Journal of Neuroimmunology 221 (2010) 32–41

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

# The hypothalamic endocannabinoid system participates in the secretion of oxytocin and tumor necrosis factor-alpha induced by lipopolysaccharide

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ARTICLE INFO

*Article history:*
Received 12 January 2010
Received in revised form 4 February 2010
Accepted 4 February 2010

*Keywords:*
Anandamide
Cannabinoid receptors
Nitric oxide synthase
Cytokines
Hypothalamus

ABSTRACT

This study investigated the participation of the hypothalamic endocannabinoid system in the response to lipopolysaccharide (LPS) challenge evaluating oxytocin (OXT) and tumor necrosis factor-alpha (TNF-*a*) plasma levels *in vivo* and their release from hypothalamic fragments *in vitro*. LPS increased OXT and TNF-α release through anandamide-activation of hypothalamic cannabinoid receptor $CB_1$, since the antagonist AM251 blocked this effect. Anandamide, through its receptors, also increased hypothalamic nitric oxide (NO) which inhibited OXT release, ending the stimulatory effect of the endocannabinoid. Our findings reveal a hypothalamic interaction between oxytocin, endocannabinoid and NO-ergic systems providing a regulation of the hypothalamic–neurohypophyseal axis under basal and stress conditions.



## 1. Introduction

The hypothalamic–neurohypophyseal (HN) axis consists of the hypothalamic supraoptic nuclei (SON) situated lateral to the optic chiasm, and the paraventricular nuclei (PVN) on each side of the third ventricle. Magnocellular neurons in those nuclei synthesize oxytocin (OXT) and vasopressin (VP) and send axonal projections to the posterior pituitary. The neuronal activity stimulates the release of these hormones into the blood, therefore reaching target organs where they regulate a number of important physiological functions, mainly reproduction and body fluid homeostasis (Leng et al., 1999). In addition, the PVN contains another group of OXT-staining cells, the parvocellular neurons, whose dendrites and axons terminate in different regions of the central nervous system (CNS) (Gimpl and Farhrenholz, 2001). Also, OXT and VP are released from perikarya, dendrites and/or axon collaterals of magnocellular neurons acting as neurotransmitters, modulating the activity of these cells in an autocrine way and also affecting more distant brain structures that do not receive direct OXT/VP innervations in a paracrine way (Leng and Ludwig, 2008).

The release of OXT and VP occurs in response to a wide variety of stimuli, including suckling, mating behavior, stress, fever and infection (McDonald et al., 2008). The HN axis plays an important role in maintaining homeostasis under a variety of stress conditions, including endotoxemia. The response of the organism to disturbance of its homeostasis caused by endotoxins includes the release of hormones such as, corticotrophin releasing hormone (CRH), as well as OXT and VP (Antunes-Rodrigues et al., 2004). The neuroendocrine response to infection can be mimicked by peripheral administration of bacterial products such as lipopolysaccharide (LPS), which is a commonly used model of immune challenge (McCann et al., 2000a). LPS activates the synthesis and release of various cytokines among which are interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α). These cytokines produced in the periphery have shown to be transported into the brain and stimulate central pathways that may contribute to the HN axis activation induced by LPS. In addition, LPS induces a central production of these cytokines. IL-1, IL-6 and TNF-α are synthesized in the brain *in situ*, mainly by microglia, astrocytes and also by neurons and endothelial cells. The cell bodies of the neurons that produce cytokines are located particularly in the PVN that has been recognized as an integrating center for brain reaction evoked by immune challenge (Besedovsky and Del Rey, 1996; Turnbull and Rivier, 1999; McCann et al., 2000b). Peripheral administration of LPS has been shown to increase plasma levels of OXT and VP (Kasting, 1986; Giusti Paiva et al., 2003, 2005). However, the mechanism involved in the activation of OXT and VP producing neurons by an immune challenge has not been well elucidated yet.

The endocannabinoid system (ECS) is actually considered as a major neuromodulatory system of the brain that modulates several neuroendocrine axes which are involved in many pathophysiological processes and whose main function is to maintain homeostasis. The ECS includes at least two endocannabinoid receptors, endogenous ligands named

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doi:10.1016/j.jneuroim.2010.02.006

endocannabinoids (eCBs), the enzymes that synthesize and degrade them as well as a transporter that is not well characterized (Freund et al., 2003; Howlet et al., 2004; Pertwee, 2006). eCBs are lipophilic arachidonic acid derivatives produced in brain and peripheral tissues. At least five eCBs have been identified, being anandamide (AEA) the first discovered and the most characterized (see in Pertwee, 2006). AEA is synthesized on demand and its formation via energy-independent condensation of arachidonic acid and ethanolamine is attributed to an enzymatic activity termed "anandamide synthase". This reaction is catalyzed by fatty acid amide hydrolase (FAAH), the enzyme that hydrolyzes AEA acting in reverse. Another pathway of AEA biosynthesis involves the hydrolysis of a phospholipid precursor catalized by phospholipase D. Depolarization of the postsynaptic cells generates AEA that acts retrogradely to inhibit subsequent neurotransmitter release by its binding to a seven transmembrane $G_{i/o}$-coupled cannabinoid receptor. Two subtypes of cannabinoid (CB) receptors, $CB_1$ and $CB_2$ have been identified and cloned. The $CB_1$ receptor is the predominant subtype in the CNS, whereas the $CB_2$ receptor is primarily expressed in immune cells, although it is also found in glial cells of the CNS (Steiner and Wotjak, 2008). In addition, AEA has affinity for the transient potential vanilloid type 1 receptor (TRPV1) which can participate in physiological processes such as blood pressure control, pain sensation and airway responsiveness (van der Stelt and Di Marzo, 2004; Cristino et al., 2006). The AEA action is terminated by its removal from its sites of action by reuptake transport system and metabolized mainly by the enzyme FAAH (Fezza et al., 2008).

Recent data suggest that the ECS may play a pivotal role in the regulation of the HN axis activity at hypothalamic level (Steiner and Wotjak, 2008). In 1991, Herkenham et al. (1991) first reported that CB receptors are localized in the PVN of the hypothalamus as well as in anterior and posterior pituitary lobes. eCBs have also been found in these tissues (Pagotto et al., 2006). It has been reported that eCBs are released as retrograde messengers in the SON by magnocellular neurones (Murphy et al., 1998) and that $CB_1$ receptors are localized within the SON (Wenger and Moldrich, 2002), suggesting that eCBs could modulate the physiology of magnocellular neurons, in fact it was reported that there is an interaction of ECS with central hormone release in the modulation of magnocellular SON neurons synaptic physiology (Sabatier and Leng, 2006; McDonald et al., 2008).

In a previous work, we demonstrated the increase of AEA synthase activity in hypothalami obtained from adult male rats peripherally injected with LPS (Fernandez-Solari et al., 2006). It is known that TNF-α is one of the cytokines that mediate the effects attributed to LPS on the hypothalamic pituitary axis. TNF-α is the first cytokine to be produced in large quantities, reaching high levels in circulation 30 min following LPS injection. TNF-α activates PVN hypothalamic neurons and triggers the release of CRH and other neuropeptides such as OXT and VP (Mastronardi et al., 2001). The intracerebroventricular (i.c.v.) administration of TNF-α and the incubation of hypothalamic fragments with this cytokine also increased hypothalamic AEA synthase activity (Fernandez-Solari et al., 2006).

