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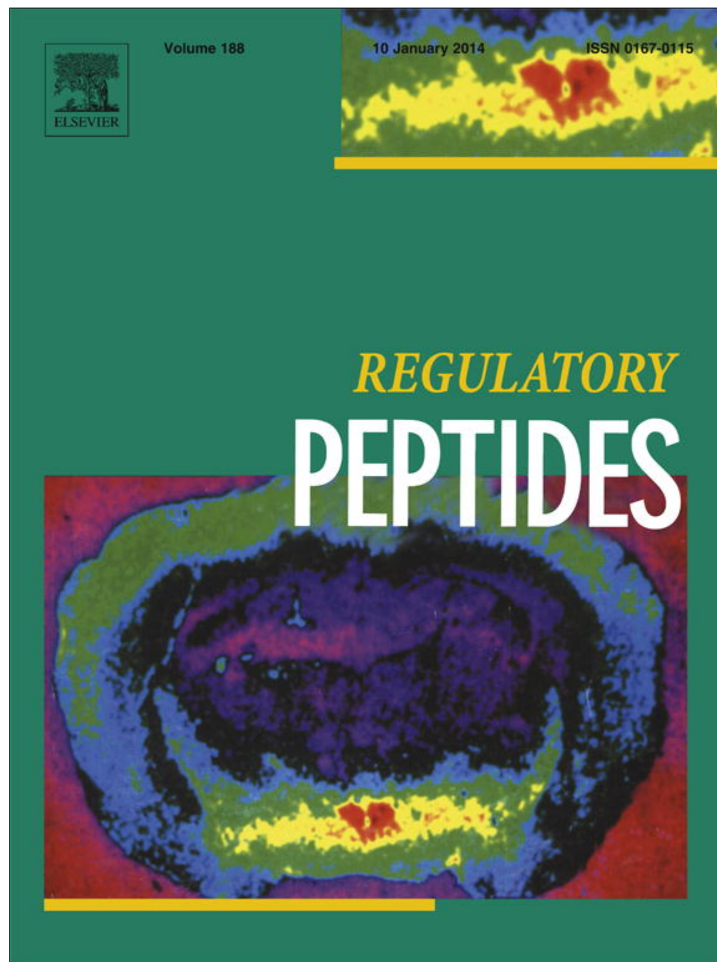

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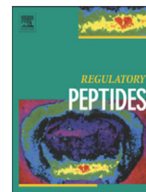

The inhibitory effect of anandamide on oxytocin and vasopressin secretion from neurohypophysis is mediated by nitric oxide

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ABSTRACT

The neurohypophyseal hormones oxytocin (OT) and vasopressin (VP) are involved in behavioral, autonomic and neuroendocrine functions. Both peptides are synthesized in magnocellular neurons of paraventricular and supraoptic nuclei at hypothalamic level whose axons terminate in the neurohypophysis (NH), from where OT and VP are released into the systemic circulation. NH contains abundant nitric oxide (NO) synthase suggesting that NO plays a role in the release of these neuropeptides. The endocannabinoid system is present in magnocellular neurons of the hypothalamic neurohypophyseal system, and we have previously demonstrated that endocannabinoids modulate OT secretion at hypothalamic level.
In the present work, we investigated the *in vitro* effect of the endocannabinoid anandamide (AEA) on OT and VP release from NH of untreated adult male rats and the involvement of NO in this action.
Our results showed that AEA decreased OT and VP secretion from NH. AEA action was mediated by NO, since the inhibition of NO synthesis completely blocked this inhibitory effect. We found that cannabinoid receptor type 2 (CB_2) and transient receptor potential cation channel subfamily V member 1 (TRPV1) are involved in the inhibitory effect of AEA because AM630 and capsazepine, CB_2 and TRPV1 antagonists respectively, but not AM251, a CB_1 antagonist, blocked AEA effect at neurohypophyseal level.
These findings revealed an interaction between endocannabinoid, nitric oxide and oxytocin/vasopressin systems that could be involved in the modulation of homeostatic, behavioral and reproductive processes.
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1. Introduction

The hypothalamo-neurohypophyseal system is a complex interactive brain structure that coordinates responses to perturbations in the balance of physiological processes. It consists of the hypothalamic supraoptic nuclei (SON) situated laterally to the optic chiasm, and the paraventricular (PVN) nuclei on each side of the third ventricle. Magnocellular neurons in those nuclei synthesize oxytocin (OT) and vasopressin (VP) and send axonal projections to the neurohypophysis (NH) from where those peptidic hormones are released into the systemic circulation. Also, magnocellular neurons release OT and VP from their perikarya, dendrites, and/or collateral axons to several areas in the central nervous system (CNS) where OT and VP have neurotransmitter functions, thus controlling complex neuroadaptive processes. In addition, parvocellular OT- and VP-containing cells are found in the hypothalamus and project into the external zone of the median eminence where these peptides are released into the portal vessel system to regulate anterior pituitary function [1]. Both hormones regulate relevant functional processes that range within the maintenance of body fluid homeostasis, modulation of neuroendocrine, immune and stress responses, memory and also social behaviors [2]. In addition to its fundamental role in reproduction, parturition and lactation, OT also participates in fluid and electrolyte balance, fever, inflammation and stress response [3,4]. It has been demonstrated recently that OT has alleviating and preventing effects on the subsequent health problems caused by excessive hypothalamic–pituitary–adrenal (HPA) axis activation during chronic stress or inflammatory responses [5]. Vasopressin also participates in healing responses of febrile illness since VP co-secreted with CRH from parvocellular neurons acts also as a releasing factor for ACTH secretion [6]. Moreover, VP secreted from neurohypophysis to the peripheral circulation also contributes to the effects during stress and immune responses [7].

