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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

experienced the same stress.

2.3.1.3. Treatments. The experiments were performed in conscious, freely moving rats (n = 5 rats *per* group). The rats received an injection of saline (5 µl, icv) that was also used as vehicle to dissolve all other drugs administered, or AEA (50 ng/5 µl) or cannabinoid receptors antagonists (AM251, 500 ng/5 µl alone or together with AM630, 500 ng/5 µl) or FAAH inhibitor (URB597, 50 µg/5 µl), depending on the experiment. These injections were made in control rats or 15 min previous to the restraint stress (previously described) with a Hamilton syringe during a period of 1 min. The adequate location of the cannula in the ventricle was confirmed by injecting a solution of methylene blue icv and confirming its position in histological sections only in a couple of random selected animals by experiment.

In another set of experiments, control and stressed rats received the drugs that modulate endocannabinoid system components *via* intraperitoneal (ip). Rats received methanandamide, a non hydrolizable analogous of anandamide (MetAEA, 2.5 mg/kg, ip), AM251 (2 mg/kg, ip), URB (0.3 mg/kg or 0.9 mg/kg, ip) or vehicle 15 min previous to the restraint stress.

Rats were sacrificed by decapitation and trunk blood collected. Blood was allowed to coagulate and then centrifuged 20 min 3000 rpm at 4 °C and the serum separated for corticosterone determinations. The brains were immediately removed, and medial basal hypothalami (MBH) were dissected by making frontal cuts 1 mm rostral to the optic chiasm. A horizontal cut extended from this point caudally to just behind the pituitary stalk, where another frontal cut was made. Longitudinally, cuts were made 1 mm lateral to the midline bilaterally. Both adrenal glands were also removed. MBH and adrenal glands were stored frozen at −80 °C to further determinations.

2.3.2. In vitro studies

Intact animals were sacrificed and their adrenal glands were excised and freed of pericapsular fat. Each adrenal gland was bisected; both halves were placed in the same tube (n = 10 adrenal/group) and preincubated for 15 min in a Dubnoff shaker (60 cycles per min) at 37 °C in an atmosphere of 95% O2–5% CO2 in 1 ml of Krebs–Ringer bicarbonate buffer (KRB) (118.46 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.18 mM NaH2PO4, 1.18 mM MgSO4, 24.88 mM NaHCO3, pH 7.4) containing 10 mM glucose and 0.1 mM bacitracin. Then, the medium was discarded and replaced with fresh medium alone (control) or containing the substances to be tested: ACTH (10^{-9} M) alone or with AEA (10^{-9} M), URB597 (3uM), AM251 (10^{-5} M) o CPZ (10^{-5} M), and the tissues were incubated further for 30 min. Then, the media were frozen at −70 °C for subsequent determination of corticosterone released into the medium by RIA. The activity of nitric oxide synthase (NOS) was immediately determined in tissues.

In another set of experiments, adrenal glands (n = 8 glands/group) were incubated with fresh medium alone (control) or ACTH (10^{-9} M) for 90 min. Then, the tissues were frozen at −70 °C for subsequent RT-PCR determination of CB1 and TRPV1 mRNAs.

2.4. Determinations

2.4.1. RT-PCR

Adrenal gland tissues were homogenized in RNAzol Reagent and frozen at −80 °C until used. Total RNA was isolated according to the manufacturer's recommendations (Molecular Research Center Inc., Cincinnati, OH, USA). Following extraction, RNA was quantified and cDNA was synthesized from total RNA (3 µg) using M-MLV RT, random primers and ribonuclease inhibitor. The specific primers to performed PCR amplifications were designed using the Primer 3 Software. The primers sequences are: β-actin forward 5′ACCCGCCGAGTACAACCTTC 3′ and reverse 5′ATGCCGTGTTCAATGGGGTA 3′ (94 °C 5 min; 35 cycles of: 94 °C 40s, 58 °C 30s, 72 °C 1 min; 72 °C 5 min); TRPV1 forward 5′ GACATGCCACCCAGCAGG 3′and reverse 5′ TCAATTCCCACACACCTCC 3′ (94 °C 5 min; 35 cycles of: 94 °C 40s, 57 °C 30s, 72 °C 1 min; 72 °C 5 min), CB1 forward 5′ GGAGAACATCCAGTGTGGG 3′ and reverse 5′ CATTGGGGCTGTCTTTACGG 3′ (94 °C 5 min; 35 cycles of: 94 °C 40s, 56 °C 30s, 72 °C 1 min; 72 °C 5 min) CB2 forward 5′ CTTGACTGAGCACCAGGACA 3′and reverse 5′ TAACAAGGCACA GCATGGAG 3′ (94 °C 5 min; 35 cycles of: 94 °C 40s, 60 °C 30s, 72 °C 1 min; 72 °C 5 min). Products were loaded onto 2% agarose gel and stained with ethidium bromide. Bands were visualized on a transilluminator under UV light. Photographs were taken with a digital camera (Olympus C-5060) and analyzed with the Image J software package. The relative mRNA level was normalized to β-actin and results were expressed as arbitrary units (au) of relative optical density.