There is abundant evidence that nitric oxide (NO) function as a local modulator of magnocellular neuronal activity. Neuronal NOS (nNOS) is expressed densely in the SON and PVN, where it is localized in OXT synthesizing neurons. Furthermore, its expression is functionally regulated, since nNOS mRNA can be up regulated after osmotic stimuli and hypovolemia and down regulated during late pregnancy and parturition (Stern and Ludwig, 2001; Xiao et al., 2005). In our previous works, we demonstrated that NO donors reduce OXT secretion from the neural pituitary lobe (Rettori et al., 1997) and also that Neurokinin A increases neurohypophyseal NOS activity and that this NO inhibits OXT secretion from this tissue (De Laurentiis et al., 2000). Electrophysiological studies have shown that the NO donor, sodium nitroprusside (NP) and the NO precursor, L-arginine, inhibit SON neurons, whereas the NOS inhibitor, nitro-L-arginine methyl ester (L-NAME) and the NO scavenger hemoglobin, excite them. These data suggest that NO is a major inhibitory regulator of SON neurons (Stern and Ludwig, 2001; Stern and Zhang, 2005). Although the involvement of NO in the regulation of OXT release has been investigated, the complicated mechanisms involved remain unclear (Rettori et al., 2009).

The aim of the present work was to study the participation of the hypothalamic ECS in the effects of LPS on OXT and TNF-α plasma levels and the interplay between the endocannabinoid and NOergic systems on OXT release from hypothalamus.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats weighing 200–250 g were used. The animals were fed with lab chow and water ad libitum and kept under controlled conditions of light (12 h light/dark) and temperature (20–25 °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.

### 2.2. Drugs

Most of the drugs were purchased from Sigma Co. (St. Louis, MO, USA) including bacterial LPS (Escherichia coli serotype 0055:B5), except: OXT standard and antisera from Bachem (California, CA, USA), AM251 [N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] and AM630 [6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone] from Tocris™ (Ellisville, MO, USA), URB597 from Cayman Chemical Company (Miami, USA) and $^{125}$Iodine and $^{14}$C-Arginine from PerkinElmer Life and Analytical Science (Boston, MA, USA).

### 2.3. Experimental protocols

#### 2.3.1. In vivo studies

Animals were injected intraperitoneally (i.p.) with LPS dissolved in pyrogen-free isotonic saline at a dose of 5 mg/kg rat or saline (control) and sacrificed by decapitation 1 or 3 h later.

To evaluate the participation of the hypothalamic endocannabinoid system on the LPS effect, one week prior to the day of the experiment, an indwelling cannula was implanted into the lateral cerebral ventricle by using a stereotaxic instrument while the rats were anesthetized with tribromoethanol (3.5% in saline, 1 ml/100gr animal body weight, i.p.). The coordinates relative to the interaural line (AP-0.6 mm, L-2 mm, DV-3.2 mm) were taken from the stereotaxic atlas of Pellegrino et al. (1979). A week later, the experiments were performed in conscious, freely moving rats. The control group of rats received an injection of saline (5 μl, i.c.v.) that was also used as vehicle to dissolve all other drugs administered. The experimental groups of rats received an injection of LPS (5 mg/kg rat, i.p.) alone or together with i.c.v. administration of 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, 20 μg/5 μl), depending on the experiment. All i.c.v. injections were made with a Hamilton syringe during a period of 1 min. Rats were decapitated 1 and 3 h after the injections and trunk blood collected into chilled heparinized tubes, centrifuged 20 min 3000 rpm at 4 °C and the plasma separated for OXT and TNF-α determinations. The adequate location of the cannula in the ventricle was confirmed by injecting a solution of methylene blue i.c.v. and confirming its position in histological sections.

### 2.4. Incubation of hypothalamic fragments

After decapitation and removal of the brain from normal untreated rats, a hypothalamic fragment including medium basal hypothalamus, SON and PVN was dissected by making a transversal cut just forward of the optic chiasm, extending dorsally 2.5 mm. A horizontal cut

extended from this point caudally to behind the pituitary stalk, where another frontal cut was made. Bilateral longitudinal cuts were made at the hypothalamic sulci. To investigate OXT and TNF-α release, the hypothalamic fragments were preincubated individually using 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. Then, the medium was discarded and replaced with fresh medium alone (control) or containing the substances to be tested and the tissues were incubated further for 30, 60, 90 or 120 min depending on the experiment. At the end of the incubation period, the media were removed and after heating for 10 min at 100 °C were centrifuged at 10,000 rpm for 10 min. The supernatants were stored at −70 °C for further determination of OXT and TNF-α levels. The tissues were immediately frozen in dry ice and stored at −70 °C for further determination of NOS activity.

### 2.5. Oxytocin determination

OXT concentration in plasma and in the incubation medium was measured on duplicate samples by radioimmunoassay using $^{125}$I-oxytocin as tracer and anti-oxytocin rabbit antiserum (final dilution 1:20,000) as described elsewhere (Elias et al., 1997). OXT was used as standard preparation 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. Oxytocin levels were expressed as pg/ml of plasma and ng/ml of incubation media.

### 2.6. TNF-α determination

Plasma and media TNF-α concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISA) using antibodies and standards obtained from BD Biosciences, San Diego, CA, USA. Briefly, 96-well MaxiSorp microtitre plates (Nalge Nunc International, New York, USA) were coated with anti-rat TNF-α antibodies diluted 1:250 in sodium carbonate buffer 0.1 M; pH 9.5, overnight at 4 °C. Plates were then washed five times with wash/dilution buffer (0.05% Tween-20 in PBS, pH 7.0) and blocked at room temperature for 1 hr using 200 µl of assay diluent (PBS with 10% fetal bovine serum heat inactivated, pH 7.0). Following five washes, 100 µl aliquots of samples or standards (0–2000 pg/ml) were added and plates were incubated overnight at 4 °C. After five washes, 100 µl of specific biotinylated anti-rat TNF-α antibody (1:250, diluted in assay diluent) was added to each well. A further incubation was carried out for 1 hr at room temperature. After five washes, 100 µl of horseradish peroxidase conjugated to streptavidin (1:250, diluted in assay diluent) was added to each well and plates were incubated at room temperature for 1 hr. Following seven washes, 100 µl of tetramethylbenzidine substrate solution was added per well and the plates were incubated for 30 min at room temperature in dark. At the end of the incubation period, 50 µl of 1 M $H_3PO_4$ was added per well to stop the reaction and to facilitate colour development. Absorbance was read immediately at 450 nm on a microplate reader (Model 3550, BIO-RAD Laboratories, California, USA). TNF-α levels in plasma and media were expressed as pg/ml.

### 2.7. NOS activity determination

NOS activity of all isoforms of the enzyme (total NOS) was determined by conversion of [$^{14}$C]arginine to [$^{14}$C]citrulline, using a modification of the method of Bredt and Snyder (1989). Briefly, the hypothalamic fragments were preincubated for 15 min in 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 the substances to be tested as previously described. The tissues 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 $CaCl_2$. The reaction was started by adding 20 µl (0.1 µCi) of L-[U-$^{14}$C]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. [$^{14}$C]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 NO/min.