The secretion of these hormones is regulated both at the level of cell bodies in the hypothalamus and at the axon terminal level in the neurohypophysis [8,9]. The activity of magnocellular neurons is regulated by several intrinsic factors within the SON and PVN [1,2]. In addition, OT synthesizing neurons express high levels of neuronal nitric oxide (NO) synthase (nNOS) and the production of NO in these neurons participate in auto- and/or cross-regulation of OT [10] as well as VP [11] secretion. Plenty of literature point out that NO is an important inhibitory

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regulator of SON neurons [12–16]. In fact, we have previously demonstrated that NO inhibits OT release from hypothalamic explants in basal conditions and that NO is a key mediator of the enhancement of OT release from hypothalamus following an immune challenge [17]. Hypothalamic magnocellular NOS-positive neurons contain the enzyme not only in the cell bodies but also in the axon terminals in the neurohypophysis [18]. The rat neural lobe of the pituitary is one of the richest regions containing NOS, with a predominance of the nNOS isoform [19,20]. However, there is scarce evidence about the role of NO in this tissue. A report, published twenty years ago, shows that NO donors reduce both OT and VP secretion from the neural pituitary lobe [21]. We then demonstrated that NO donors reduced OT secretion from the NH and also that Neurokinin A increases NOS activity in this tissue therefore inhibiting OT secretion [22].

The endocannabinoid system (ECS) is a neuromodulatory system widespread distributed throughout the CNS as well as in peripheral organs. Recently, the endocannabinoid system has been recognized as a major neuromodulatory system whose main function is the maintenance of body homeostasis [23]. Components of the ECS are present throughout the hypothalamic-pituitary axis and several evidences suggest that both systems interact extensively [24]. The endocannabinoids (eCB) are lipophilic arachidonic acid derivatives being anandamide (AEA) the most studied. AEA is produced "on demand" and removed from its sites of action by a reuptake transport system, and finally metabolized mainly by the fatty acid amide hydrolase (FAAH) enzyme [23,25]. The eCB released into intercellular space binds to two well characterized cannabinoid receptor subtypes, CB_1 and CB_2 [26]. CB_1 receptors are highly expressed in the brain and by certain nonneuronal cells and tissues such as the pituitary gland where they are found at the central and peripheral nerve terminals to mediate inhibition of transmitters, neuropeptides and hormone release [27–30]. The CB_2 receptor has been found particularly associated with immune tissues and in the CNS on neurons and microglial cells [31,32].

Interestingly, AEA behaves as a partial agonist of CB_1 receptors but is a full agonist at the rat and human transient receptor potential cation channel subfamily V member 1 (TRPV1) [33]. TRPV1 is a member of a family of six "vanilloid-like" receptors that traduces the nociceptive and hyperalgesic effects of capsaicin [34]. TRPV1 is abundantly located in terminals of several peptidergic neurons [35] including vasopressinergic [36] and they behave as nonselective channels for several cations. Anandamide binds at TRPV1 that generates ion currents probably controlling neuropeptide release. AEA induces TRPV1-mediated vasodilatation and calcium influx in neurons, suggesting that endogenous AEA is involved through TRPV1 in physiological processes such as blood pressure and pain sensation. Therefore, their physiopathological relevance still needs to be elucidated [37,38].

The main effect of cannabinoids on anterior pituitary hormone secretion is inhibitory, suppressing, for example, luteinizing hormone, growth hormone and prolactin secretion [39–43]. Exogenous cannabinoids have also been shown to exert a potent inhibitory effect on posterior pituitary hormone secretion. Marijuana consumption or application of Δ^9-tetrahydrocannabinol (THC) results in increased diuresis, which is thought to be mediated by central inhibitory cannabinoid actions on VP release [44]. Also, THC blocked suckling-induced milk ejection mediated by the secretion of OT in lactating rats [45]. Furthermore, it was shown that chronic exposure to THC down-regulates OT expression [46] and that endogenous cannabinoids are released as retrograde messengers in the SON by magnocellular neurons [47]. All these findings are in concordance with our previous study in which we reveal a hypothalamic interaction between oxytocin, endocannabinoid and NOergic systems in the regulation of the hypothalamic-neurohypophyseal axis under basal and stress conditions confirming that endocannabinoids modulate OT and VP neurons at hypothalamic level [17]. However, the participation of endocannabinoids on OT and VP release at neurohypophyseal level, to the best of our knowledge, has not been elucidated. Therefore, the primary aim of the present study was to determine the effect of the endocannabinoid AEA on basal and potassium (K^+)-stimulated OT and VP release from incubated neurohypophysis and to establish whether NO is involved in these actions.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 200–250 g 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 treated according to the NIH Guide for the Care and Use of Laboratory Animals from the National Academy Press, Washington D.C., 1996. 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), L-[U-14C] Arginine with specific activity: 11.26 GBq/mmol (Amersham Int., Buckinghamshire, UK), OT and VP standards and antisera (Bachem Americas Inc., Torrance, CA, USA), AM251 [N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-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).

2.3. Incubation of neurohypophysis

On the day of the experiment, animals were decapitated between 9.00 and 11.00 a.m., the brain and the pituitary were removed from the skull and the neurohypophysis (NH) was carefully dissected by separation from anterior pituitary gland.

The NH (6–10 per group) were pre-incubated individually (1 per tube) for 15 min in a Dubnoff shaker (60 cycles per min) at 37 °C in an atmosphere of 95% O_2–5% CO_2 in 1 ml of Krebs–Ringer bicarbonate buffer (KRB) (118.46 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM NaH_2PO_4, 1.18 mM $MgSO_4$, 24.88 mM $NaHCO_3$, pH 7.4) containing 10 mM glucose, 0.1 mM bacitracin. When the experiment was performed using a NOS enzyme inhibitor or hemoglobin or cannabinoid receptor antagonists, the drug was added from the beginning of the pre-incubation period. After 15 min of pre-incubation, the medium was discarded and replaced with fresh medium alone or containing the substances to be tested and the tissues were incubated for 30 min. In basal release, medium was removed from the tissues and then were incubated further for 30 min in KRB containing 40 mM K^+, balanced by reducing Na^+ concentration (K^+-evoked release). At the end of each incubation period, the media were removed, heated for 10 min at 100 °C and then centrifuged at 10,000 $\times g$ for 10 min. The supernatants of these media were stored at −70 °C for further determination of OT and VP. The tissues were rapidly frozen on dry ice and homogenized in distilled water for NOS activity determination.