2.4.2. Corticosterone radioimmunoassay

The corticosterone concentration of serum and released into the incubation media were measured by RIA as described previously (Mohn et al., 2011). The samples were incubated with rabbit anti- corticosterone for 30 min at room temperature. Then, [3H]corticosterone was added as a tracer and incubated for 1 h at 37 °C. The reaction was stopped by the addition of cold dextran-coated charcoal suspension and after the incubation for 10 min at 0 °C; the tubes were centrifuged at 2000 × g for 15 min at 4 °C. Scintillation mixture was added to the supernatant, and the amount of radioactivity was determined in a beta counter. The sensitivity of the assay was 30 pg *per* tube.

2.4.3. Nitric oxide synthase (NOS) activity

NOS activity of all isoforms of the enzyme (total NOS) was determined by conversion of [14C]arginine to [14C]citrulline, using a modification of the method of Bredt and Snyder described previously (De Laurentiis et al., 2000). Briefly, the hypothalamic fragments or adrenal glands were immediately homogenized in 0.5 ml of 20 mM Hepes, pH 7.4, containing 0.4 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM dithiothreitol, 0.45 mM CaCl2. The reaction was started by adding 20 µl (0.1 µCi) of L-[U-14C]arginine to the homogenate. After 20 min of incubation at 37 °C, the reaction was stopped by centrifugation at 10,000 rpm for 10 min. The supernatants were applied to 1.5 ml columns of Dowex AG 50 W-X8 in 20 mM Hepes, pH 7.4, loaded with 20 µl of 100 mM L-citrulline. [14C]citrulline was eluted with 3 ml of distilled water and the radioactivity quantified by liquid scintillation spectroscopy of the flow-through. The results were expressed as pmoles de NO/min* mg of protein.

2.5. Statistics

Experiments were performed at least three times (N = 3) and figures represent results of individual experiments. Data were expressed as mean ± SEM. Analysis of variance followed by the Tukey's test for multiple comparison or unpaired "*t*" tests were used to determine statistical significance (95%; $P < 0.05$). Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene test, respectively. Statistical analysis was performed using the software Infostat (Córdoba, Argentina).

3. Results

3.1. In vivo experiments

3.1.1. Hypothalamic anandamide blocks the stress-induced-increase of corticosterone levels

We conducted experiments in which animals were subjected to restraint stress for 30 min and subsequently immediately euthanized to determine corticosterone (CORT) serum levels. This acute stress paradigm causes a robust production of corticotropin-releasing hormone (CRH), ACTH, and CORT. Our data shows that serum corticosterone content was approximately 6 fold greater in rats subjected to one

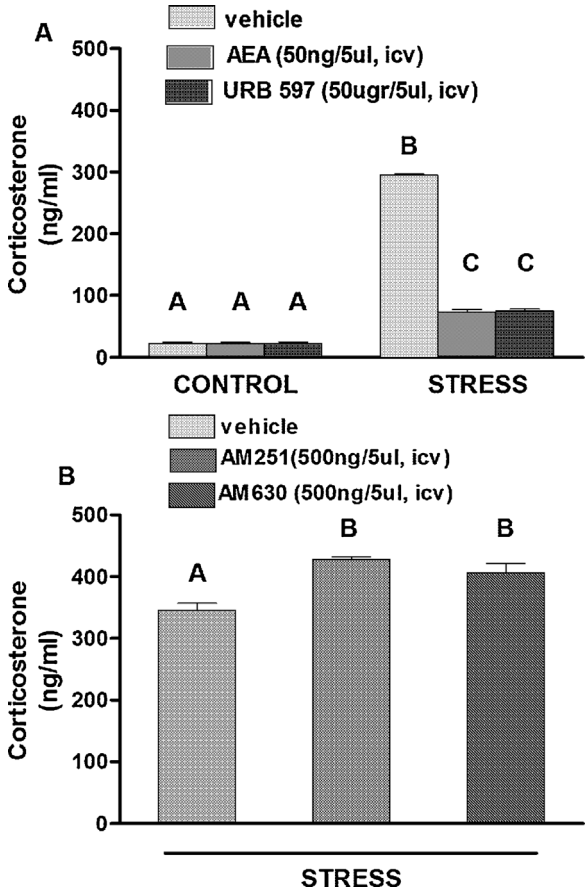

Fig. 1. A. Acute restraint for 30 min significantly increased corticosterone plasma levels. Both, anandamide (AEA, 50 ng/5ul, icv) and the inhibitor of the fatty acid amide hydrolase (FAAH), the enzyme that degrades AEA (URB597, 50ugr/5ul, icv) blocked the stress-induced increase of corticosterone. B. Stressed rats received CB1 (AM251, 500 ng/5ul, icv) or CB2 (AM630, 500 ng/5ul, icv) receptors antagonists into the 3° cerebral ventricle. The blockade of both cannabinoid receptors subtypes significantly increased corticosterone plasma levels. Bars represent mean ± SE (n = 6–8). Statistics: A ≠ B ≠ C, $p < 0.05$.