### 2.8. Expression of endocannabinoid receptors

#### 2.8.1. Quantitative Real Time PCR (q-PCR)

Hypothalamic fragments from control and LPS-treated rats, 1 hour after the i.p. injection of saline or LPS (5 mg/kg) were homogenized in 1 ml of Trizol® (Invitrogen, CA, USA). Total RNA was extracted according to manufacturer recommendations, dissolved in RNA storage solution (Ambion, TX, USA), UV quantified by a Nano Drop® (Eppendorf, Hamburg, Germany), and stored at −80 °C. RNA aliquots (6 µg) were digested by RNAse-free DNAse I (Invitrogen deoxyribonucleic acid [DNA]-free™ kit) in a 20-µl final volume reaction mixture, to remove contaminating genomic DNA. After DNAse digestion, concentration and purity of RNA samples were evaluated by the RNA-6000-Nanodrop®, using a 2100 Bioanalyzer® equipped with a 2100-Expert-Software® (Agilent Technologies Inc., CA, USA), following the manufacturer instructions. For all samples tested, the RNA integrity number was greater than 6 (relatively to a 0–10 scale). Four micrograms of total RNA was reverse transcribed in a 25-µl reaction mixture containing: 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM deoxyribonucleotide triphosphate, 20 U of RNAse inhibitor, 0.125 A260 units of hexanucleotide mixture for random priming and 200 U of MoMuLV Superscript® III reverse transcriptase (Invitrogen, CA, USA). The reaction mixture was incubated in a termocycler CR Corbett Research® (Corbett Life Science, Qiagen Ltd., UK) for a 5 min at a 55 °C step, followed by a rapid chilling of 2 min at 4 °C. The protocol was stopped at this step and the MoMuLV reverse transcriptase was added to the samples, excepting the negative controls (−RT). The incubation was resumed by two thermal steps: 10 min at 20 °C followed by 90 min at 50 °C. Finally, the reaction was terminated by heating at 95 °C for 10 min. Quantitative real-time PCR was performed by an CR Corbett Research Cycler® in a 25-µl reaction mixture containing: 1× SYBR®-Green-Supermix (Bio-Rad), 20 ng of complementary DNA (cDNA; calculated on the basis of the retro-transcribed RNA), and 330 nM for each primer. The amplification profile consisted of an initial denaturation of 2 min at 94 °C and 40 cycles of 30 s at 94 °C, annealing for 30 s at optimum annealing temperature and elongation for 45 s at 68 °C. Fluorescence data were collected during the elongation step. A final extension of 7 min was carried out at 72 °C, followed by melt-curve data analysis. Optimized primers for SYBR®-Green analysis were designed by the REST® 08 Rotor-Gene 6000 software 1.7 version (Corbett Life Science, Qiagen Ltd., UK) and were synthesized (high-performance liquid chromatography purification grade) by Invitrogen, CA, USA. Assays were performed in triplicate (maximum ΔCt of replicate samples less than 0.5), and a standard curve from consecutive fivefold dilutions (100 to 0.10 ng) of a cDNA pool representative of all samples was included, for PCR efficiency determination. Relative expression analysis, correct for PCR efficiency and normalized with respect to reference gene β-actin, was performed by REST® 08 for group wise comparison and statistical analysis. The amount of $CB_1$ or $CB_2$ transcripts was normalized to

those of controls using the ΔΔCT method. The following primers were used in RT-PCR:

$CB_1$ 5′-GGA GAA CAT CCA GTG TGG GG-3′sense
5′-CAT TGG GGC TGT CTT TAC GG-3′antisense
$CB_2$ 5′-CTT GAC TGA GCA CCA GGA CA-3′sense
5′-TAA CAA GGC ACA GCA TGG AG-3′antisense
β-actin 5′-AGC CAT GTA CGT AGC CAT CC-3′sense
5′-CTC TCA GCT GTG GTG GTG AA-3′antisense

*2.8.2. Western-blot*

Hypothalamic fragments from control and LPS-treated rats, 1 h after the i.p. injection of saline or LPS (5 mg/kg), respectively were homogenized in lysis buffer (1:2 w/v) containing 0.5 M β-glycerophosphate, 20 mM $MgCl_2$, 10 mM ethylene glycol tetraacetic acid, and supplemented with 100 mM dithiothreitol and protease/phosphatase inhibitors (100 mM dimethylsulfonyl fluoride, 2 mg/ml apronitin, 2 mM leupeptin, and 10 mM $Na_3VO_4$). Homogenates were centrifuged at 3000 rpm for 5 min at 4 °C; the supernatants were collected and centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were determined using the method of Bradford. For Western blot analysis, lysate aliquots containing 50 μg of proteins were denatured, separated on a 12% sodium dodecyl sulfate–polyacrylamide gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, UK) using a Bio-Rad Transblot Mini II (90 V constant voltage for 1 h at 4 °C). Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma, Co., MO, USA) and washed in phosphate-buffered saline (PBS). Membranes were blocked at 4 °C in milk buffer (5% non fat dry milk in PBS Tween 0.1%) and then incubated overnight at 4 °C with polyclonal antibodies for $CB_1$ receptors (BIOMOL International, Enzo Life Sciences International, Inc., PA, USA) and $CB_2$ receptors (Abcam Inc., Cambridge, MA, USA). The rabbit polyclonal anti-$CB_1$ and anti-$CB_2$ were used at 1:1000 dilution in milk buffer (5% non fat dry milk in PBS Tween 0.1%). Subsequently, the membranes were incubated for 1 h at room temperature with 1:2000 anti-rabbit IgG–horseradish peroxidase-conjugated secondary antibodies (Abcam Inc.). After washing with PBS Tween 0.1%, the membranes were analyzed by enhanced chemiluminescence (Amersham Biosciences, UK). The optical density (OD) of the bands on autoradiographic films was determined by an image analysis system. Film analysis was conducted by quantitative densitometry with a computerized image processing system (Image Quantun). After stripping, the membranes were probed with anti-β-actin antibody, to normalize the results.

*2.9. 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. 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. In vivo experiments*

*3.1.1. Effect of LPS administration on plasma oxytocin and TNF-α levels*

One hour after the administration of LPS (5 mg/kg, i.p.) the levels of plasma OXT were increased significantly ($p<0.001$) as compared to saline treated (control) rats. These increased levels returned almost to control values 3 h after LPS administration (Fig. 1A). The same pattern was observed when we determined TNF-α plasma levels. LPS significantly ($p<0.01$) increased TNF-α 1 h after its administration, these levels were much lower 3 h later (Fig. 1B). The TNF-α plasma levels were undetectable in control rats.

*3.1.2. Effect of central AEA administration on plasma oxytocin and TNF-α levels*

Both OXT and TNF-α plasma levels remained unchanged one hour after the central administration of the endocannabinoid AEA (50 ng/5 μl, i.c.v) to control rats, untreated animals in basal conditions (*not shown*).

*3.1.3. Effect of central administration of CB receptors antagonists on plasma oxytocin and TNF-α levels with and without LPS*

The observed increase in plasma OXT levels after 1 h of LPS (5 mg/kg, i.p.) was partially blocked when a $CB_1$ receptor antagonist (AM251, 500 ng/5 μl, i.c.v) was centrally administered at the same time than LPS injection; and completely blocked, when both a $CB_1$ (AM251, 500 ng/5 μl, i.c.v) and a $CB_2$ (AM630, 500 ng/5 μl, i.c.v) receptor antagonists were centrally co-administered (Fig. 2A).

Similarly, the increase on TNF-α plasma levels produced after 1 h of LPS injection was partially blocked when AM251 (500 ng/5 μl, i.c.v) was injected alone or together with AM630 (500 ng/5 μl, i.c.v) at the same time than LPS (5 mg/kg, i.p.) (Fig. 2B).

*3.1.4. Effect of central administration of FAAH inhibitor on plasma oxytocin and TNF-α levels in LPS treated rats*

The OXT and TNF-α plasma levels observed after 1 h of LPS (5 mg/kg, i.p.) were higher ($p<0.05$) when a FAAH inhibitor (URB597, 20 μg/5 μl, i.c.v) was centrally administered at the same time than LPS (Fig. 3). OXT and TNF-α levels were undetectable in rats without LPS injection and URB injected in these rats did not modified OXT nor TNF-α levels (data not shown).