2.4. Oxytocin and vasopressin determination

OT and VP concentration in the incubation medium were measured on duplicate samples by radioimmunoassay using I^{125}-oxytocin and I^{125}-vasopressin as tracers and anti-oxytocin rabbit antisera with no crossreaction with other related peptides (cat. # T-4084, Bachem Americas Inc., CA, USA) and anti-vasopressin rabbit antisera (cat. # T-4563, Bachem Americas Inc., CA, USA) which recognizes Arg^8-vasopressin, Arg^8-vasotocin and $[d(CH_2)^5$, D-Ile^2, Ile^4,$Arg^8]$-vasopressin (100%)

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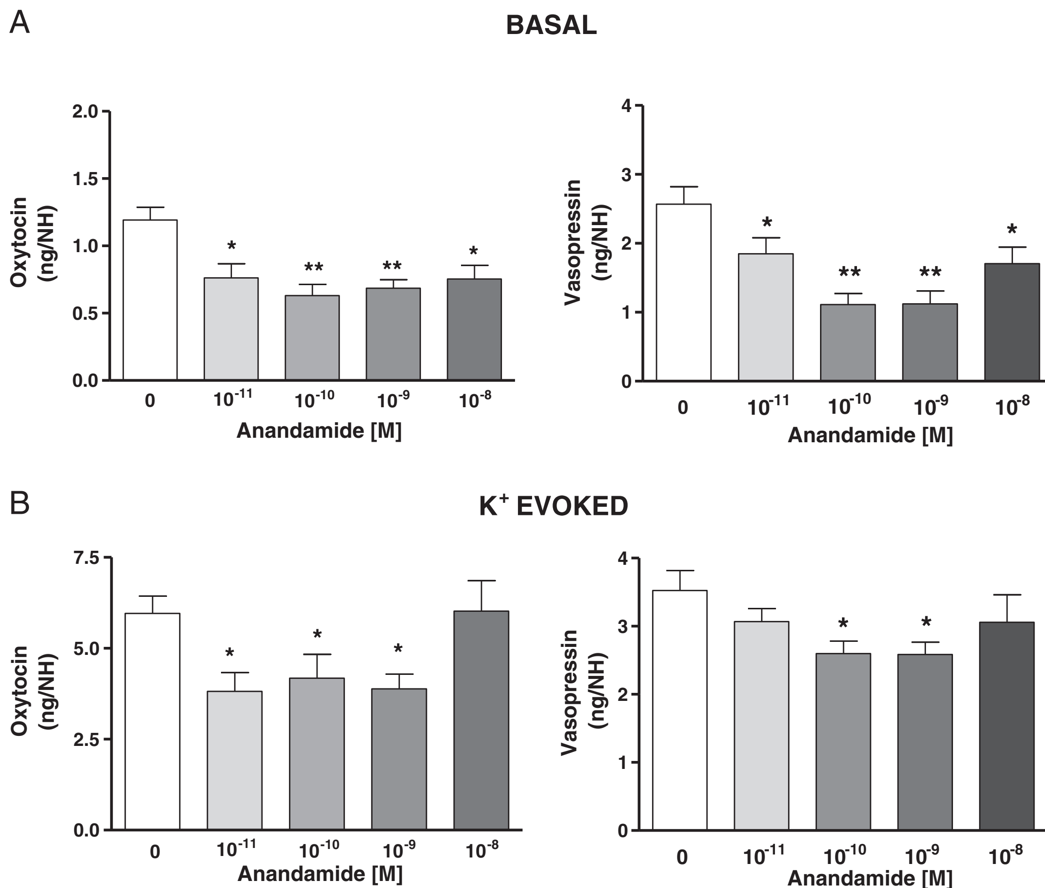

Fig. 1. Effect of anandamide [10^{-11}]–[10^{-8} M] on basal (A) and potassium-evoked (B) release of oxytocin and vasopressin from neurohypophysis (NH). Values represent mean ± SEM, n = 7–9 per group. Data were evaluated by one-way ANOVA followed by Dunnett's test. *p < 0.05, **p < 0.01 *vs.* control group.

and crossreacts with Lys^8-vasopressin (38%) (final dilutions 1:20000 and 1:50000 respectively). OT (cat. # H-2510, Bachem Americas Inc., CA, USA) and VP (cat. # H-1780, Bachem Americas Inc., CA, USA) were used as standard preparations as well for iodination with ^{125}I. The reaction was stopped with cold 96% ethanol. All samples from animals tested within a specific experimental paradigm were measured in the same RIA to avoid interassay variability. The intrassay coefficient of variation was lower than 9%, and assay sensitivity was 0.5 pg/tube.

2.5. NOS activity determination

NOS activity was determined by measuring the conversion of [^{14}C] arginine to [^{14}C]citrulline, since this product is formed in equimolar quantities with NO. We used a modification of the method of Bredt and Snyder [48]. Briefly, the tissues were preincubated for 10 min in 1 ml of KRB at 37 °C in an atmosphere of 95% O_2–5% CO_2 and then incubated for 30 min with fresh medium in the presence of AEA (10^{-9} M). The tissues were immediately homogenized in 0.5 ml of 20 mM Hepes, pH 7.4, containing 0.4 mM NADPH, 1 mM dithiothreitol, 0.45 mM CaCl2. The reaction was started by adding 20 μl (0.1 μCi) of L-[U-^{14}C] arginine to the homogenate. After 10 min of incubation at 37 °C, the reaction was stopped by centrifugation at 10,000 ×g 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. [^{14}C]citrulline was eluted with 2 mL of distilled water and the radioactivity quantified by liquid scintillation spectroscopy of the flow-through. Because NOS converts arginine into equimolar quantities of NO and citrulline the results were expressed as pmoles of NO produced in 1 min by NH.

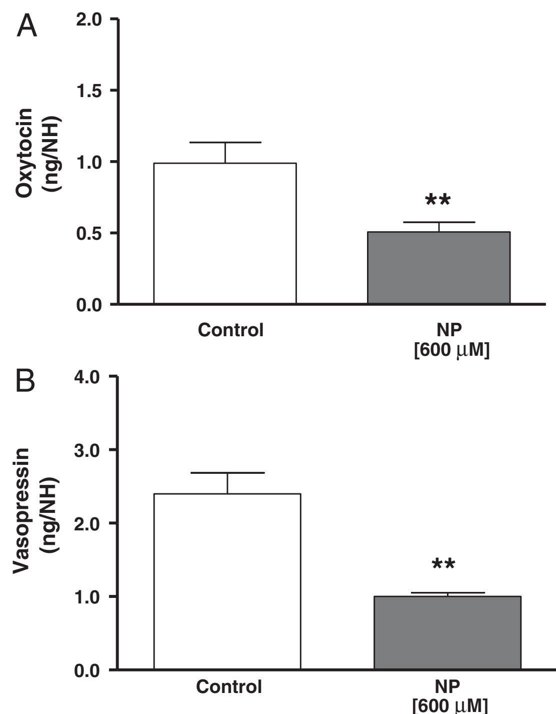

Fig. 2. Effect of sodium nitroprusside (NP) [600 μM] on oxytocin (A) and vasopressin (B) basal release from neurohypophysis (NH). Values represent mean ± SEM, n = 6–9 per group. Data were evaluated by Student's *t*-test. **p < 0.01 *vs.* control group.