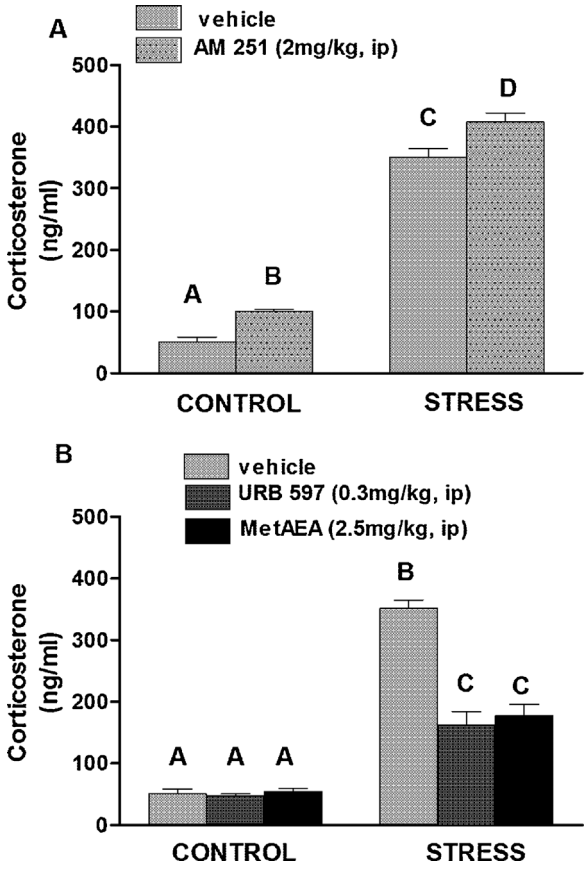

Fig. 2. A. Intraperitoneal (ip) injection of FAAH inhibitor (URB597, 0.3 mg/kg, ip) or methanandamide (MetAEA, 2.5 mg/kg, ip), a non-hydrolysable analogue of AEA, significantly decreased the stress-induced corticosterone plasma levels. Bars represent media ± SE (n = 6). B. CB1 receptors antagonist (AM251, 2 mg/kg, ip) increased corticosterone plasma levels in both stressed and no stressed rats. Bars represent mean ± SE (n = 6). Statistics: A ≠ B ≠ C ≠ D, $p < 0.05$.

restraint episode compared to non-stressed control rats (Fig. 1A).

The importance of eCBs in directly affecting synaptic function in the hypothalamus led us to investigate the effects of centrally administered pharmacological compounds that modify eCB system components. The central administration of anandamide (AEA, 50 ng/5 µl, icv) or the inhibitor of fatty acid amide hydrolase (FAAH), the enzyme that degrades AEA (URB597, 50 µg/5 µl, icv), significantly blocked the stress-induced increase of corticosterone (Fig. 1A) (ANOVA $F(5, 24) = 1519.67$, $P < 0.0001$, n = 5).

3.1.2. Blockade of hypothalamic cannabinoid receptors potentiates restraint-induced corticosterone levels

To determine whether eCB hypothalamic signaling modulates the activity of the HPA axis, we administered the CB1 or CB2 receptor specific antagonists and determined serum CORT concentrations immediately after 30 min of restraint stress. Control and stressed rats received antagonists of the CB1 (AM251, 500 ng/5 µl) or CB2 (AM630, 500 ng/5 µl) cannabinoid receptors into the 3° cerebral ventricle (icv) 15 min before the induction of stress. None of the used antagonists modified basal corticosterone levels in control rats (data not shown). However, the blockade of any of the two cannabinoid receptor subtypes studied significantly increased serum corticosterone levels in stressed rats (Fig. 1B) (ANOVA, $F(2.12) = 14.74$, $P = 0.0006$,n = 5).

3.1.3. Peripheral anandamide blocked the stress-increased corticosterone levels

As previously demonstrated, the inhibitory effect of AEA on stress response is supposedly centrally mediated. However, additional effects on peripheral endocrine glands such as pituitary and adrenals cannot be entirely ruled out. Therefore we explored the action of systemic administered cannabinoids agonists and antagonists on HPA activation by stress. The intraperitoneal (ip) injection of FAAH inhibitor (URB597, 0.3 mg/kg, that increases endogenous levels of AEA) or methanandamide (MetAEA, 2.5 mg/kg, a non-hydrolysable analogue of AEA) significantly decreased the stress-induced CORT serum levels (Fig. 2A) (ANOVA, $F(5.23) = 80.97$, $P < 0.0001$, n = 5). Moreover, the systemic administration of CB1 receptors antagonist (AM251, 2 mg/kg, ip) increased CORT serum levels in both stressed and no stressed rats (Fig. 2B) (ANOVA 2 factors, F interaction $(1.16) = 0.09$, $P = 0.765$; F stress $(1, 16) = 24.05$; F AM251 $(1.16) = 785.52$, $P < 0.0001$; n = 5). These pharmacological effects could be mediated at pituitary or adrenal levels; however a central action of these compounds should be taken into account for at least two reasons. One is that we cannot rule out that these peripherally injected compounds can cross the blood-brain barrier and the other is our observation that hypothalamic NOS activity was inhibited by the ip administration of the FAAH inhibitor during stress. Moreover, this effect is more evident with the non-hydrolysable cannabinoid agonist methanandamide. Stress *per se* did not modify hypothalamic NOS activity (Fig. 3) (ANOVA, $F(4.20) = 17.42$, $P < 0.0001$, n = 5). No dose of URB597 or methanandamide modified hypothalamic NOS activity in control non-stressed rats (data not shown).

