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Participation of hypothalamic CB1 receptors in reproductive axis disruption during immune challenge

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Immune challenge inhibits reproductive function and endocannabinoids (eCB) modulate sexual hormones. However, no studies have been performed to assess whether the eCB system mediates the inhibition of hormones that control reproduction as a result of immune system activation during systemic infections. For that reason, we evaluated the participation of the hypothalamic cannabinoid receptor CB1 on the hypothalamic-pituitary-gonadal (HPG) axis activity in rats submitted to immune challenge. Male adult rats were treated i.c.v. administration with a CB1 antagonist/inverse agonist (AM251) (500 ng/5 μL), followed by an i.p. injection of lipopolysaccharide (LPS) (5 mg/kg) 15 minutes later. Plasmatic, hypothalamic and adenohypophyseal pro-inflammatory cytokines, hormones and neuropeptides were assessed 90 or 180 minutes post-LPS. The plasma concentration of tumour necrosis factor α and adenohypophyseal mRNA expression of *Tnf*α and *Il1*β increased 90 and 180 minutes post i.p. administration of LPS. However, cytokine mRNA expression in the hypothalamus increased only 180 minutes post-LPS, suggesting an inflammatory delay in this organ. CB1 receptor blockade with AM251 increased LPS inflammatory effects, particularly in the hypothalamus. LPS also inhibited the HPG axis by decreasing gonadotrophin-releasing hormone hypothalamic content and plasma levels of luteinising hormone and testosterone. These disruptor effects were accompanied by decreased hypothalamic *Kiss1* mRNA expression and prostaglandin E2 content, as well as by increased gonadotrophin-inhibitory hormone (*Rfrp3*) mRNA expression. All these disruptive effects were prevented by the presence of AM251. In summary, our results suggest that, in male rats, eCB mediate immune challenge-inhibitory effects on reproductive axis at least partially via hypothalamic CB1 activation. In addition, this receptor also participates in homeostasis recovery by modulating the inflammatory process taking place after LPS administration.

KEYWORDS
endocannabinoid system, immune challenge, reproductive axis

1 | INTRODUCTION

Reproductive physiology is controlled mainly by the hypothalamic-pituitary-gonadal (HPG) axis in adult individuals. In the medial basal hypothalamus (MBH), gonadotrophin-releasing hormone (GnRH) is released to a specific region of the pituitary, the adenohypophysis (AH), which induces the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) to the circulatory system. LH is responsible for stimulating the secretion of sex steroids (such as testosterone, oestradiol and progesterone) from the gonads of both sexes, whereas

FSH is the main gametogenic hormone. GnRH neurones are influenced by a complex context in which GnRH release is modulated by gonadal steroids, neurotransmitters (opioids, dopamine, GABA, histamine, glutamate, serotonin, etc.), as well as recently discovered hypothalamic neuropeptides, such as gonadotrophin-inhibitory hormone (GnIH)/RFamide-related peptides (RFRPs) and kisspeptin. GnIH was first discovered in birds and is able to suppress the HPG axis at both hypothalamic and AH level.[1] In mammals, two GnIH orthologues are known as RFRP-1 and RFRP-3.[2] RFRP-3 inhibits GnRH and LH synthesis and secretion.[3] On the other hand, GnRH synthesis and secretion are stimulated by kisspeptin, a peptide whose expression is regulated by the *Kiss1* gene.[4]

Inflammatory diseases and systemic infections are often associated with an impaired reproductive function.[5] Lipopolysaccharide (LPS), a potent inducer of the release of pro-inflammatory cytokines, prostaglandins and catecholamines, is widely used to induce immune challenge, which in turn disrupts reproductive capability. Parenteral administration of LPS is known to inhibit GnRH release from hypothalamic neurones, mainly via the effects of pro-inflammatory cytokines such as interleukin (IL)1β, and tumour necrosis factor (TNF)α.[6,7]