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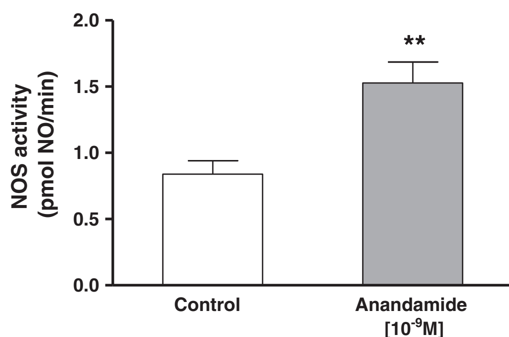

Fig. 3. Effect of anandamide [10^{-9} M] on nitric oxide synthase (NOS) activity in neurohypophysis (NH) expressed as pmol NO/min. Values represent mean ± SEM, n = 6–9 per group. Data were evaluated by Student's *t*-test. **p < 0.01 *vs.* control group.

2.6. Statistics

The results were expressed as mean ± SEM. The significance of the differences between means was determined by Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison against the control group or Student–Newman–Keuls multiple comparison tests or two way ANOVA. Differences were considered significant when p < 0.05. All experiments were performed at least twice. Figures represent results of individual experiments.

3. Results

3.1. Effects of AEA on OT and VP release from NH in vitro

In order to investigate the effects of AEA on OT and VP release from the NH, tissues were incubated with AEA (10^{-11}–10^{-8} M). AEA showed, at all cases, a downregulation of OT and VP basal release in a bell shaped dose response curve when compared to control. Conversely, only some of the tested doses of AEA decreased OT and VP release from NH under K^+-stimulated conditions. Moreover, these inhibitory effects were less evident; therefore we performed the following set of experiments in basal conditions (Fig. 1).

3.2. Involvement of NO in the inhibitory effects of AEA on OT and VP basal release

We first confirmed the inhibitory effect of NO on OT and VP basal release from the NH *in vitro* in our experimental conditions. NH tissues were incubated with sodium nitroprusside (NP) which spontaneously releases NO. NP (600 μM) significantly reduced (p < 0.01) both OT and VP basal release from the NH (Fig. 2). Then, we evaluated the possible participation of NO in the inhibitory effect of AEA on the basal release of both neuropeptides. In a first approach, we determined that AEA (10^{-9} M) significantly stimulated (p < 0.01) NOS activity in the NH (Fig. 3). Since AEA increased NO production, and NO decreased OT and VP release from NH; then we studied the effect of AEA on OT and VP basal release in the presence of hemoglobin (Hb), a NO scavenger or L-NAME, a competitive NOS inhibitor. Hb (40 μg/ml) and L-NAME (1 mM) did not affect OT or VP release *per se*, but significantly blocked the inhibitory effect of AEA on OT and VP basal release from the NH (Fig. 4).

3.3. Study of cannabinoid receptors

We studied if the inhibitory effects of AEA on OT and VP release were caused by its interaction with CB_1, CB_2 and/or TRPV1 receptors. To identify which receptor was implicated, NH tissues were incubated in the presence of AEA (10^{-9} M) alone or together with each receptor antagonist. AM251 (10^{-5} M), a CB_1 antagonist; AM630 (10^{-5} M), a CB_2 antagonist or Capsazepine (CPZ, 10^{-5} M), a TRPV1 receptor antagonist were separately added to the media from the beginning of the incubation period. Any of them had effect *per se*. AM251 did not modify AEA

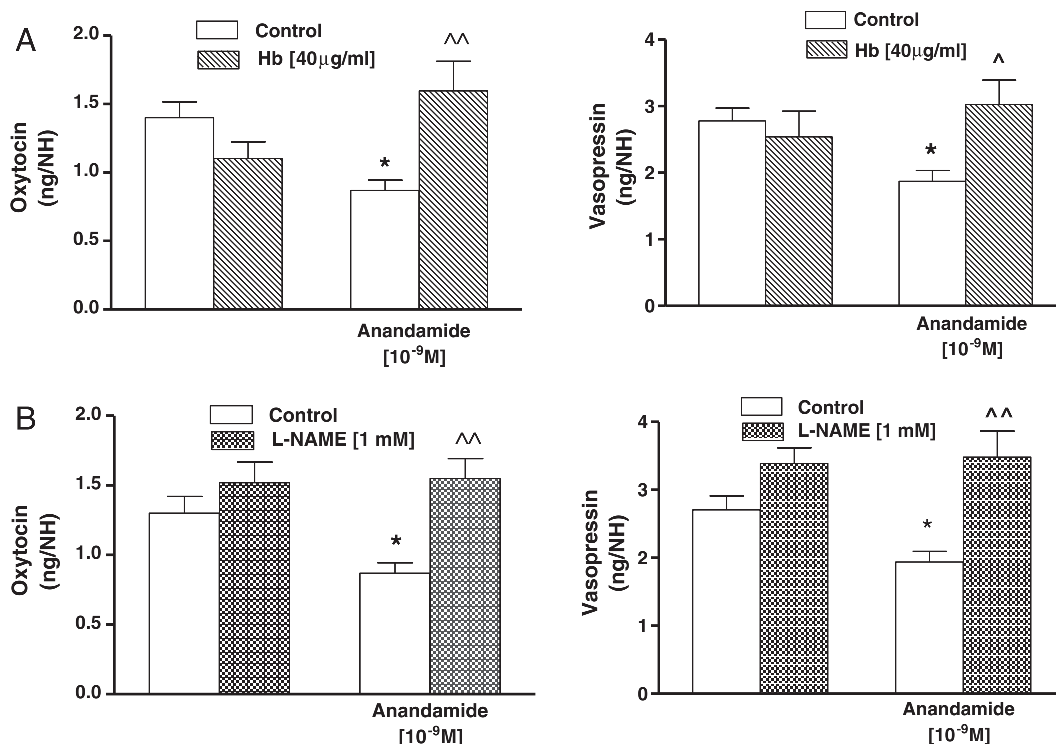

Fig. 4. Effect of anandamide [10^{-9} M] on oxytocin and vasopressin basal release from neurohypophysis (NH) in the presence of: (A) hemoglobin (Hb) [40 μgr/ml] or (B) L-NAME [1 mM]. Values represent mean ± SEM, n = 7–9 per group. Data were evaluated by two-way ANOVA. *p < 0.05 *vs.* control without anandamide; ^p < 0.05 and ^^p < 0.01 *vs.* control group with anandamide.