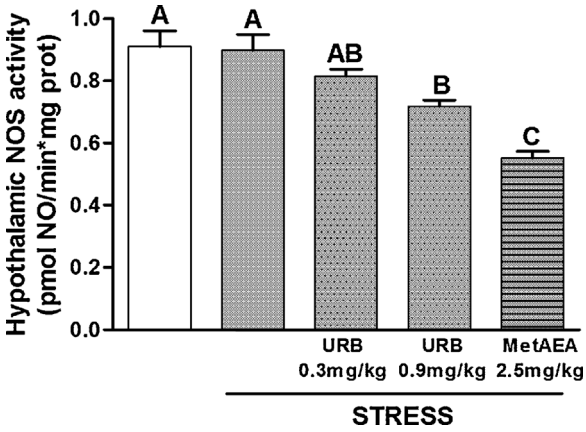

Fig. 3. Hypothalamic NOS activity was inhibited by the administration of the FAAH inhibitor (URB597, ip) during stress. This effect is more evident with the agonist methanandamide (MetAEA, 2.5 mg/kg, ip). Bars represent mean ± SE (n = 5). Statistics: A ≠ B ≠ C, $p < 0.05$.

3.1.4. Cannabinoid and TRPV1 receptors mRNA expression

It has been shown that moderate CB1 receptor densities are found in the hypothalamus and that chronic stress can increase the expression and binding of this receptor (Hillard, 2014). In our present study, acute restraint stress also increased CB1 mRNA in medial basal hypothalamus (MBH) without affect TRPV1 mRNA expression when the tissue was excised from rats sacrificed 4 hs post stress (Fig. 4A) (P = 0.0013, n = 6).

We have previously demonstrated that the normal human adrenal cells and the human adrenocortical cell line NCI-H295R express CB1 but not CB2 receptors (Ziegler et al., 2010). In the present study RT-PCR data revealed that normal rat adrenal glands express mRNA of both, the endocannabinoid receptor CB1 and vanilloid TRPV1. Furthermore, stress significantly increased TRPV1 mRNA levels in adrenal glands (P = 0.0257, n = 4) with no changes in CB1 mRNA levels in this tissue (Fig. 4B) (P = 0.0945, n = 6). CB2 expression was not detected in hypothalamus or in adrenal gland evaluated in our experimental conditions (data not shown).

3.2. In vitro experiments

The aforementioned presence of CB1 and TRPV1 in adrenal gland tissue suggests an influence of cannabinoid agonists on adrenocortical function. To determine the local effects of eCB at adrenal level we performed *in vitro* experiments and we therefore tested whether AEA influences corticosterone release. As we had previously demonstrated (Mohn et al., 2005), ACTH (10^{-9} M) highly stimulated the release of corticosterone over 30 min of incubation of intact adrenal glands. The presence of AEA (10^{-9} M) or the inhibition of its degrading enzyme by URB597 (3uM) prevented the ACTH-induced increased incorticosterone release (Fig. 5A) (ANOVA, F(5.24) = 32.26, $p < 0.0001$, n = 5). Since anandamide completely abolished the stimulation of ACTH on corticosterone release from adrenal gland, we sought to investigate which receptor is involved in this effect. The presence of the CB1 antagonist (AM251, 10^{-5} M) showed no effect *per se* in absence of ACTH and partially reverts AEA blockade of ACTH-induced corticosterone release (Fig. 5B) (ANOVA, F(4.67) = 40.43, $P < 0.0001$, n = 10–20). On the other hand, the TRPV1 antagonist (capsazepine, CPZ, 10^{-5} M) showed a stimulatory effect on corticosterone release even in absence of ACTH and completely reversed inhibitory effect of AEA. These results revealed an endogenous inhibitory tone of AEA on corticosterone release produced *via* TRPV1 and also the main role of these receptors in the control of adrenal gland corticoid secretion (Fig. 5C) (ANOVA, F(4.67)