The endocannabinoid system (ECS) is one of the main neuromodulatory systems acting in the central nervous system (CNS). The most thoroughly studied cannabinoids (CB) ligands to date are the endogenous cannabinoids, or endocannabinoids (eCB), such as anandamide (AEA) and 2-arachidoylglycerol (2-AG),[8,9] and the exogenous cannabinoids isolated from *Cannabis sativa*, such as Δ-9-tetrahydrocannabinol (THC). To date, two cannabinoid receptors have been identified and characterised[10-12]: cannabinoid receptor 1 (CB1), which is one of the most abundant receptors in the brain[13]; and cannabinoid receptor 2 (CB2), found mainly on immune cells and peripheral organs.[14,15] However, the potency of anandamide for activating the transient potential vanilloid type-1 channel (TRPV1) suggests that TRPV1, a non-selective cation channel expressed widely in the periphery and in the CNS, could act as a functional anandamide receptor as well.[16,17]

The effects of CB on hormones that control reproduction are also well known. THC has been shown to inhibit GnRH release with the consequent inhibition of gonadotrophins secretion by the AH not only in laboratory animals, but also in humans.[18,19]

Moreover, it has been suggested that the main effect of CB on HPG axis is in the hypothalamic area since CB1 are appreciably expressed in this region.[20] Indeed, the suppressive effects on ovulation and gonadotrophin secretion induced by i.p. injection of THC were prevented by exogenous GnRH administration, suggesting that CB act mainly on the hypothalamus, impairing reproductive physiology.[21] Additionally, previous studies performed by our group showed that AEA (at physiological concentration) decreased NMDA-induced GnRH release from the MBH of male rats in vitro, and this effect was totally reversed by AM251, a specific CB1 antagonist/inverse agonist.[22,23] Furthermore, numerous studies show the mediating effects of CB1 and TRPV1 on GnRH modulators, such as oestradiol, GABA, ghrelin and glucagon-like peptide-1.[24-27]

Previous studies have shown that LPS administration stimulates CB1 mRNA and protein expression, as well as eCB synthesis in the MBH of rats,[28,29] which suggests a possible role of the ECS mediating the disrupting effects of systemic infections on the reproductive axis. However, few studies have investigated the effects of their interplay on reproductive physiology. Our group has previously shown that AM251 prevents TNFα inhibitory effect on forskolin-induced GnRH release from the MBH in vitro, suggesting a CB1 participation on inflammation-induced HPG axis disruption[29]; however, these results have not been confirmed in any in vivo experiment.

Given that cannabinoids inhibit reproductive activity, and also that immune challenge promotes ECS activation and inhibits reproductive physiology, the present study aimed to assess the hypothesis of an involvement of the hypothalamic CB1 in reproductive axis disruption taking place in vivo during an immune challenge in male rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Sprague-Dawley rats weighing 250-300 g were used in the present study. Animals had free access to food and water and were maintained under a 12 : 12 hour light/dark cycle at 20-25°C. The animals were kept under veterinary supervision for the duration of the experiment and were treated in accordance with the NIH Guide for the Care and use of Laboratory animals (NIH 8th edition). Protocols were approved by the Ethical Commission of the Faculty of Dentistry, University of Buenos Aires.

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 for AM251 [*N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] from Tocris (Ellisville, MO, USA); GnRH for iodination and standards from Peninsula Laboratories, Inc., Division of Bachem (San Carlos, CA, USA); LH for iodination and standards from Dr A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA);^{125}Iodine from PerkinElmer Life and Analytical Science (Boston, MA, USA); and [^{3}H] PGE2 from New England Nuclear Life Science Products (Boston, MA, USA). For each experiment, LPS was dissolved in a sterile saline solution to reach a final concentration of 5 mg mL^{-1} and was then injected i.p. in a volume of 1 μL mg^{-1} rat weight, which is the equivalent to 5 mg kg^{-1} rat weight. AM251 was dissolved in ethanol and then sterile saline was added to reach a final concentration of 100 ng μL^{-1}.