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effect; however AM630 and CPZ completely prevented the inhibition of AEA on OT and VP basal release (Fig. 5).

Finally, the incubation of neurohypophysis in the presence of capsaicin (10^{-11}–10^{-7} M), the selective agonist for TRPV1 receptors significantly inhibits both OT and VP basal release, mimicking AEA inhibitory effect and confirming the participation of TRPV1 receptors on OT and VP release from NH (Fig. 6).

In summary, these results based on pharmacological studies using specific receptor agonists and antagonists and together with the previous evidence of the presence for CB_2 and TRPV1 receptors in the neurohypophysis, suggest that these two receptors aforementioned are actively involved in the anandamide reduced OT and VP basal release. Furthermore, NO mediates these inhibiting pathways.

4. Discussion

Oxytocin and vasopressin importance underlies in its fundamental roles in homeostasis, neuroendocrine functions related to reproduction and stress response, memory and also to social behavior [49]. Endogenous cannabinoids are important signaling molecules in the neuroendocrine control of homeostatic and reproductive functions. Hence, we sought to determine the role of the endocannabinoid system in controlling the physiological levels of OT and VP. Cannabinoid receptors are localized on the soma of OT- and VP-containing hypothalamic neurons that project to the NH and on the pituitary [50]. Oxytocin and endocannabinoids cooperate regulating the physiological function of these neurons [51]. Also, CB_1 cannabinoid and OT receptors interact in PVN and SON hypothalamic nuclei to regulate food and water intake [52]. Finally, our previous *in vivo* work provides a direct evidence for cannabinoid-mediated enhancement of OT release from hypothalamus, following immune challenge [17]. However, to date, the endocannabinoids action and function in the NH and its participation in OT and VP release from this tissue is unknown.

Our study shows for the first time that the endocannabinoid system controls OT and VP secretion to periphery at neurohypophyseal level. The endocannabinoid AEA, acting through CB_2 and/or TRPV1 receptors, inhibits the release of both neuropeptides and concomitantly increases the activity of nitric oxide synthase in this tissue. Furthermore, nitric oxide is a key mediator on AEA inhibitory action at neurohypophyseal level.

Our results indicate that the endocannabinoid AEA decreases basal OT and VP secretion from incubated neurohypophysis at all tested

