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Pharmacological augmentation of endocannabinoid signaling reduces the neuroendocrine response to stress

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ABSTRACT

Activation of the hypothalamic-pituitary-adrenal axis (HPA) is critical for survival when the organism is exposed to a stressful stimulus. The endocannabinoid system (ECS) is currently considered an important neuromodulator involved in numerous pathophysiological processes and whose primary function is to maintain homeostasis. In the tissues constituting the HPA axis, all the components of the ECS are present and the activation of this system acts in parallel with changes in the activity of numerous neurotransmitters, including nitric oxide (NO). NO is widely distributed in the brain and adrenal glands and recent studies have shown that free radicals, and in particular NO, may play a crucial role in the regulation of stress response.

Our objective was to determine the participation of the endocannabinoid and NOergic systems as probable mediators of the neuroendocrine HPA axis response to a psychophysical acute stress model in the adult male rat. Animals were pre-treated with cannabinoid receptors agonists and antagonists at central and systemic level prior to acute restraint exposure. We also performed *in vitro* studies incubating adrenal glands in the presence of ACTH and pharmacological compounds that modifies ECS components.

Our results showed that the increase in corticosterone observed after acute restraint stress is blocked by anandamide administered at both central and peripheral level. At hypothalamic level both cannabinoid receptors (CB1 and CB2) are involved, while in the adrenal gland, anandamide has a very potent effect in suppressing ACTH-induced corticosterone release that is mainly mediated by vanilloid TRPV1 receptors. We also observed that stress significantly increased hypothalamic mRNA levels of CB1 as well as adrenal mRNA levels of TRPV1 receptor. In addition, anandamide reduced the activity of the nitric oxide synthase enzyme during stress, indicating that the anti-stress action of endocannabinoids may involve a reduction in NO production at hypothalamic and adrenal levels.

In conclusion, an endogenous cannabinoid tone maintains the HPA axis in a stable basal state, which is lost with a noxious stimulus. In this case, the ECS dampens the response to stress allowing the recovery of homeostasis. Moreover, our work further contributes to *in vitro* evidence for a participation of the endocannabinoid system by inhibiting corticosterone release directly at the adrenal gland level.

1. Introduction

An acute and adaptive endocrine response to stress is necessary for survival (Chrousos and Gold, 1992). Stress neuroendocrine response is triggered by the secretion of hypothalamic-releasing hormones and is characterized by the activation of the hypothalamic–pituitary–adrenal (HPA) axis that culminates in increased circulating corticosterone (CORT) (Belda et al., 2004; Buynitsky and Mostofsky, 2009; Dal-Zotto et al., 2003; Hsu et al., 1998; Mora et al., 2012). Restraint may be considered a psychological stressor in which a potent stress response may not result from physical noxious stimuli (Gądek-Michalska et al., 2016). Activation of the HPA axis by stress is generally beneficial for the organism but should not be excessive or prolonged. An appropriate regulatory control of the axis includes several brain structures and mediators and also is modulated by endocannabinoid (eCB) signaling (Crowe et al., 2014; Evanson et al., 2010; Hill et al., 2011, 2010; Hill

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and McEwen, 2010; Roberts et al., 2014; Steiner and Wotjak, 2008; Tasker et al., 2015). eCBs are a class of neuroactive lipids that include N-arachidonoyl-etanolamine (AEA, anandamide), which is a partial agonist of the G- protein-coupled cannabinoid receptors named CB1 and CB2 (Howlett et al., 2002; Lu and MacKie, 2016; Pertwee et al., 2010). Moreover, the potency of AEA to activate the transient potential vanilloid type-1 channel (TRPV1) suggests that TRPV1, a nonselective cation channel widely expressed in the periphery as well as in the central nervous system, is a functional AEA receptor. Intracellular AEA induces the opening of TRPV1 channel which contributes to many of the non-CB-mediated effects of AEA (Cristino et al., 2006; Hillard, 2015; Ross, 2003; Smart et al., 2000; th et al., 2009, 2005; th et al., 2009, 2005).

Concerning the regulation of the stress response, the main physiological role of AEA is modulating the release of various neurotransmitters through the activation of presynaptic CB1 cannabinoid receptors located on axon terminals throughout the brain, mainly at the hypothalamus (Hillard, 2014; Roberts et al., 2014). Furthermore, CB1 receptors are also located at pituitary and adrenal levels (Howlett et al., 2002; Mechoulam and Parker, 2013; Pagotto et al., 2001). However, the available literature regarding the participation of pituitary CB1 receptors in the control of the HPA axis remains very scarce and suggests that the stress response is modulated at other levels besides the pituitary (Rabasa et al., 2015). We found that human adrenal cortex cells expressed CB1 receptors and that synthetic cannabinoids directly inhibited steroidogenesis and cortisol release (Ziegler et al., 2010). However, evidence is lacking regarding the effects of anandamide on rat adrenal glands *via* CB1 or other receptors in either basal or stress conditions.

The pharmacological activity of AEA is limited by its intracellular hydrolysis by fatty acid amide hydrolase (FAAH). Pharmacological inhibition of this enzyme specifically augments AEA-mediated eCB signaling and has been shown to reduce anxiety behaviors in rodents and represents a promising approach to the treatment of stress disorders (Bluett et al., 2014; Fowler, 2012; Hill et al., 2010; Pertwee et al., 2015).