2.3 | Experimental design

To evaluate the participation of the hypothalamic CB1 on HPG axis disruption by immune challenge, 1 week prior to the day of the experiment, an indwelling cannula was implanted into the lateral cerebral ventricle by using a stereotaxic instrument when the rats were anaesthetised i.p. with a cocktail consisting of ketamine HCL (70 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The coordinates relative to the interaural line (anteroposterior -0.6 mm, mediolateral -2 mm, dorsoventral -3.2 mm) were taken

from the stereotaxic atlas of Paxinos and Watson.[30] The correct location of the cannula in the lateral ventricle was confirmed at the end of the experiment by injecting methylene blue (5 μL) via the cannula followed by brain slicing using a razor blade and direct examination of the dye in the hypothalamus. One week later, the experiments were performed in conscious, freely moving rats. Two types of experiments, using different animals, were performed: in one of them, animals received i.c.v. injections of 5 μL of the cannabinoid receptor antagonist/inverse agonist (AM251, 100 ng μL^{-1}) or vehicle (1% ethanol in saline). Fifteen minutes after i.c.v. administration, animals were given an i.p. injection of LPS dissolved in pyrogen-free isotonic saline at a dose of 5 mg kg^{-1}, or vehicle, and another i.c.v. administration was given 45 minutes post i.p. injection to ensure CB1 blockade during the experiment. Animals were killed by decapitation 90 minutes post i.p. injection. In the other experiment, animals were submitted to the same procedure, although they also received an additional i.c.v. administration of AM251 or vehicle 105 minutes post i.p. injection of LPS or vehicle, and were killed by decapitation at 180 minutes. All i.c.v. injections were made with Hamilton syringes during a period of 1 minute. Thereby, rats were divided into four groups: (i) control group receiving saline i.p. and vehicle i.c.v.; (ii) AM251 control group receiving saline i.p. and AM251 i.c.v.; (iii) LPS-treated group receiving LPS i.p. and vehicle i.c.v.; and (iv) LPS+AM251 group receiving LPS i.p. and AM251 i.c.v. Each experiment was repeated three times.

Following decapitation (90 or 180 minutes post LPS, depending on the experiment performed), trunk blood was collected into chilled heparinised tubes, centrifuged for 20 minutes 3625 g at 4°C, and the plasma was separated and stored frozen at −20°C for biochemical determination. Brains were immediately removed and the MBH was dissected by making frontal cuts 2 mm rostral to the optic chiasm, a posterior coronal cut at the posterior border of the mammillary bodies, parasagittal cuts along the hypothalamic fissures and a dorsal cut at 2.5 mm from the ventral surface.[31] Pituitary glands were dissected out and the AH was carefully separated from the neurohypophysis. MBH and AH were stored frozen at −80°C until further use. All the of the procedure described after decapitation was performed in both types of experiment.

Alternatively, only for the 180 minutes post LPS experiment, MBH were incubated individually with 1 mL of Krebs-Ringer bicarbonate buffer containing 10 mmol L^{-1} glucose, 0.1 mmol L^{-1} bacitracin and high K+ concentration (56 mmol L^{-1}) to deplete GnRH.[32] The incubation was made using a Dubnoff shaker (60 cycles min^{-1}) for 15 minutes at 37°C in an atmosphere of 95% O_2-5% CO_2. At the end of the incubation period, media was heated for 10 minutes at 100°C and centrifuged at 11 057 g for 10 minutes. Finally, supernatants were stored at −70°C for further determination of GnRH.

2.4 | TNFα determination

Plasma TNFα concentrations were determined using specific rat enzyme-linked immunosorbent assay (ELISA) using antibodies and standards obtained from BD Biosciences (San Diego, CA, USA). The assay was performed in accordance with the manufacturer's instructions as described previously.[28] The sensitivity and coefficient of variation of the assay was 31.25 pg mL^{-1} and 9.1%, respectively. TNFα levels in plasma are expressed as pg mL^{-1}.

2.5 | Testosterone determination

Plasma testosterone concentration was determined using a specific rat ELISA, with antibodies and standards obtained from DRG Instruments GmbH (Marburg, Germany). Briefly, microtitre wells coated with a monoclonal antibody available for a single site for testosterone binding were treated with 25 μL of Standard (0-16 ng mL^{-1}) or aliquots of samples. Then, 200 μL of testosterone conjugated to a horseradish peroxidase was added to each well for 60 minutes at room temperature. After enzyme conjugate disposal, plates were washed three times with 400 μL wash/dilution buffer (0.05% Tween-20 in phosphate-buffered saline, pH 7.0) and 200 μL of tetramethylbenzidine was added to each well for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 μL of 0.5 mol L^{-1} H_2SO_4, and absorbance was immediately determined at 450 nm on a microplate reader (Model 3550; Bio-Rad, Hercules, CA, USA). The sensitivity and coefficient of variation of the assay were 0.083 ng mL^{-1} and 3.59%, respectively. Plasma testosterone levels are expressed as ng mL^{-1}.