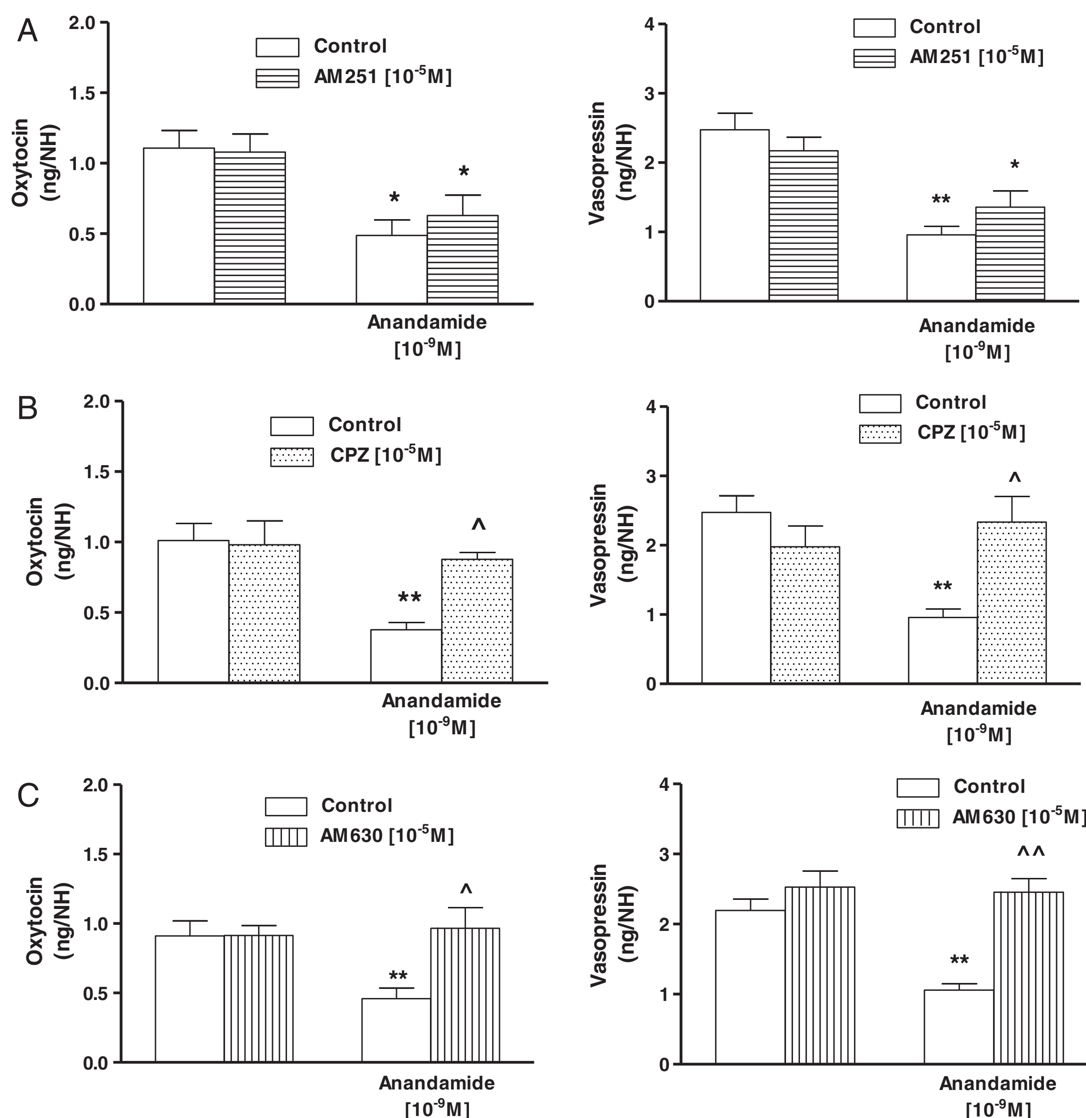

Fig. 5. Effect of the blockade of cannabinoid or vanilloid receptors on the inhibitory effect of anandamide [10^{-9} M] on oxytocin and vasopressin basal release from neurohypophysis (NH). (A) Blockade of CB_1 receptor with the antagonist AM251 [10^{-5} M]; (B) Blockade of TRPV1 receptor with the antagonist capsazepine (CPZ) [10^{-5} M]; (C) Blockade of CB_2 receptor with the antagonist AM630 [10^{-5} M]. Values represent mean ± SEM, n = 7–9 per group. Data were evaluated by two-way ANOVA. *$p < 0.05$ and **$p < 0.01$ *vs.* respective control group without anandamide. ^$p < 0.05$ and ^^$p < 0.01$ vs control group with anandamide.

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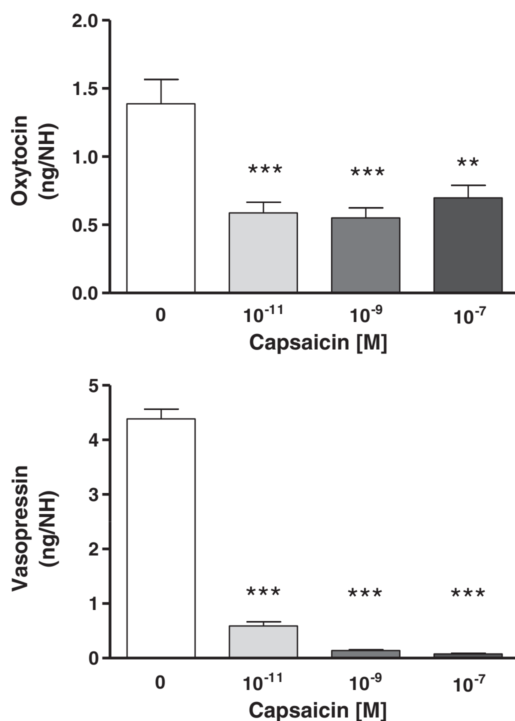

Fig. 6. Effect of capsaicin $[10^{-11}]$–$[10^{-7}$ M] on oxytocin and vasopressin basal release from neurohypophysis (NH). Values represent mean ± SEM, n = 6–9 per group. Data were evaluated by one-way ANOVA followed by Dunnett's test. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* control group.

doses in a bell shaped dose–response curve. This dose–response effect of AEA was also found by other groups in animal models where cannabinergic drugs have been shown to possess biphasic effects depending on the dose [53]. This effect could be explained by the necessity to achieve a minimum level of endocannabinoid to activate the receptors, however *in vitro* concentrations reached after exogenous AEA administration were larger than those found under physiological conditions and higher doses became ineffective [54]. Similarly, another study suggests that high doses of AEA overcome a rapid and massive inactivation by FAAH and show less effect since brain physiological low levels of AEA are sufficient to activate cannabinoid receptors [55]. Other authors suggest that this bell-shaped dose curve response effect of AEA sometimes reflects interactions between cannabinoid with TRPV1 [56], which also agree with our model. Finally, we choose the dose of 10^{-9} M of AEA in all further experiments because this was the optimal dose that modified LHRH, dopamine and OT release at hypothalamic level in previous studies of our group [17,40,41].

Since the release of VP and OT strongly depends on the action potentials generated at the hypothalamus and propagated to the nerve endings at the neurohypophysis, the experiments with depolarizing conditions are good attempts to mimic the action potential discharge during *in vitro* experiments. Then we also tested the effect of AEA in depolarizing conditions by increasing extracellular $[K^+]$ that leads to an increase in intracellular $[Ca^{++}]$ and in the total amount of neuropeptides released to the media. We used 40 mM of extracellular $[K^+]$ [22,57] and we observed that depolarization increases the total amount of OT and, in less extent, VP released to the media. However, AEA inhibitory effect on K^+-induced OT and VP release was less significant than in basal conditions. Since the endocannabinoid system is a fine tuning modulator of several systems, the potent depolarization acquired with this high concentration of K^+ is probably difficult to be reversed or blocked with the weak modulator AEA.

Other possible explanation is based on the ultrastructural morphology of neurohypophysis. This gland consists of nerve endings of hypothalamic neurons and pituicytes, glial-like cells surrounding these terminals. Pituicytes are the resident glia and the major cell type in the neurohypophysis [58]. The pituicyte is considered to be a special type of astrocyte and it has recently become clear that the activity of OT and VP neurons is strongly influenced by this glial cell being a critical regulator of the neurohypophyseal hormone output [59]. These cells are large and partially envelop some nerve endings but their morphological location around the terminal and functions vary throughout different stimuli. Ultrastructural studies have shown that pituicytes engulf or completely surround neurosecretory axonal endings under basal conditions; however they release these neural processes when conditions require increased hormone output. The presence of pituicyte interposed between the neurosecretory terminal and the basal lamina constitutes a potential physical barrier to secreted peptide entering the circulation. It is also possible that the pituicytes could control secretion disallowing the depolarization of the terminal with the consequent decrease in peptides release. Moreover, pituicytes could be sites of OT and VP gene expression and these peptides are produced and released by those cells in isolated NH tissue cultures [60]. Modification of the microenvironment alters the structure and function of pituicytes, in a similar way as astroglia, and neuroactive substances such as GABA, other neurotransmitters and ionic species such as K^+ and Ca^{2+} are likely to be involved in the retractile mechanism and also in the secretion of OT and VP from these cells [58]. Our supposition is that CB_2 receptors could be located on pituicytes since they are astrocyte-like cells and because the morphology and function of these cells are modified by high potassium concentration, this could be another explanation about the different response to AEA during each condition. Based on these results, all experiments were performed in basal conditions.