*3.1.5. Cannabinoid receptors mRNA quantification and protein determination in hypothalamic fragments*

The relative expression analysis, evaluated by quantitative RT-PCR (q-PCR), of $CB_1$ and $CB_2$ mRNA of hypothalamic fragments from saline

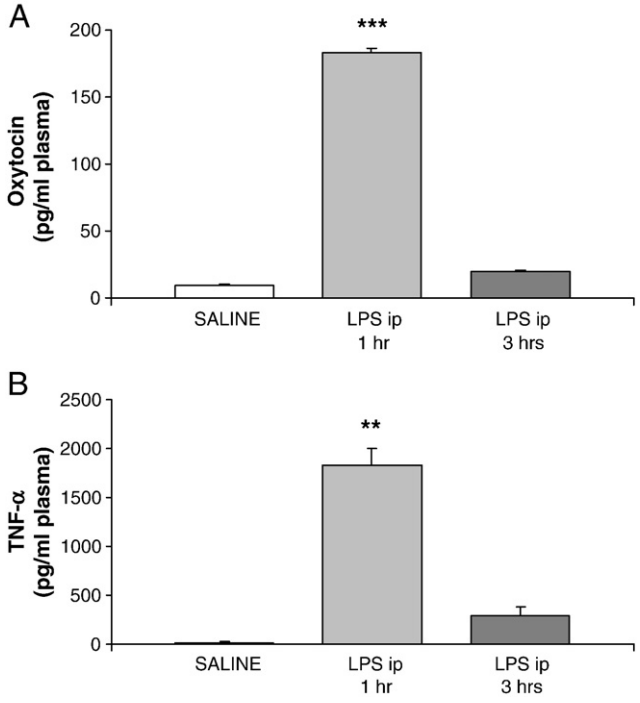


**Fig. 1.** *In vivo* effect of LPS (5 mg/kg rat) or saline i.p. administration on OXT (A) and TNF-α (B) plasma levels 1 and 3 h post injection. Values represent mean ± SEM, $n=6$–8 per group. Data were evaluated by one-way ANOVA followed by Dunnett´s test (**$p<0.01$, ***$p<0.001$ vs saline).

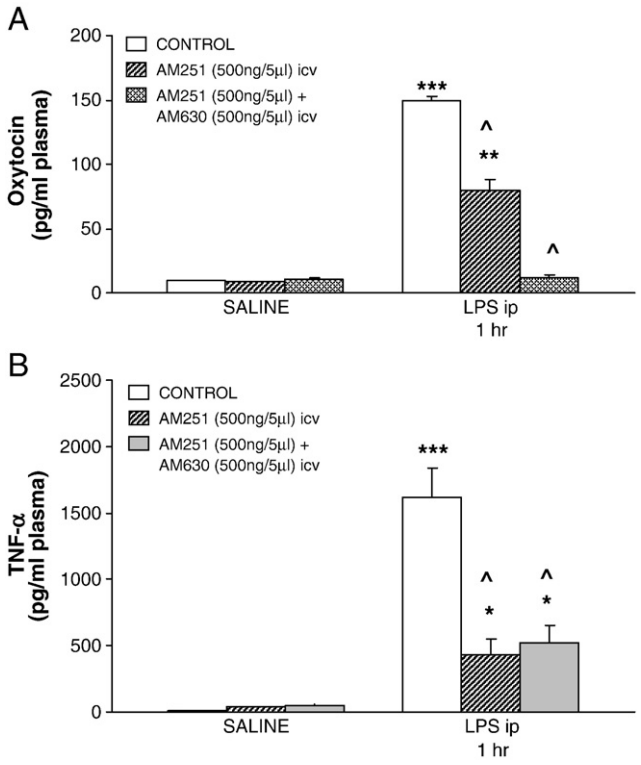


**Fig. 2.** Effect of intracerebroventricular (I.C.V.) injection of $CB_1$ receptors antagonist (AM251, I.C.V., 500 ng/5 µl) alone or coadministered with $CB_2$ receptors antagonist (AM630, I.C.V., 500 ng/5 µl) or saline (control) injected at the same time than LPS (i.p., 5 mg/kg rat) or saline on OXT (A) and TNF-α (B) plasma levels 1 h post injections. Values represent means ± SEM, $n = 6–8$ per group. Data were evaluated by two-way ANOVA. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs respective control without LPS and $^{\wedge}p<0.05$ vs respective control without antagonist.

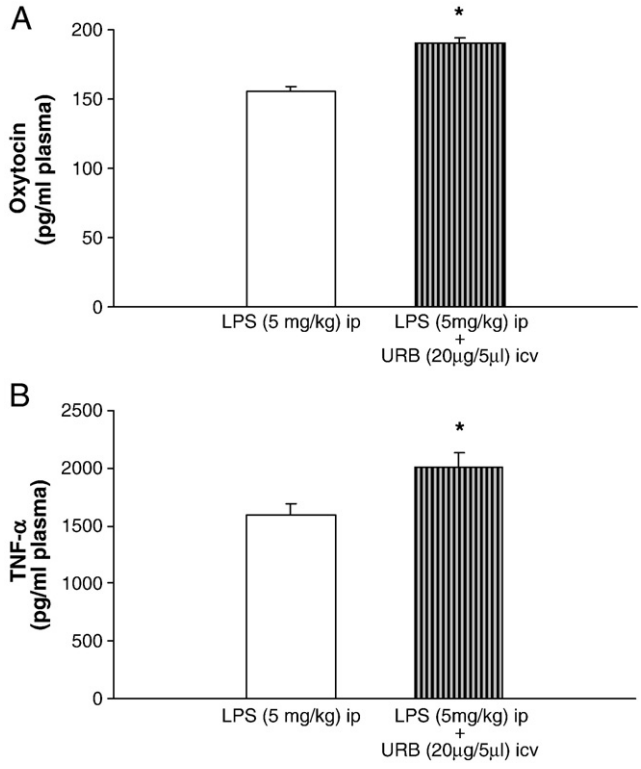


**Fig. 3.** Effect of intracerebroventricular (I.C.V.) injection of a FAAH inhibitor (URB597, I.C.V., 20 µg/5 µl) or saline injected at the same time than LPS (i.p., 5 mg/kg rat) on OXT (A) and TNF-α (B) plasma levels 1 hr post injections. Values represent means ± SEM, $n = 6–8$ per group. Data were evaluated by Student's $t$ test. $^{*}p<0.05$ vs LPS.

(control) and LPS-treated rats, is depicted in Fig. 4A. All targets were well detectable in the linearity range of analysis ($CB_1$ mean cycle threshold = 24.5; $CB_2$ mean cycle threshold = 24; β-actin mean cycle threshold = 18.5). The amplification reaction efficiencies were comparable, ranging from 96 to 108%. Hypothalamic fragments showed significantly ($p<0.05$) elevated expression of $CB_1$ mRNA receptor (relatively to the housekeeping gene). On the contrary, no variation of $CB_2$ expression levels was detected.

The LPS administration significantly ($p<0.05$) increased $CB_1$ receptor protein expression in hypothalamic fragments, but any change was observed in the expression of $CB_2$ receptor subtype protein (Fig. 4B).

### 3.2. In vitro experiments

#### 3.2.1. Effect of AEA on OXT release from hypothalamic fragments

The hypothalamic fragments were incubated in the presence of AEA ($10^{-10}$ M to $10^{-6}$ M) for 30 min. AEA ($10^{-9}$ M) significantly increased ($p<0.01$) basal OXT release from the tissues (Fig. 5A). The presence of AM251 ($10^{-5}$ M), a $CB_1$ receptor antagonist, completely prevented the stimulatory effect of AEA ($10^{-9}$ M) (Fig. 5B).

The tissues were incubated with the FAAH inhibitor, URB597 ($10^{-11}$–$5.10^{-9}$ M). The presence of URB597 $10^{-10}$ and $5.10^{-10}$ M significantly increased ($p<0.01$) basal OXT release from hypothalamic fragments (Fig. 5C).