2.6 | Radioimmunoassay (RIA)

2.6.1 | Corticosterone

Plasma corticosterone was determined by RIA by incubating plasma samples with rabbit anti-corticosterone for 30 minutes at room temperature and then with [^{3}H] corticosterone as a tracer for 1 hour at 37°C. The reaction was stopped by the addition of cold dextran-coated charcoal suspension and, after incubation for 10 minutes at 4°C, the tubes were centrifuged at 2000 ***g*** for 15 minutes at 4°C. Scintillation cocktail was added to the supernatant and the amount of radioactivity was determined. The sensitivity and coefficient of variation of the assay was 30 pg $tube^{-1}$ and <15%, respectively. The values are expressed as ng mL^{-1}.

2.6.2 | LH and GnRH

Plasma LH was measured by RIA using rat LH antiserum (NIDDK-anti-rLH-S-II), antigen (NIDDK-rLH-I) and reference preparation (NIDDK-rLH-RP-3) purchased from Dr A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases). The inter-assay variations for these assays were 6.6% and the intra-assay variations were 3.6%. All samples were measured in duplicate and the results are expressed as ng LH mL^{-1} plasma. The sensitivity of the assay was 0.0244 ng mL^{-1}. GnRH released from MBH to the incubation media was measured by RIA utilising a highly specific GnRH antiserum kindly provided by Ayala Barnea (University of Texas Southwestern Medical Center, Dallas, TX, USA). The intra-assay coefficient of variation was 7.3% and the inter-assay coefficient of variation was 8.9%. The sensitivity of the assay was 0.2 pg 100 μL^{-1}. Data are expressed as pg GnRH released/MBH.

TABLE 1 Primer data table

Primer	Sequence	Annealing temperature (°C)	Accession number	Product size (bp)
Tnfα forward	GAT CGG TCC CAA CAA GGA GG	60	NM_012675.3	137
Tnfα reverse	CTT GGT GGT TTG CTA CGA CG			
IL1β forward	ACC CAA GCA CCT TCT TTT CCT T	60	M98820.1	106
IL1β reverse	TGC AGC TGT CTA ATG GGA ACA T			
GnRH forward	TGG TAT CCC TTT GGC TTT CAC A	60	NM_012767.2	188
GnRH reverse	CTC CTC CTT GCC CAT CTC TTG			
Kiss1 forward	CTG AAC CCA CAG GCC AAC AGT	60	NM_181692.1	253
Kiss1 reverse	AAG GAG TTC CAG TTG TAG GCT G			
Rrpv3 forward	CTG AAC CCA CAG GCC AAC AGT	60	NM_023952.1	93
Rrpv3 reverse	AAG GAG TTC CAG TTG TAG GCT G			
Trpv1 forward	GTG AGA CCC CTA ACC GTC AT	60	NM_031982.1	200
Trpv1 reverse	CCC AAC GGT GTT TTT CAG CTT			
Cb1 forward	GGA GAA CAT CCA GTG TGG GG	60	NM_012784.4	184
Cb1 reverse	CAT TGG GGC TGT CTT TAC GG			

2.6.3 | PGE2

To quantify PGE2 content by RIA, each MBH was weighed (approximately 100 mg), then homogenised in 1.5 mL of absolute ethanol and, following centrifugation, the supernatant was collected and dried in a Speedvac (Thermo Fisher, Waltham, MA, USA) at room temperature. The residues were then resuspended in buffer. A specific antiserum from Sigma-Aldrich (St Louis, MO, USA) was used as described by Faletti et al.[33] The sensitivity of the assay was 12.5 pg tube^{-1}. The cross-reactivity of PGE2 and PGE1 was 100%, whereas cross-reactivity with other prostaglandins was 0.1%. The intra- and inter-assay coefficients of variation for PGE2 were 8.2% and 12%, respectively. Results are expressed as pg mg tissue^{-1}.