Nitric oxide (NO) may function as a modulator in the brain *per se* or by influencing the functioning of other neural networks. Under physiological conditions, neuronal NO synthase (nNOS) is constitutively expressed, and it constitutes the major isoenzyme in the brain. Inducible NO synthase (iNOS) is undetectable under basal conditions, and it is upregulated in response to various stimuli such as inflammation and stress (Gadek-Michalska et al., 2013; Gądek-Michalska et al., 2012; Nelson et al., 2006). Recent studies have shown that NO clearly has a role during the stress response (Gądek-Michalska et al., 2016; Gulati et al., 2015; , 2009). Its production is modified during diverse stress conditions and NO can modulate the release of stress hormones such as CRH, ACTH and corticosterone (Gadek-Michalska et al., 2013; Karanth et al., 1993; McCann et al., 2005; Mohn et al., 2011, 2005; Rettori et al., 2009) playing an important regulatory role in stress response (Chakraborti et al., 2011; Esch et al., 2002). However, conflicting findings abound in literature. It has been proposed that NO has opposite effects on different components of the HPA axis and that the effects of this molecule also depends on the type and the duration of stressful stimulus (Gulati et al., 2009). Thus, the ultimate effect of NO mediated regulation of stress axis is still unknown. Moreover, very scarce information is available about the interaction between NOergic and endocannabinoid systems. In this regard, we previously revealed a cooperation between both systems providing a regulation of the hypothalamic–neurohypophyseal axis under basal and inflammatory conditions (Luce et al., 2013).

Consequently, in the present study we hypothesized that eCB signaling inhibits acute restraint stress- induced HPA axis activation. Therefore, cannabinoid agonists, antagonists or FAAH inhibitor were microinjected intracerebroventricularly (icv) into the third cerebral ventricle or intraperitoneally (ip) to male rats to determine the effects on plasma corticosterone concentrations. We also evaluated the effect of restraint on cannabinoids receptors expression at hypothalamic and adrenal levels. Furthermore, the possible interaction between endocannabinoid and NOergic systems in the control of stress response was examined by nitric oxide synthase activity determination at hypothalamus and adrenal glands.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 200–250 g purchased from the School of Veterinary Sciences of the University of Buenos Aires were used. The animals were fed lab chow and water *ad libitum* and kept under controlled conditions of light (12 h light/dark) and temperature (19–23 °C). The animals were kept in group cages (five rats *per* cage) in our animal room for 7 days before the experiment. The animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals from the National Academy Press, Washington D.C., 8th Edition, 2011. All the procedures were in compliance with the Institutional Committee of Care and Use of Experimental Animals (CICUAL) from the School of Medicine, University of Buenos Aires (Res. (CD) No. 2831/10).

2.2. Drugs

All materials were purchased from Sigma Co. (St. Louis, MO, USA), except Dowex AG 50W-X8 resin (Bio- Rad Laboratories, CA, USA), ACTH was obtained from Elea Laboratories (Buenos Aires, Argentina), L-[U- 14C]Arginine with specific activity: 11.26 GBq/mmol (Amersham Int., Buckinghamshire, UK), [1,2,6,7- 3H]Corticosterone from NEN Life Science Products (Boston, USA); AM251 [N-(piperidin-1-yl)-1-(2,4- dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1Hpyrazole-3-carboxamide], AM630 {6-Iodo-2-methyl-1- [2-(4-morpholinyl)ethyl]-1H-indol-3-yl}(4-methoxyphenyl) methanone, capsaicin and capsazepine N-[2-(4-chlorophenyl) ethyl]-1, 3, 4, 5-tetrahydro-7, 8-dihydroxy-2H-2-benzazepine-2-carbothioamide were obtained from Tocris™ (Ellisville, MO, USA).

The doses of the compounds were chosen in accordance with literature and our previous studies demonstrating neuroendocrine effects following icv microinjection, ip injection or incubation with them (Fernandez-Solari et al., 2009, 2006; Luce et al., 2013).

2.3. Experimental protocols

2.3.1. In vivo studies

2.3.1.1. Surgery. To evaluate the participation of the hypothalamic endocannabinoid system, one week prior to the day of the experiment, an indwelling cannula was implanted into the third cerebral ventricle by using a stereotaxic instrument while the rats were anesthetized *via* ip with a cocktail consisting of Ketamine HCL (70 mg/kg), Xylazine (10 mg/kg). The coordinates relative to the interaural line (AP- 0.6 mm, L–2 mm, DV- mm) were taken from the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 2007).

2.3.1.2. Restraint stress procedure. Animals were acclimated to the testing room for 24 h prior to experimentation. Animals were restrained only once in transparent acrylic tubes with numerous air holes to increase ventilation. Animals in the tube were placed on the bench top for the restraint period of 30 min. Control animals were left undisturbed in their home cages. Rats were euthanized immediately after the restraint period in a different room than the one in which the stress was carried out and in a random order within each cage. Although sequential euthanasia could have resulted in social stress among the cage mates that remained, the time required to euthanize an entire cage of rats was 10 min and both restrained and control groups