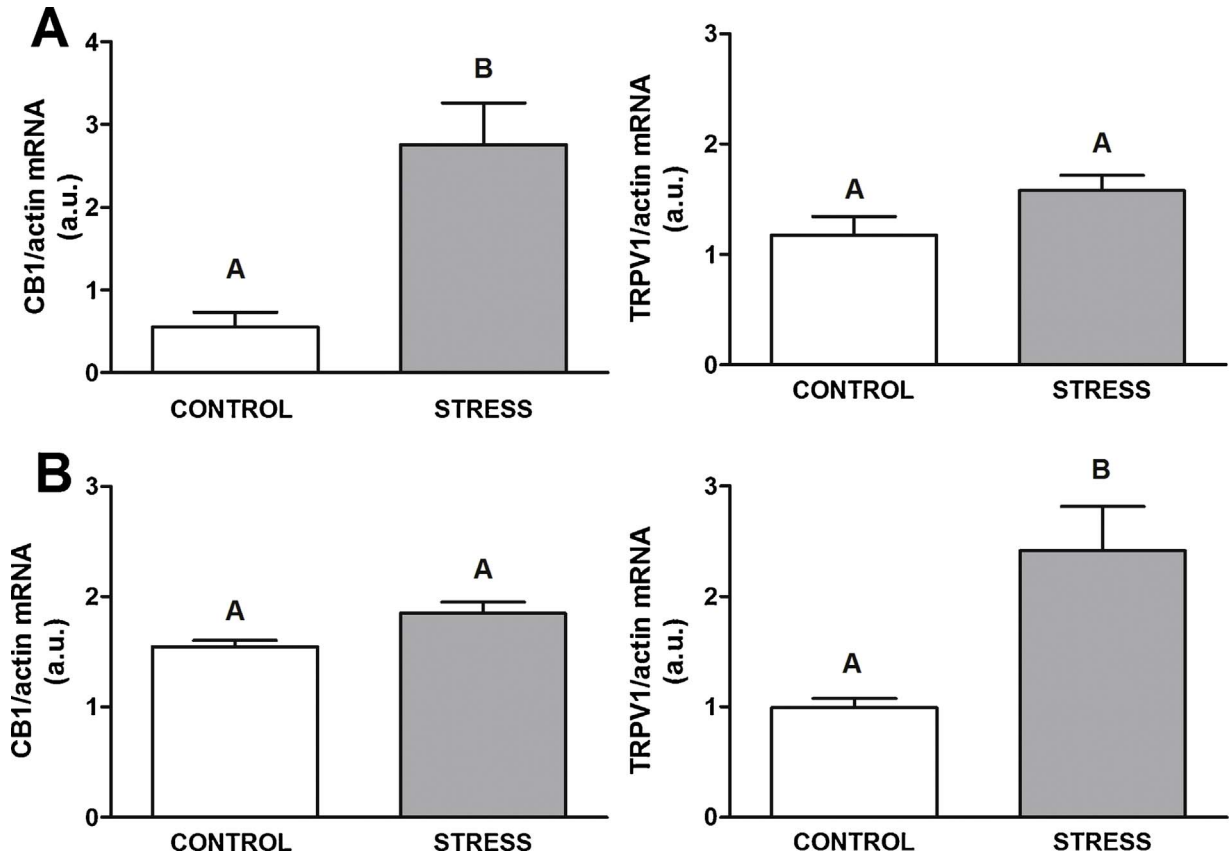

Fig. 4. CB1 and TRPV1 mRNA expression were assessed by RT-PCR and normalized to β-actin mRNA expression (CB1/β-actin or TRPV1/β-actin). A. Medial basal hypothalamic (n = 6) and B. adrenal glands (n = 6) from control and stressed rats. Results are expressed as relative optical density in arbitrary units (a.u.). Statistics: A ≠ B, $p < 0.05$.

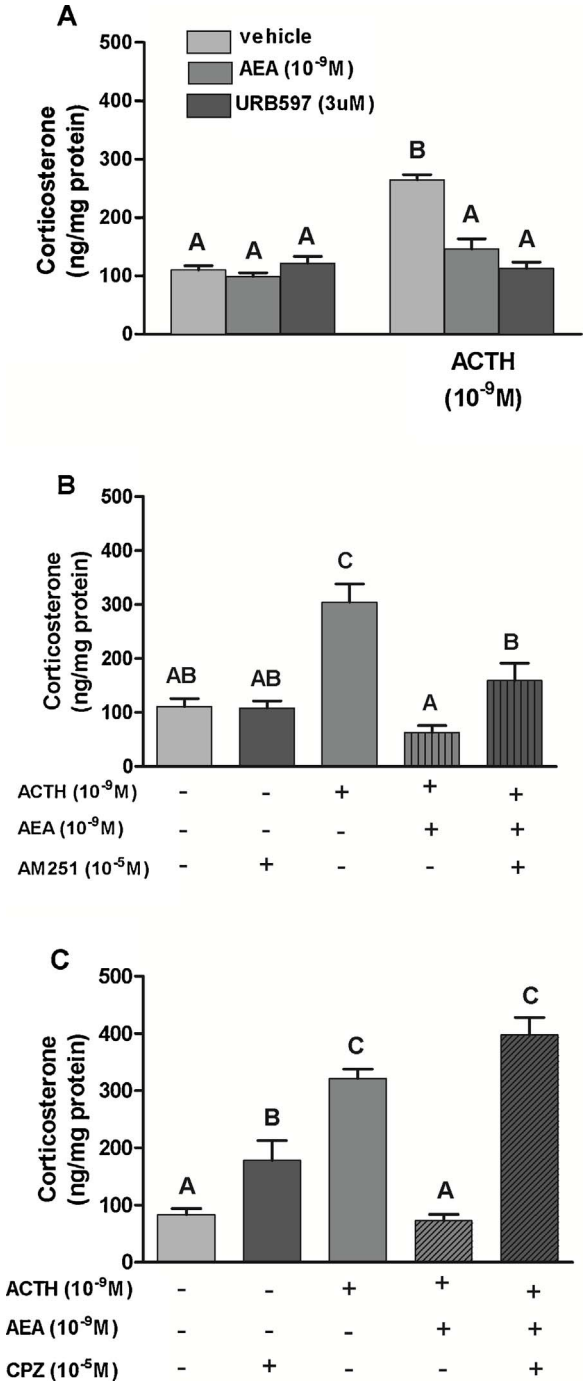

Fig. 5. A. ACTH (10^{-9} M) increased corticosterone release from adrenal glands *in vitro*. The presence of AEA (10^{-9} M) or the FAAH inhibitor (URB597, 3 uM) prevented the ACTH-induced increased in corticosterone. B. CB1 antagonist (AM251, 10^{-5} M) partially prevented AEA (10^{-9} M) blockade. C. TRPV1 antagonist (capsazepine, CPZ, 10^{-5} M) completely prevented AEA inhibitory effect and also unmask ACTH-free induced corticosterone levels. Bars represent mean ± SE (n = 10–20). Statistics: A ≠ B ≠ C ≠ D, $p < 0.05$.