Nitric oxide acts as an important modulator of magnocellular neuronal activity since the enzyme that produces NO is localized in both the soma and the neurohypophyseal terminals of these neurons [19,20]. It has been demonstrated that NO act as an inhibitory regulator of OT and VP [21,22,61]. Our study confirmed these previous evidences, since the incubation of NH in the presence of sodium nitroprusside reduced basal OT and VP release from the tissue.

It was shown that AEA, binding to cannabinoid receptors leads to NO production in several cell types [62,63]. In concordance with these findings, our study showed that AEA increased NOS activity in the NH. Moreover, the action of AEA appears exclusively mediated by NO, since the scavenging of NO by hemoglobin and the inhibition of NO synthesis by L-NAME completely blocked the inhibitory effect of AEA on OT and VP secretion. Furthermore, expression of TRPV1 channels occurs in magnocellular neurons [34]. Since the putative binding site for an endocannabinoid ligand to TRPV1 is located intracellularly, then AEA must be up taken in order to reach that site. Interestingly, it has been shown that NO can stimulate AEA uptake [63]. These observations reinforce our findings that NO is necessary to mediate AEA inhibitory action on OT and VP release from NH. On the other hand, since enhanced CB receptor stimulation results in prolonged pharmacological activity, it is probably that when AEA activates NOS, NO potentiates the endocannabinoid transporter protein, which clears AEA from the extracellular space ending this signal transduction pathway as shown in endothelial cells [63].

It has been well established that gamma aminobutyric acid (GABA) inhibits OT release from hypothalamus and that the administration of the endocannabinoids AEA and 2-AG in the SON and PVN suppress GABA release [47]. This data is in complete agreement with our previous study in which AEA stimulates OT release from hypothalamic fragments and we proposed that GABA could mediate this action [17]. The tuberohypophysial GABAergic system, which originates in the hypothalamus, projects towards the neural and intermediate lobes of the pituitary [64] and GABAergic terminals in the neurohypophysis have an inhibitory role in OT and VP release [65,66]. We can speculate that GABA could be involved in AEA inhibitory effect on OT release. However, in a previous work we demonstrate that NO inhibits the release of GABA from neurohypophysis. Furthermore, the inhibition of NOS activity

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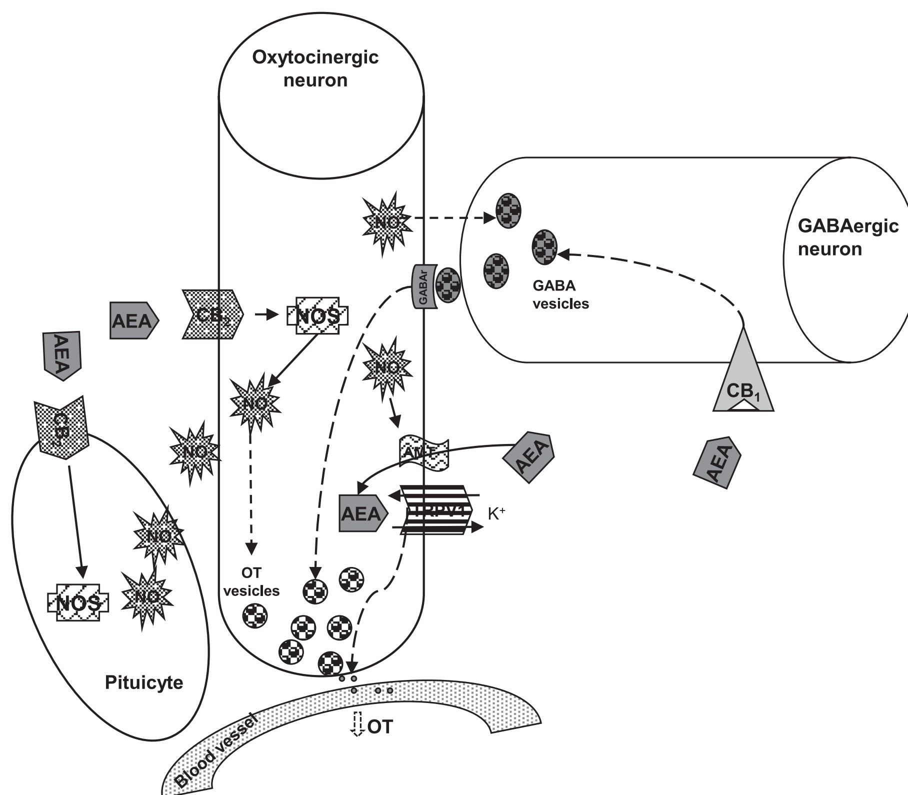

Fig. 7. Scheme showing the possible mechanism of the inhibitory action of AEA on OT release from neurohypophysis based in the present data and previous works of our group. Nerve ending of oxytocinergic neuron is schematically shown surrounded by a glial cell (Pituicyte). Also a GABAergic neuron is represented with gamma aminobutyric acid (GABA) vesicles. Nitric oxide (NO) and nitric oxide synthase (NOS), anandamide (AEA), Cannabinoid receptor type 1 (CB_1) and type 2 (CB_2), transient receptor potential channel subfamily V member 1 (TRPV1) and AEA membrane transporter (AMT) are also schematized. Arrows with solid lines indicate stimulation and dotted lines indicate inhibitory action. Briefly, AEA could bind to CB_2 receptors located on pituicyte and oxytocinergic terminal activating NOS. NO produced can directly inhibit OT vesicles release and also acts promoting AEA transport inside the axon allowing its binding to TRPV1. This channel activation by AEA causes alteration in membrane polarization inhibiting OT release. Cross-talk between TRPV1 and CB_2 receptors co-expressed in the same cells might also include reciprocal influences on receptors activation. AEA could also bind to CB_1 receptors probably located on GABAergic neurons inhibiting GABA release.

increased GABA release indicating that endogenous NO has an inhibitory effect on GABAergic activity in this tissue. Moreover, previously [22] and in the present study we demonstrate that NO inhibits OT secretion from neurohypophysis. However, since NO decreased both OT and GABA release from the neurohypophysis and GABA was reported to inhibit OT release from nerve terminals of the neural lobe it is unlikely that GABA mediates the inhibitory effect of NO on OT release from neurohypophysis. It is possible that NO might influence the release of OT through GABA at the level of the cell bodies and by different mechanisms on nerve terminals of the neurohypophysis. It could be possible that other factors were involved since the release of OT and VP is also regulated by other peptidergic and aminergic neurotransmitters, such as dopamine [67], serotonin [68], galanin [69] and histamine [70] at neurohypophyseal level.