2.7 | Real-time polymerase chain reaction (PCR)

Following decapitation, MBH were collected as described above and each AH was removed and separated from each neurohypophysis. The tissues were then harvested in RNAzol Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and frozen at −80°C until used. Total RNA from cells was isolated in accordance with the manufacturer's recommendations (Molecular Research Center Inc., Cincinnati, OH, USA). Following RNA extraction, genomic DNA was digested with DNAse for 10 minutes at room temperature. Next, DNAsa was inactivated and RNA was quantified. cDNA was synthesised from total RNA (3 μg) using Moloney murine leukemia virus reverse transcriptase, random primers and ribonuclease inhibitor. The cDNA amounts present in each sample were determined by a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the KAPA SYBR® FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems, Wilmington, MA, USA) in accordance with the manufacturer's instructions. The specific primers to perform PCR amplifications were designed using PRIMER3 (http://primer3.sourceforge.net/). Polymerase chain reaction product detection was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA. Primer sequences, annealing temperature, accession number and product size are shown in Table 1. Each real time-PCR quantification experiment was performed in duplicate. All the reactions were subjected to a heat dissociation protocol following the final cycle of PCR to confirm that the SYBR Green dye detected only one PCR product. Quantification of the target gene expression was performed using the comparative cycle threshold (Ct) method.[34] An average Ct value was calculated from the duplicate reactions and normalised to the expression of β-actin and the 2(−ΔΔCt) value was calculated. No changes in the reference gene were found by treatments (data not shown).

2.8 | Statistical analysis

The results were expressed as the mean±SEM. All data were processed using STATISTICA (StatSoft, Inc., Tulsa, OK, USA). Normality and homogeneity of variance for the dataset were tested using the Shapiro-Wilk test and Levene's test, respectively. The significance of the differences between means was determined by two-way ANOVA followed by Tukey's test. $P<.05$ was considered statistically significant. All measurements were performed at least in duplicate. Images represent the results of individual experiments.

3 | RESULTS

3.1 | Participation of central CB1 on plasma corticosterone and TNFα levels altered by the immune challenge

Plasma corticosterone was measured to determine the stressful effect induced by LPS and its possible modulation by hypothalamic CB1. Two-way ANOVA analysis 90 minutes post-LPS injection revealed a nonsignificant LPS/AM251 interaction ($F_{1,16}=3.98$, $P=.063$). A main