= 33.32, P < 0.0001, n = 10–20).

Regulation of adrenal function by ACTH may involve NO, which has been identified as a major biological signaling molecule exerting both inter- and intracellular effects in this gland (Cymeryng et al., 2002). Our results showed that the stimulatory effect of ACTH (10^{-9} M) on adrenal NOS activity was significantly inhibited by AEA (10^{-9} M) even to significantly lower levels than control. When CB1 receptors were blocked by AM251 (10^{-5} M), the AEA inhibitory effect still partially persisted, but is completely prevented when vanilloid receptors were blocked by CZP (10^{-5} M) (Fig. 6A) (ANOVA, F(4.25) = 16.41, P < 0.0001, n = 6). The presence of ACTH (10^{-5} M) in the incubation media during 90 min induced a significantly increase in mRNA expression of TRPV1 channel in adrenal glands (P < 0.0001, n = 5) with no effect on CB1 messenger expression (Fig. 6B) in concordance with *in vivo* results.

4. Discussion

Previous studies employing acute stressors that include novelty stress, immobilization, forced swimming and tail suspension demonstrated that inhibition of ECS results in increased circulating corticosterone concentrations following stress exposure (Evanson et al., 2010; Newsom et al., 2012; Patel et al., 2004; Pertwee et al., 2015). Our findings are also paralleled with pharmacological studies showing that systemic administration of the CB1 receptor antagonist rimonabant potentiates stress-induced increases in circulating levels of corticosterone (Finn, 2010). Our present study is consistent since we demonstrate that eCB signaling negatively modulates the activation of the HPA axis in a restraint stress model. Our data indicates that CB1 receptor and TRPV1-endocannabinoid signaling functions to buffer or dampen the endocrine effects of acute stress. The endocannabinoid system in general, and anandamide in particular, could be part of a negative system that limits the neuroendocrine acute stress response. Our data support the role of ECS as a homeostatic mechanism that inhibits unnecessary HPA axis activation and promotes the recovery of the HPA axis to baseline after the threat has ended. We investigated the importance of endocannabinoid signaling in the acute stress response by using pharmacological agents that modulate the eCB system activity both *in vivo* and *in vitro*.

Our first aim was to find out whether brain and peripheral anandamide could affect corticosterone plasma levels after 30 min of restraint. Both central and systemic anandamide blocked the stressed induced increase of corticosterone levels. These findings are in agreement with those of other authors who have demonstrated that the pharmacological augmentation of eCB signaling significantly reduces immobilization- (Evanson et al., 2010) and loud noise- (Newsom et al., 2012) induced corticosterone release. Our results contrast with the work of Roberts *et al.* where URB597 intraperitoneal treatment had no significant effect on corticosterone concentrations at any time before or after the restraint stress in either male or female mice (Roberts et al., 2014). This difference could be attributed to the dose of the FAAH inhibitor used, with ours being 3- fold higher. Moreover, this interpretation is strengthened by the dose response observed in the present work. Furthermore, our results are in concordance with previous findings indicating that pharmacological elevation of AEA after stress by the blockade of FAAH enzyme with URB597 (Viveros et al., 2005) or PF-3845 (Bluett et al., 2014) produces anxiolytic-like effects typically exhibited after exposure to an intense acute stressor (Hill et al., 2013). These results indicate that anandamide participates in the modulation of emotional states and point to FAAH inhibition as an innovative approach to anti-stress therapy.

Since the central administration of CB1 and CB2 receptor specific antagonists produced a small, but significant potentiation of the HPA axis activation induced by acute restraint stress, we suggest that both brain cannabinoid receptors participate equally in the anandamide signaling that negatively modulates the activation of the HPA axis. In CNS, CB2 receptors present in brain areas related to the HPA axis are mainly expressed by microglia and astrocytes, whereas the extent of CB2 expression in neurons remained questionable (Atwood and MacKie, 2010). Although there is still controversy regarding the role of the CB2 receptor in the brain, its expression in stress-responsive neural circuits, such as the hippocampus, amygdala and hypothalamus (Gong et al., 2006), indirectly suggests that CB2 receptor activation could regulate the neuroendocrine response (Onaivi et al., 2012). However,