Very little is known about the expression and function of cannabinoid receptors in the neurohypophysis. All the modulatory properties on hormone release mediated by endocannabinoid receptors activation have been mainly attributed to an action of the ligands at hypothalamic nuclei, rather to a direct action on the receptors at pituitary gland. Only a few reports have highlighted the possibility of a direct interaction of cannabinoids at the level of the pituitary by describing the presence of CB_1 in rat anterior pituitary [27,28]. Some studies described that CB_1 are present in the anterior pituitary gland and, much less in the intermediate lobe whereas they were not found in the neural lobe. These findings are in concordance with the pharmacological results presented here. In our study, while we blocked CB_1 receptor with its antagonist (AM251), the inhibitory effect of AEA on OT and VP release persisted suggesting that CB_1 did not participate in these AEA effects. Since AM251 is a CB_1 specific antagonist and the Ki of this antagonist and AEA for this receptor are 7.49 nM and 89 nM, respectively [71], the dose of antagonist used in our study exceeded at least 1000 times the agonist and was present since pre-incubation period ensuring the complete blockade of these receptors. Furthermore, we previously observed that this dose of CB_1 antagonist is effective by preventing an inhibitory effect of AEA on LHRH and OT release from the hypothalamus [17,40,72]. On the other hand, Herkenham et al. [50] demonstrated cannabinoid receptor binding in the neural lobe using a potent synthetic radiolabeled cannabinoid that can precisely localize and quantify cannabinoid receptors in sections of rat brain and pituitary. Densitometry studies showed a low quantity of specific binding in neural lobe in comparison to other areas such as substantia nigra with very dense binding; however neural lobe density value is twice greater than anterior pituitary binding. Moreover, since the hypothalamus has high density of CB_1 receptors at PVN and SON nuclei it is possible to speculate that neural lobe cannabinoid receptor binding is localized on the axons whose perikarya arises from those hypothalamic nuclei mentioned above [73]. Moreover, we found that TRPV1 selective agonist, capsaicin mimics AEA effects and that its specific antagonist, capsazepine, completely blocked AEA inhibitory effect on OT and VP release. Since CB_1 and TRPV1 stimulation causes opposing effects on intracellular calcium concentrations [74] is not unreasonable to think that both receptors could not coexist functionally in the same terminal. One speculative possibility is that CB_1 could be located on GABAergic terminals while the TPRV1 could be present at the oxytocinergic ones.

Despite quite little studies indicating that CB_2 receptors might be present in the CNS, the expression of CB_2 receptors has been much less well established and characterized in comparison to the expression of CB_1 receptors. Only one previous study addressed that CB_2

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immunoreactivity was not observed in any part of the pituitary gland [28]. However, our present results are in disagreement since the CB_2 antagonist AM630 blocked AEA effects. One possible explanation about the dependence of both TRPV1 and CB_2 receptors is based on the ultrastructural morphology of neurohypophysis. We could speculate that in the nerve endings TRPV1 receptors are probably present since they are abundantly located in terminals of several peptidergic neurons including vasopressinergic [64,65]. Moreover, TRPV1 receptors contribute to osmoreception in vasopressin secreting neurons [65]. On the other side, CB_2 receptors, which are mainly located on glial cells, could probably be expressed on pituicytes [31,32]. At present, we can speculate that a substance, such as AEA, released at these axo-glial synaptoid contacts between pituicytes and nerve endings could bind to CB_2 receptors on pituicytes and also be internalized into the terminal to bind TRPV1. CB_2 activation probably affects pituicyte morphology around the terminal leading to changes in neurotransmitter release while TRPV1, which behaves as nonselective channel for several cations, alters the membrane polarization and the subsequent exocytose of neuropeptide vesicles.

Therefore, based on our previous and present data we postulate that the inhibitory effect of AEA on OT and VP release from NH necessarily involved NO and with AEA binding to both CB_2 and/or TRPV1 receptors. In the diagram is shown the structure of the neurohypophysis with the oxytocinergic and GABAergic terminals and the glia (pituicyte) and the proposed location of cannabinoid and TRPV1 receptors in basal conditions (Fig. 7). AEA could bind to CB_2 receptors located both on the pituicyte and on oxytocinergic/vasopressinergic terminals activating NOS with the concomitant increase in NO production. NO directly inhibit OT/VP vesicles release from the terminal and also acts promoting AEA transport inside the axon allowing its binding to TRPV1. This channel activation by AEA causes alteration in membrane polarization inhibiting OT/VP release. One fact that we could not explain is why the blockade of these TRPV1 reverts the inhibitory action of AEA on CB_2 receptors. Probably both should be active to inhibit the neuropeptide release. Cross-talk between TRPV1 and CB_2 receptors co-expressed in the same cells might also include reciprocal influences on receptor activation. In fact, it was demonstrated for CB_1 and TRPV1 that seem to share the same signaling pathway in some biological systems [75]. On the other side, AEA could bind to CB_1 receptors probably located on GABAergic neurons inhibiting GABA release, for that reason with the blockade of CB_1 receptors we still observed inhibition of OT/VP release.

5. Conclusion

The results of the present study demonstrate that endocannabinoids can act directly on the neurohypophysis to inhibit both OT and VP release and, that this effect involves necessarily the participation of NO. These findings reveal an interaction between endocannabinoid, nitric oxide and oxytocin/vasopressin systems at neurohypophyseal level and this intercommunication could be a relevant piece in the network for modulation of homeostatic and reproductive processes.

Author contributions

Dr Andrea De Laurentiis was responsible as study director for the conception of the research plan, the design of the experimental trial and the preparation of the manuscript. Experiments and measurements were carried on by Valeria Luce. Analysis and interpretation of data were conducted by Javier Fernandez Solari. Valeria Rettori critically revised the manuscript for important intellectual content.

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