We also studied the effect of depolarizing conditions on OXT release incubating the hypothalamic fragments in KRB containing 40 mM $K^+$ balanced by reducing $Na^+$ concentration ($K^+$evoked release) and comparing this release with KRB with 5 mM of $K^+$ (basal release). The depolarization induced a dramatic increase in OXT release, about 10 folds (data not shown). Therefore all *in vitro* experiments of the present study were performed in KRB with 5 mM $K^+$, therefore evaluating the basal release of OXT.

#### 3.2.2. Participation of NO in the effect of AEA on OXT release from hypothalamic fragments

AEA ($10^{-9}$ M) significantly increased ($p<0.01$) total NOS activity in hypothalamic fragments incubated for 30 min (Fig. 6A). Sodium nitroprusside (NP, 600 µM), a NO donor, significantly decreased ($p<0.05$) OXT release from the hypothalamic fragments (Fig. 6B). In addition, the presence of hemoglobin (Hb, 40 µg/ml), a scavenger of NO, augmented the increase of OXT release induced by AEA (Fig. 6C).

#### 3.2.3. Effect of LPS on TNF-α and oxytocin release from hypothalamic fragments

In order to determine if LPS *in vitro* was able to induce TNF-α release from hypothalamic fragments we incubated the tissues in the presence of LPS (10 µg/ml) together with interferon gamma (INF-γ, 100 ng/ml), since LPS alone was without effect. LPS + INF-γ increased TNF-α release after 60 ($p<0.05$), 90 ($p<0.01$) and 120 min ($p<0.001$) of incubation (Fig. 7).

The release of OXT in the presence of LPS (10 µg/ml) together with INF-γ (100 ng/ml) remained unchanged at 30 min (not shown), but was significantly increased ($p<0.01$) after 60 min of incubation. This increase of OXT release was completely prevented by the presence of AM251 ($10^{-5}$ M) in the incubation media (Fig. 8A). Similarly, when the hypothalamic fragments were incubated in the presence of TNF-α (50 ng/ml), there was a significant ($p<0.05$) increase in OXT release at 30 min, that was completely prevented by the presence of the $CB_1$ receptor antagonist (AM251, $10^{-5}$ M) in the media (Fig. 8B).

#### 3.2.4. Effect of LPS and TNF-α on NOS activity in hypothalamic fragments

LPS (10 µg/ml) together with INF-γ (100 ng/ml) (Fig. 9A) and TNF-α (50 ng/ml) (Fig. 9B) significantly increased total NOS activity in hypothalamic fragments incubated for 30 min ($p<0.01$ and $p<0.001$, respectively). There is a considerable difference in the effect between

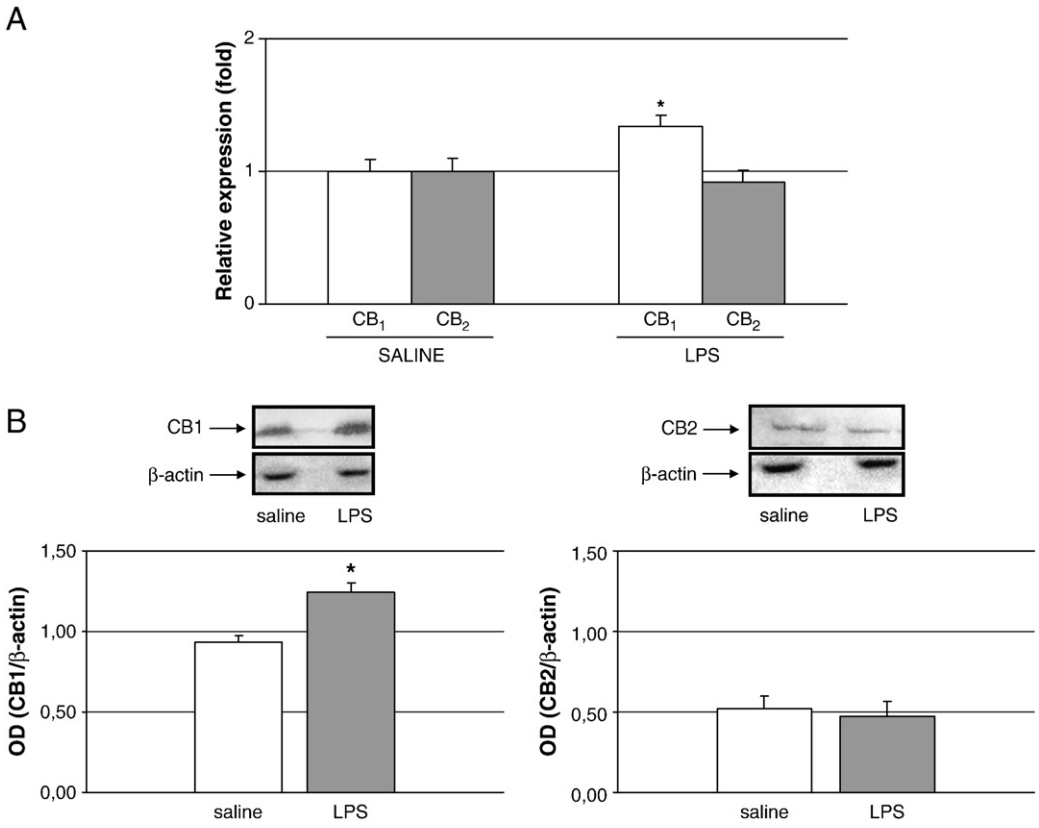


**Fig. 4.** (A) Relative expression analysis of $CB_1$ receptors ($CB_1$) and $CB_2$ receptors ($CB_2$) mRNAs in hypothalamic fragments from saline and lipopolysaccharide (LPS)-treated rats. The rats were sacrificed 1 h after the i.p. injection of LPS (5 mg/kg rat) or saline. Total RNA extracted from hypothalamic fragments was subjected to quantitative (real-time) RT-PCR analysis as described in Materials and methods. Data were analyzed by Rotor Gene software for group wise comparisons and statistical analysis. Data are mean ± SEM of $n = 3$ determinations from three independent experiments and are expressed as fold changes of mRNA expression in the hypothalamic fragments from LPS-treated rats vs. saline (considered as 1). Refer to results for expression fold data. Data were evaluated by Student's *t*-test. $^*p<0.05$ vs. saline. (B) Representative Western blot analysis (shown in the top part of B). Arrows indicate the immunoreactive bands of the $CB_1$ and $CB_2$ receptors as well as β-actin. Relative proteins levels of $CB_1$ and $CB_2$ receptors in hypothalamic fragments from saline and LPS-treated rats (LPS, i.p., 5 mg/kg rat) were quantified by densitometric scanning and normalized to β-actin and expressed as Optic Density (OD), shown in the bottom part of B. Data are expressed as mean ± SEM of five independent experiments. Data were evaluated by Student's *t*-test. $^*p<0.05$ vs. saline.

both treatments being TNF-α much more potent in the activation of NOS. The increased NOS activity induced by both, LPS and TNF-α, were partially prevented when the $CB_1$ antagonist AM251 ($10^{-5}$ M) was present in the incubation media (Fig. 9).

## 4. Discussion

The systemic administration of LPS can induce endotoxic shock with pathophysiological consequences, including changes in hormone secretion. Thus, the hypothalamus is activated, particularly PVN and SON nuclei, and neurohypophyseal hormone secretion is increased (Matsunaga et al., 2000; Xia and Krunkoff, 2003). The present study is in concordance with previous reports that have shown that LPS causes an increase in OXT secretion (Kasting, 1986; Giusti Paiva et al., 2005). We observed, in our acute model of intraperitoneal LPS administration, that OXT plasma levels were increased after 1 h of LPS injection and returned almost to basal levels after 3 hr.