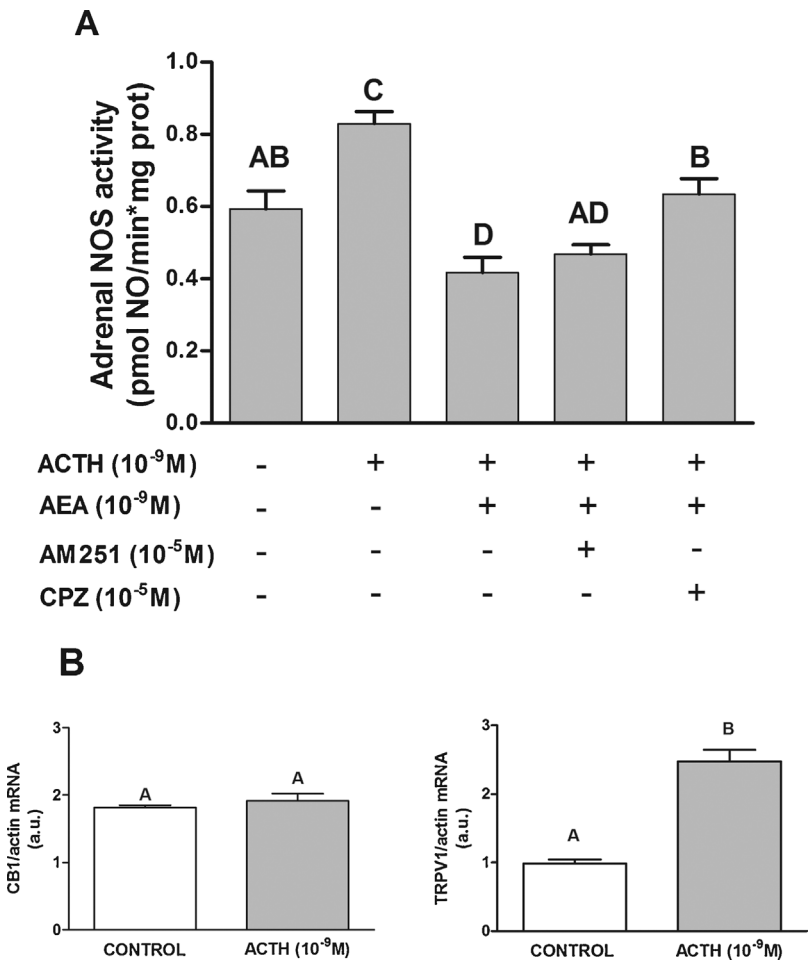

Fig. 6. A. The stimulatory effect of ACTH (10^{-9} M) on adrenal NOS activity was not observed in the presence of AEA (10^{-9} M). This effect was completely reversed when vanilloid receptors were blocked by capsazepine (CPZ, 10^{-5} M). However, CB1 receptors blockade by AM251 (10^{-5} M) did not avoid significantly AEA inhibitory effect. Bars represent mean ± SE (n = 6). Statistics: A ≠ B ≠ C ≠ D, $p < 0.05$. B. CB1 and TRPV1 mRNA expression were assessed by RT-PCR and normalized to β-actin mRNA expression (CB1/β-actin or TRPV1/β-actin in adrenal glands from intact rats incubated in the presence of buffer (CONTROL) or ACTH (10^{-9} M) for 90 min (n = 8). Results are expressed as relative optical density in arbitrary units (a.u.). Statistics: A ≠ B, $p < 0.05$.

our results differ from the results found in a sub-chronic immobilization and acoustic stress protocol used by Zoppi *et al.* that excluded a role of CB2 receptors in the regulation of corticosterone levels (Zoppi et al., 2014). These differences could be explained by differences in the duration of the stress. Nonetheless, we cannot rule out that AM630 could activate CB1. Even though this drug behaves as a potent and selective CB2 receptor antagonist, with a Ki of 32.1 nM, it has been shown to act as a weak partial CB1 inverse agonist (Ross et al., 1999).

The aforementioned data suggest that the brain eCB system is an important regulator of the central hypothalamic stress response however, we found that systemic anandamide also blocked the increase of corticosterone levels. Furthermore, the CB1 antagonist unmasked a strong interaction of endogenous cannabinoids with these receptors located peripherally, at least at pituitary and/or adrenal glands during basal and stress conditions. Although we cannot rule out that peripherally injected anandamide and cannabinoid receptor antagonists can cross the blood-brain barrier, the systemic effect was confirmed by the incubation of adrenal glands with these compounds.

Also, our results are in concordance with a previous works showing that intraperitoneal AM251 treatment increased the control and stress-induced levels of HPA-axis activity (Hill and McEwen, 2010; Newsom et al., 2012; Patel et al., 2004). These data suggest that endocannabinoid signaling constrain the activation of the HPA axis and, if this signaling is disrupted, the axis is activated even in absence of a stressful stimuli, with an exaggerated response occurring after a stress challenge.

The cellular and molecular mechanisms underlying the effect of endocannabinoids on HPA axis regulation have only begun to be deciphered. However, based in our present results, we suggest that anandamide could act at least *via* CB1 and CB2 receptor mediating the modulation of hypothalamic factors that regulate the secretion of neuroendocrine cells. Among these factors it is known that NO generated by nNOS and iNOS in brain structures is involved in HPA axis regulation. The brain NO produced may act as an important regulator of the hypothalamic neurons function. In fact, it has been demonstrated an excitatory role of vascular NO on CRH secretion in the PVN and median eminence after acute hypoxia (Coldren et al., 2017). During chronic stress response, the induced expression of iNOS generate larger NO production that is implicated in the pathophysiology of chronic stress-induced inflammation (Gadek-Michalska et al., 2013). However, we demonstrate in our acute experimental conditions that 30 min of restraint stress is not able to modify hypothalamic NOS activity. This is probably because we analyzed the joint activity of both hypothalamic NOS isoforms and in this short time iNOS is possibly still inactive. In a previous study, we found that anandamide increased basal hypothalamic NOS activity *in vitro* (De Laurentiis et al., 2010). Conversely, in the present study the hypothalamic NOS activity was attenuated in stressed animals treated with URB597 and markedly inhibited with the non-hydrolysable anandamide analogue. One possible explanation for these opposite effects could be that in the previous *in vitro* study we incubated the hypothalamic fragments from naive animals in the presence of only one dose of AEA (10^{-9} M) that produced an stimulatory effect on NOS activity. This effective dose of AEA was immediately removed from the site of action and hydrolyzed by the degrading enzyme present in the tissue (De Laurentiis et al., 2010). In the present study, we evaluate the AEA effects by the systemically injection of a non-hydrolysable metabolically stable analogue of the endocannabinoid anandamide or by the blockade of AEA degrading enzyme allowing an exacerbated signal by permanent high levels of AEA in the whole stressed animal. In conclusion, the effect observed in the

