bertoglio et al., 2006) its dorsal portion of the

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Abbreviations: AEA, anandamide; AI, antinociception index; CB1, cannabinoid-1; EPM, elevated plus maze; FAAH, fatty acid amide hydrolases; THC, Δ9-tetrahydrocannabinol; VCT, Vogel conflict test; VHC, ventral hippocampus.

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hippocampus would be more involved with memory processes (Bannerman et al., 2004; Bertoglio et al., 2006). The precise role of endocannabinoids on anxiety modulation in the ventral hippocampus (VHC), however, is still unclear. Whereas Rubino et al. (2008) reported that unilateral microinjection of THC, a partial CB1 receptor agonist, into the VHC produced anxiolytic-like effects in the EPM test, Roohbakhsh et al. (2009) showed anxiogenic effects after FAAH inhibition in this region.

Both the endocannabinoid system and the hippocampal formation are significantly influenced by stressful stimuli. Haller et al. (2004), for example, verified that the differences in open arm exploration of the EPM between CB1 receptor knock-out and wild-type mice occurred only when the intensity of light was very high. In this situation the genetic deletion of CB1 receptor promoted an anxiogenic-like effect. Moreover, hippocampal CB1 receptors are known to be sensitive to stressful environments (Hill et al., 2005).

Based on these pieces of evidence, the present study was conducted to investigate a possible interference of stressful experience on the role of endocannabinoid signaling and CB1 receptors in the VHC on the control of anxiety-like behaviors.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats weighing 250–270 g at the beginning of each experiment were housed in groups of four animals/box in a temperature-controlled room (24±1 °C) under standard laboratory conditions with free access to food and water and a 12 h light/12 h dark cycle (lights on at 06:30 h AM). Animals used in the EPM study were moved from group to individual housing conditions 24 h before the stress session. 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. The experimental protocol was approved by the local Ethical Committee. All efforts were made to minimize animal suffering.

Apparatus

Restraint stress. Animals were restrained in a wire chamber (6.3–19.3 cm) with an adjustable roof. Immobilization took place from 8:00 to 10:00 AM (Padovan and Guimaraes, 1993).

Elevated plus maze. The EPM consisted of two opposite open arms (50×10 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 and a 1-cm high edge made of Plexiglas surrounded the open arms to prevent falls. Rodents naturally avoid the open arms of the EPM and anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed arm entries (Carobrez and Bertoglio, 2005; File, 1992). The Ethovision software (V. 1.9, Noldus, Netherlands) was employed for behavioral analysis in the EPM. It detects the position of the animal in the maze and calculates the number of entries and time spent in the open and enclosed arms. For these calculations, a 6-cm-large exclusion zone was added between the center of the maze and each arm so that most of the animal's body should be in the open or enclosed arm for an entry to be registered.

The experiments took place in a sound-attenuated, temperature-controlled (25±1 °C) room, illuminated with three 40 W fluorescent bulbs placed 4 m above the apparatus.

Vogel conflict test (VCT). The VCT was performed in a Plexiglas box (length: 42 cm, width: 25 cm, height: 20 cm) with a stainless grid floor. A metallic spout of a drinking bottle containing water was projected into the box. 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, Ohio, USA) that recorded the number of licks on the metallic spout. Every 20th lick produced a 0.5-mA shock for 2 s. The apparatus registered the total number of licks and shocks delivered during the test period. The whole apparatus was located inside a sound-attenuated cage.

Tail flick test. The apparatus consisted of an acrylic platform with a niquelchrome wire coil (EFF 300, Insight Instruments, Ribeirão Preto, Brazil) maintained at room temperature (24–26 °C). The coil temperature can be raised at 9 °C/s by the passage of electric current. The system had a cut-off time of 6 s to prevent tissue damage when the coil temperature approached 80 °C.

Drugs

N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM404, TOCRIS, Ellisville, MO, USA), an anandamide transporter inhibitor (Beltramo et al., 1997), was dissolved in TOCRIS solvent. The CB1 cannabinoid receptor antagonist N-(piperidin-1yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1Hpyrazole-3 carboxamide (AM251, TOCRIS, Ellisville, MO, USA) was dissolved in DMSO 10% (saline, NaCl 0.09%).

Surgery

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (10 mg/kg, i.p.) and fixed in a stereotaxic frame. Stainless steel guide cannulae (0.6 mm OD) were implanted bilaterally aimed at the ventral hippocampus (coordinates: AP=5 mm from Bregma, L=5.2 mm; D=4.0 mm; θ=0 °). The cannulae were attached to the bones with stainless steel screws and acrylic cement. An obturator inside the guide cannulae prevented obstruction.

Procedures

The experiments took placed 7 days after surgery. Intracerebral injections were performed with a thin dental needle (0.3 mm OD) introduced through the guide cannula until its tip was 3.0 mm below the cannula end. A volume of 0.5 μL was injected in 30 s using a microsyringe (Hamilton, Reno, NV, USA) connected to an infusion pump (Kd Scientific, model 200, Holliston, MA, USA). A polyethylene catheter (PE 10) was interposed between the upper end of the dental needle and the microsyringe.

Experiment 1. The animals received intra-VHC injections of vehicle or AM404 (5–50 pmol) and were placed, 10 min later, in the center of the EPM facing an enclosed arm. The number of entries and time spent in the open and enclosed arms were recorded for 5 min.

Experiment 2. Similar to experiment 1 except that 24 h before the EPM test the animals were submitted to restraint stress for 2 h.

Experiment 3. The animals received intra-VHC injections of vehicle or AM251 (0.1–100 pmol) and were placed, 10 min later, in the center of the EPM facing an enclosed arm. The number of entries and time spent in the open and enclosed arms were recorded for 5 min.