Acute LPS administration has long been associated with increases in plasma pro-inflammatory cytokines (Roche et al., 2006). TNF-α is a cytokine that is found in high levels in circulation during endotoxic shock (McCann et al., 2000b). This cytokine is produced in sufficient quantity to accumulate in blood, which carries it to the brain where acts directly on the central nervous system (CNS). Furthermore, this cytokine could be produced in the brain (Watkins et al., 1995a,b). Because TNF-α is known to be released rapidly after LPS administration and mediates a number of effects attributed to LPS administration in the hypothalamic pituitary axis (Dunn, 1993; McCann et al., 1994, 2000b), we decided to evaluate the production of this cytokine in our model. In agreement with the above mentioned literature, in our experimental conditions, we observed highly increased TNF-α plasma levels 1 h after LPS injection and these levels were much lower after 3 h. Moreover, it was reported that LPS-induced TNF-α release is mainly controlled by the CNS (Mastronardi et al., 2001), then, we performed *in vivo* studies in which the hypothalamic endocannabinoid system was directly affected by different approaches in order to determine its participation in the control of OXT and TNF-α plasma levels in LPS-treated rats.

Exogenous cannabinoids exert effects on hormone secretion from pituitary gland having, predominantly, an inhibitory impact on neuroendocrine function. For example, $\delta^9$-tetrahydrocannabinol produced an increase in diuresis and suppression of milk ejection reflex by inhibiting the release of VP and OXT, respectively (Sofia et al., 1977; Tyrey and Murphy, 1988). These effects are possible due to the fact that $CB_1$ receptors are located in OXT-secreting parvocellular and magnocellular neurons of the PVN and SON nuclei (Di et al., 2005a,b).

On the other hand, it was reported the stimulatory effect of cannabinoids on stress-induced hypothalamic pituitary axis (Steiner and Wotjak, 2008), with enhanced secretion of hypothalamic-pituitary hormones (Weidenfeld et al., 1994). These results are in concordance with our present findings that endocannabinoids enhance the stimulatory effect of LPS on hypothalamo-neurohypophyseal axis since the blockade of hypothalamic cannabinoid receptors $CB_1$ and $CB_2$ prevents the LPS-increase in OXT plasma levels. This complete prevention of LPS-induced increase of OXT plasma levels due to $CB_1$ and $CB_2$ receptors blockade induced with both antagonists centrally injected indicates that, the hypothalamic ECS controls OXT

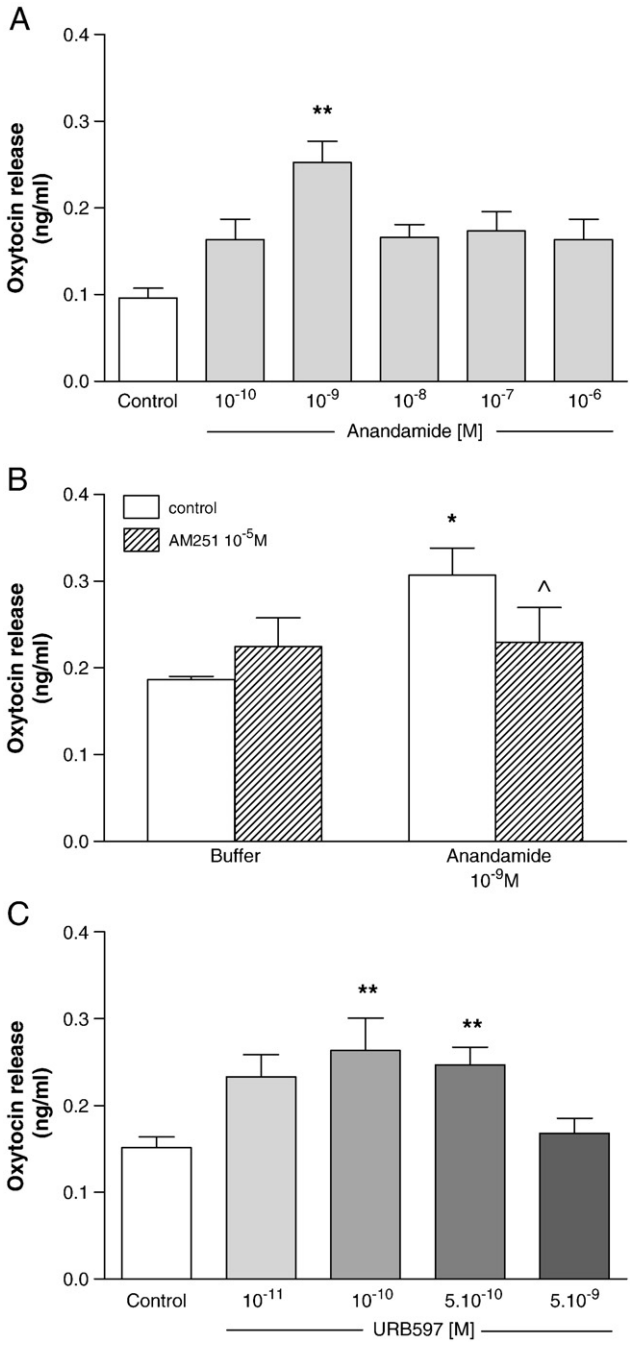


**Fig. 5.** Oxytocin (OXT) release from hypothalamic fragments incubated for 30 min in the presence of: (A) Anandamide (AEA, $10^{-10}$–$10^{-6}$ M); (B) AEA ($10^{-9}$ M) alone or in the presence of $CB_1$ receptors antagonist (AM251, $10^{-5}$ M); (C) An inhibitor of FAAH (URB597, $10^{-11}$–$5.10^{-9}$ M). In all experiments the tissues of the control group were incubated with KRB buffer. Values represent mean ± SEM, $n = 5$–9 per group. Data were evaluated by one-way ANOVA followed by Dunnett's test (A and C) and two-way ANOVA (B). $^{*}p<0.05$, $^{**}p<0.01$ vs. control and $^{\wedge}p<0.05$ vs respective control without antagonist.

release under endotoxic conditions with the participation of both subtypes of receptors. Moreover, the approach to attenuate the degradation of endocannabinoids by the FAAH inhibitor URB597 administered into the lateral ventricle, which only enhances anandamide levels thus promoting endocannabinoid signaling specifically in the brain area of our interest, further increased OXT plasma levels in LPS-challenged rats.

Endocannabinoids such as anandamide may signal through cannabinoid receptors to facilitate LPS-induced cytokine release (Di Marzo et al., 1999; Maccarrone et al., 2001, 2002). Anandamide, in addition to modulate cellular responsiveness to various cytokines, was also reported to increase the production of cytokines (Molina-Holgado et al., 1998). In a previous study we demonstrated the increase of AEA synthase activity in hypothalamic fragments removed from rats injected with LPS (Fernandez-Solari et al, 2006). Such mechanism of action would explain the inhibitory effect of central CB receptors blockade on LPS-induced TNF-α plasma levels observed in the present study. The $CB_1$ receptor antagonist (AM251) alone or together with $CB_2$ receptor antagonist (AM630) inhibited partially but highly significantly in equal amounts the LPS-increased plasma levels