present study by anandamide inhibiting NOS activity at the hypothalamic level, could be one of the pathways involved in the anti-stress effect of this endocannabinoid.

We cannot rule out that endocannabinoids actions in the hippocampus, amygdala, pituitary and adrenal glands may also contribute to the negative regulation of the HPA axis as well as to the stress neuroendocrine response. In pituitary gland, CB1 receptors are located on the human corticotroph cells (Pagotto et al., 2001) raising the possibility that cannabinoids exert a direct action on these pituitary cells. However, the effects of cannabinoids on murine corticotroph cells are minimal, and the CB1 receptors of these cells are unable to substantially affect ACTH secretion (Barna et al., 2004). Confirming this report, a recent work by Rabasa *et al.* (2015) has shown that a knock-out mice for the CB1 receptor presented similar ACTH response to stress as wild-type mice. Neither genetic deletion nor pharmacological blockade of CB1 with AM251 impeded ACTH adaptation to daily repeated exposure to two different immobilization procedures in rat and mice (Rabasa et al., 2015). These data suggest interactions of the endocannabinoids with the HPA axis at other levels than the pituitary, which prompted us to focus our study on the endocannabinoid system in the adrenal glands. Furthermore, our previous data indicated the expression of CB1 in human adrenal cortex cells and adrenocortical NCI-H295R cells; while CB2 was not detected (Ziegler et al., 2010). Moreover, we have shown that anandamide directly inhibited basal and stimulated steroidogenesis and the release of cortisol and aldosterone in cultured cells, and this late effect was reversed by Rimonabant, a CB1 antagonist (Ziegler et al., 2010). Our present results are in agreement since CB2 mRNA was not detected and CB1 mRNA was present in adrenal glands from control and stressed rats. Moreover, TRPV1 mRNA was present in this tissue and its expression was upregulated after stress.

ACTH is the major regulator of steroid synthesis and secretion from the adrenal cortex, inducing an acute response within a few minutes. Our present results show that ACTH increased corticosterone secretion 3 fold compared with glands incubated in buffer for 30 min. We determined for the first time a full inhibitory effect of anandamide on ACTH-stimulated corticosterone secretion that was partially reversed by the concomitant incubation with the CB1 receptor antagonist and completely reversed by the blockade of TRPV1 activation. In fact, likewise as we observed in adrenal glands from stressed rats, ACTH also induced an overexpression of TRPV1 mRNA in the incubated adrenals and the blockade of this channel with capsazepine even in absence of ACTH potentiated corticosterone secretion probably unmasking an endogenous tone of AEA that controls the release of corticosterone. These findings have a great impact since we have determined that stress increases the expression of this receptor and it is known that in tissues with high receptor reserve and in circumstances associated with certain disease states, such as stress, anandamide behaves as a full agonist for TRPV1 (Ross, 2003). NO also participates in signal transduction pathways that result in the release of corticosterone from the adrenal gland. We previously showed that NO leads to a rapid release of stored corticosterone from the adrenals and ACTH increased NOS adrenal activity (Mohn et al., 2005). Similarly, in the present study ACTH activates adrenal NOS. This activation was blocked by anandamide acting partially *via* CB1 and also through TRPV1. In conclusion, our data further contributes to *in vitro* evidence for a participation of the endocannabinoid and NOergic systems in adrenal steroidogenesis, inhibiting adrenal function directly at the level of the adrenal gland. This effect might, in addition to a central influence of endocannabinoids on HPA axis function, be relevant in glucocorticoid homeostasis.

5. Conclusions

In summary, our present results lead us to conclude that an endogenous cannabinoid tone maintains the HPA axis in a stable basal state, which is lost with a noxious stimulus. This complex response of central and peripheral pathways to acute stress involve a crosstalk between NO and endocannabinoid systems that regulate and turn off this response that would be potentially harmful for health. Collectively, the results presented here contribute to a better understanding of the role of CB1 and TRPV1 in the physiopathology of the HPA axis, and particularly in the adrenal gland.

Author contributions

ADL contributed to the conception and design of the study, analyses and interpretation of data and funding support.

PNS, SLG and VL performed the experiments and the acquisition of data.

ADL wrote the first draft and FC and JFS participated in the subsequent drafts revising it critically for important intellectual content.

All authors have approved the final article.

Declaration of interest

The authors declare no conflict of interest and have approved the final article.

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