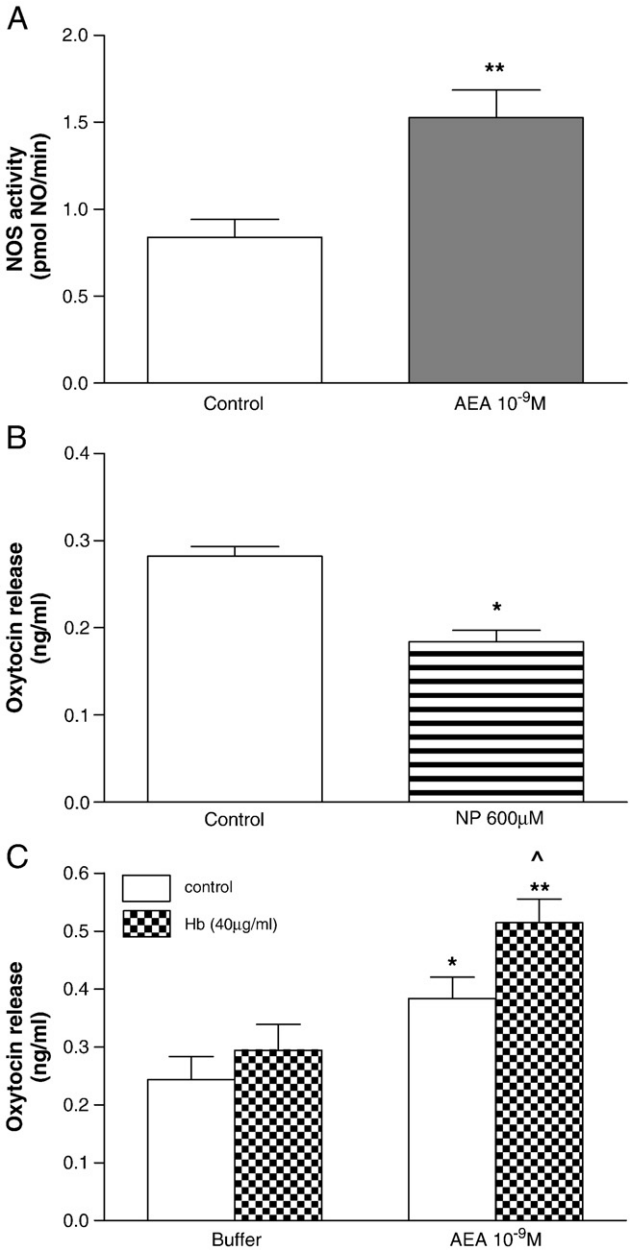


**Fig. 6.** (A) *In vitro* effect of anandamide (AEA, $10^{-9}$ M) on NO synthase (NOS) activity in hypothalamic fragments incubated for 30 min. (B) OXT release from hypothalamic fragments incubated with sodium nitroprusside (NP, 600 µM) for 30 min. Values represent means ± SEM, $n = 6$–8 per group. Data were evaluated by Student's $t$ test. $^{*}p<0.05$ and $^{**}p<0.01$ vs control. (C) OXT release from hypothalamic fragments incubated with AEA ($10^{-9}$ M) alone or together with haemoglobin (Hb, 40 µg/ml) for 30 min. In all experiments the tissues of the control group were incubated with KRB buffer. Values represent mean ± SEM, $n = 6$–7 per group. Data were evaluated by two-way ANOVA. $^{*}p<0.05$ and $^{**}p<0.01$ vs respective control and $^{\wedge}p<0.05$ vs respective control without Hb.

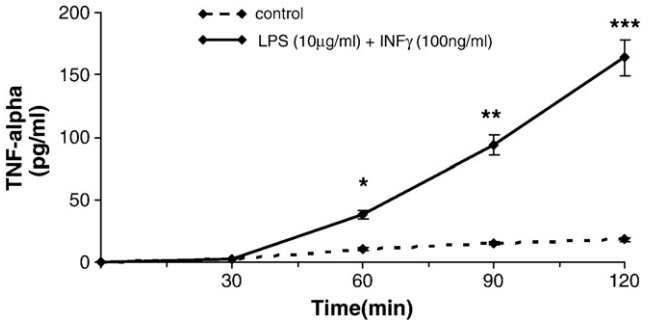


**Fig. 7.** *In vitro* effect of LPS (10 μg/ml) together with INF γ (100 ng/ml) on TNF-α release from hypothalamic fragments incubated for 30, 60, 90 and 120 min. Values represent means ± SEM, $n = 6–9$ per group. Data were evaluated by Student's $t$ test. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs control.

of TNF-α. The fact that the blockade with both antagonists was equal and partial suggest that endocannabinoids signaling through $CB_1$ subtype of receptors is sufficient to mediate LPS-induced increases in TNF-α plasma levels, however probably other receptors could be involved, such as vanilloid TRPV1 (van der Stelt and Di Marzo, 2004). This partial blockade could be explained since peripherally administered LPS activate cells of the immune system triggering the synthesis and release of TNF-α from immune cells thus increasing the amount of this cytokine in plasma.

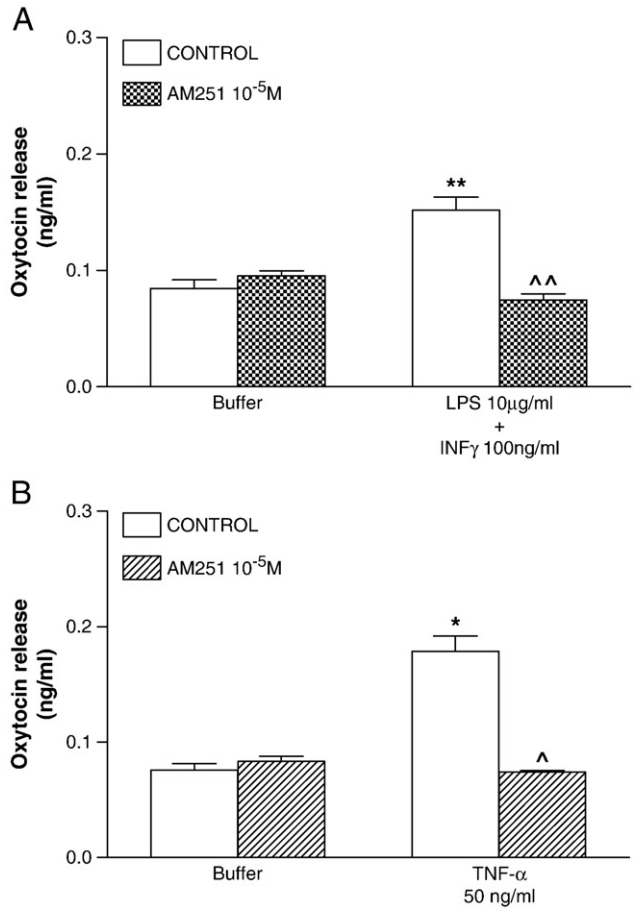


**Fig. 8.** OXT release from hypothalamic fragments incubated with: (A) LPS (10 μg/ml) together with INF γ (100 ng/ml) for 60 min and (B) TNF-α (50 ng/ml) for 30 min, with or without $CB_1$ receptors antagonist (AM251, $10^{-5}$ M). In all experiments the tissues of the control group were incubated with KRB buffer. Values represent mean ± SEM, $n = 5–9$ per group. Data were evaluated by two-way ANOVA. $^{*}p<0.05$ and $^{**}p<0.01$ vs respective control and $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$ vs respective control without antagonist.

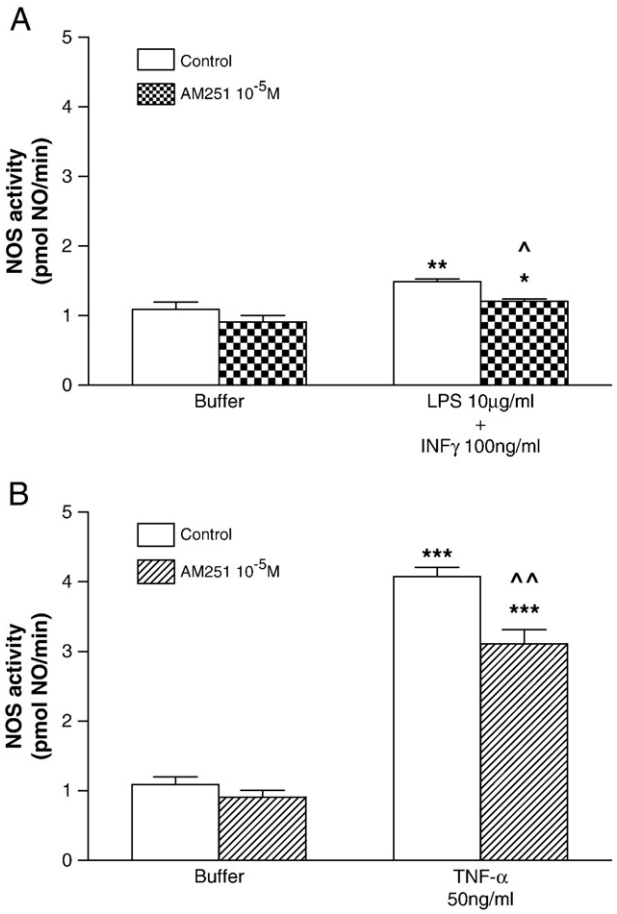


**Fig. 9.** NO synthase (NOS) activity in hypothalamic fragments incubated with: (A) LPS (10 μg/ml) together with INF γ (100 ng/ml) and (B) TNF-α (50 ng/ml), with or without $CB_1$ receptors antagonist (AM251, $10^{-5}$ M) for 30 min. In all experiments the tissues of the control group were incubated with KRB buffer. Values represent mean ± SEM, $n = 5–7$ per group. Data were evaluated by two-way ANOVA. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ vs respective control and $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$ vs respective control without antagonist.

Similarly, another study (Roche et al., 2006) described that the peripheral administration of SR141716A and SR144528, $CB_1$ and $CB_2$ receptors antagonists respectively, was sufficient to suppress central and peripheral cytokine responses. Indeed, immunosuppressive and anti-inflammatory effects of SR 141716A have previously been reported. In concordance with us, they demonstrated that both antagonists could inhibit LPS-induced increases in TNF-α plasma levels in mice (Smith et al., 2001; Croci et al., 2003). The immunosuppressive effects of the antagonists may be associated with their ability to block the effects of released endocannabinoids on CB receptors. LPS itself induces the release of endocannabinoids by macrophages and platelets (Di Marzo et al., 1999; Maccarrone et al., 2002) and inhibits endocannabinoids degradation in lymphocytes (Maccarrone et al., 2001), effects that could occur in microglial cells and astrocytes in the brain.

Both, q-PCR analyses and western blot (followed by quantitative densitometry analyses of numerous samples) revealed that the specific $CB_1$ receptor mRNA transcripts and the expression of $CB_1$ receptor protein respectively, were indeed significantly increased in hypothalamic fragments from LPS-treated rats (when compared to saline). On the contrary, $CB_2$ levels of both, mRNA and proteins, did not vary and were almost undetectable. Probably, these results explain the main participation of $CB_1$ receptor in the effect of endocannabinoids on TNF-α increased release induced by LPS observed in the present study.

Our *in vitro* results demonstrate that anandamide administered exogenously is able to increase OXT release from hypotalamic fragments and that this action is mediated by $CB_1$ subtype cannabinoid receptor since its blockade completely prevented this effect. Moreover, this effect was corroborated by the pharmacological enhancement of endogenous anandamide tone following URB597 incubation. LPS has been shown to down regulate the expression of the endocannabinoid metabolizing enzyme, FAAH (Maccarrone et al., 2001) and this is the probable mechanism responsible for the increase of AEA induced by LPS. Furthermore, it is well known that the activity of OXTergic magnocellular neurons of the hypothalamic PVN and SON nuclei are controlled by excitatory glutamatergic and inhibitory GABAergic neurons. Di et al. (2005b) had shown that LPS increased eCBs synthesis and release in magnocellular neurons that bind to presynaptically localized CB1 receptors resulting in down regulation of glutamate release. Also, GABAergic transmission might be negatively controlled by eCBs. In this case the activation of eCBs synthesis and release leads to downregulation of GABA release (Oliet et al., 2007). Endocannabinoid signaling at GABAergic synapses is expected to result in increased secretion of OXT from axon terminals. All these reports are in concordance with our findings suggesting that LPS increased hypothalamic AEA levels that binds to $CB_1$ receptors located in magnocellular neurons and/or in presynaptic GABAergic neurons (Di et al., 2005a; Oliet et al., 2007) leading to an increased OXT release from hypothalamus.

Also, LPS enhance the activity of NOS in hypothalamus and pituitary gland (McCann et al., 2005). The expression and activity of nNOS is dynamically modulated in response to LPS stimuli having a profound effect on the hypothalamic neurohypophyseal system. In the present study we found that anandamide increased hypothalamic NOS activity. The NO produced may act as an important inhibitory regulator of the magnocellular neurons function, in fact, it has been demonstrated that NO inhibits oxytocin secretion (Kadekaro, 2004). Confirming these facts we observed that the NO donor sodium nitroprusside, inhibited OXT release from hypothalamic explants. Moreover, the NO scavenger hemoglobin, enhanced anandamide-induced OXT release, with no effect in basal conditions, suggesting that NO acts when the system is activated. All these data are in accordance with the inhibitory role of NO on the OXT induced secretion during endotoxemia observed by Giusti Paiva et al. (2005). The production of NO by OXTergic neurons suggests that it may participate in auto and/or cross-regulation of OXT secretion. Indeed, it has been postulated that released NO may suppress OXT secretion through a negative feedback mechanism by which OXT stimulates the release of NO, which in turn, acts back to inhibit OXT release (Rettori et al., 1997). It has been reported that the NOS inhibitor L-NAME centrally administrated, enhanced plasma levels of both, OXT and VP (Kadekaro, 2004). Indeed, Summy-Long et al. (1993) have shown that the inhibition of NOS augments the release of OXT in dehydration conditions, in agreement with our findings.

Exposure to LPS plus interferon-γ (IFN-γ) is a strong proinflammatory stimulus for brain cells. LPS in combination with IFN-γ has been widely used in different *in vitro* and *in vivo* experimental approaches for the study of brain inflammatory diseases (Caruso et al., 2007). In the present study we used the LPS/IFN-γ combination, since LPS alone had no effect, in incubated hypothalamic fragments to mimic, at least in part, the effects of LPS-challenge. LPS/INF-γ increased TNF-α release from this tissue after 60 min reaching 150-fold the initial values by 2 h, indicating that this treatment is able to induce this cytokine production in hypothalamic fragments. Also, LPS/INF-γ increased OXT release and this effect seems to be mediated by $CB_1$ receptors activation since the presence of the antagonist AM251 prevents this stimulatory effect. This *in vitro* study corroborates the participation of CB receptors in LPS-induced OXT release observed *in vivo*. Similarly the presence of TNF-α in the incubation media increased OXT release that could be blocked by the $CB_1$ antagonist.

In conclusion, the present study provides direct evidence for cannabinoid receptor mediated enhancement of OXT release from hypothalamus, following immune challenge, been NO a mediator of this pathway. Also, endocannabinoids participates in LPS-induced TNF-α production in the brain and periphery. This effect could be mediated, at least in part, by endocannabinoid activation of hypothalamic $CB_1$ receptors, however, the potential involvement of another receptor cannot be excluded. Our findings reveal an interaction between oxytocin, endocannabinoid and NO-ergic systems at hypothalamic level and this interaction provides a mechanism of hypothalamic-neurohypophyseal activity regulation under basal and stress conditions.

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