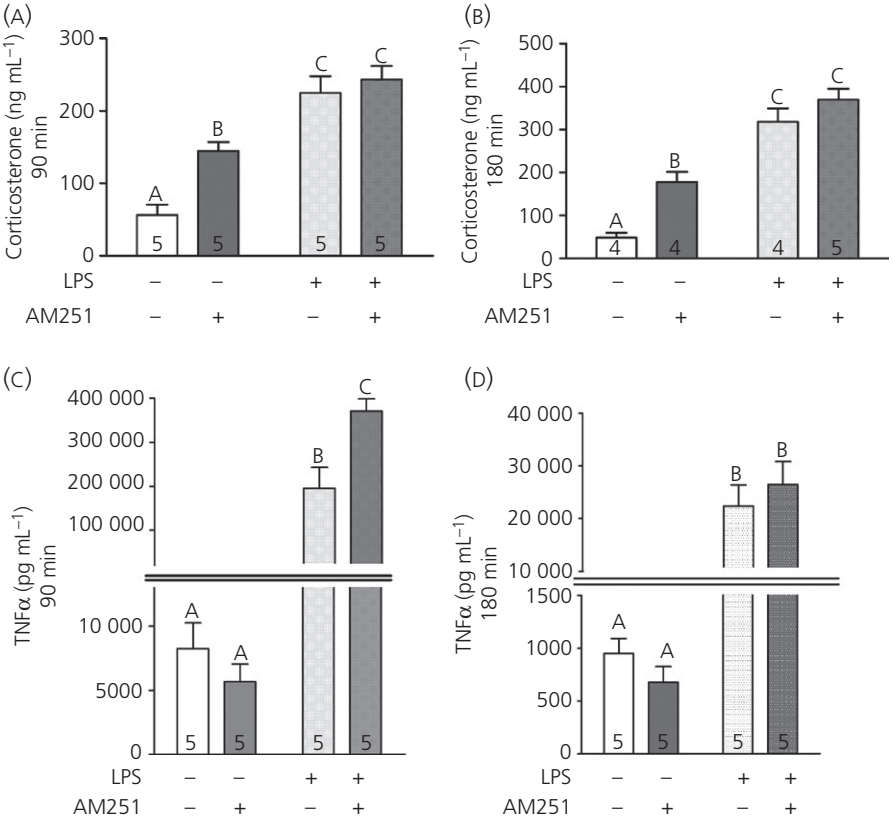

FIGURE 1 Effects of AM251 on plasma corticosterone and tumour necrosis factor (TNF)α concentration in rats subjected to immune challenge. (A) Plasma corticosterone levels at 90 (A) and 180 (B) minutes post i.p. injection, and TNFα levels at 90 (C) and 180 (D) minutes post i.p. injection of lipopolysaccharide (LPS) (5 mg kg^{-1}, i.p.) or saline, and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v. Number inside the bar is per group. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

effect of LPS ($F_{1,16}$=57.93, $P<.0001$) and AM251 ($F_{1,16}$=9.30, $P=.0077$) was observed. At 180 minutes post-LPS injection, statistical analysis also did not reveal a significant LPS/AM251 interaction ($F_{1,13}$=2.47, $P=.1398$), and a main effect of LPS ($F_{1,13}$=87.53, $P<.0001$) and AM251 ($F_{1,13}$=13.42, $P=.0029$) was observed. Post-hoc evaluation at 90 and 180 minutes revealed that LPS and AM251 per se significantly increased plasma corticosterone levels (Figure 1A,B). These results confirm the stressful condition induced by LPS and reveal an eCB control of basal corticosterone production via CB1, which is not observed in the presence of LPS. However, a possible stimulatory inverse agonist effect of AM251 on corticosterone levels should not be discounted.

TNFα is one of the main cytokines responsible for the disruptive effect on GnRH and gonadotrophins released during an immune challenge. Therefore, the plasma TNFα concentration was measured in all experimental groups at 90 and 180 minutes post-LPS administration. At 90 minutes post-LPS injection, statistical analysis showed a significant LPS/AM251 interaction ($F_{1,16}$=10.52, $P=.0051$). Post-hoc analysis showed that CB1 blockade enhanced the TNFα plasma concentration induced by LPS (Figure 1C). Conversely, at 180 minutes post-LPS injection, the statistical analysis showed a nonsignificant LPS/AM251 interaction ($F_{1,16}$=0.54, $P=.473$) and a significant main effect of LPS ($F_{1,16}$=62.86, $P<.0001$), although a nonsignificant effect of AM251 ($F_{1,16}$=13.42, $P=.0029$) was observed (Figure 1D). These results confirm the participation of central CB1 during immune challenge response as a result of the systemic administration of LPS.

3.2 | Participation of central CB1 on cytokine expression during immune challenge

Next, we evaluated *Tnf*α and *Il1*β mRNA expression in MBH (Figure 2) and AH (Figure 3) at 90 and 180 minutes after LPS administration to determine whether immune challenge alters inflammatory parameters in the main hormonal centres controlling reproduction. *Tnf*α mRNA analysis showed a significant interaction of factors at 90 minutes ($F_{1,18}$=10.74, $P=.0042$). A post-hoc test showed a significant induction of *Tnf*α mRNA levels only with LPS plus AM251 treatment (Figure 2A). Similarly, a significant interaction of factors was observed for *Tnf*α mRNA at 180 minutes ($F_{1,20}$=16.08, $P=.0007$). A post-hoc test showed significant induction of *Tnf*α mRNA for AM251, LPS and a combination of both (Figure 2B). *Il1*β analysis also showed a significant interaction of factors at 90 minutes ($F_{1,16}$=34.68, $P<.0001$). A post-hoc test showed a significant induction in *Il1*β mRNA levels only for the combination of LPS and AM251 treatment (Figure 2C). At 180 minutes, a significant interaction of factors was observed ($F_{1,16}$=64.98, $P<.0001$). A post-hoc test showed that LPS significantly augmented *Il1*β mRNA levels, which were enhanced in the presence of AM251 (Figure 2D).

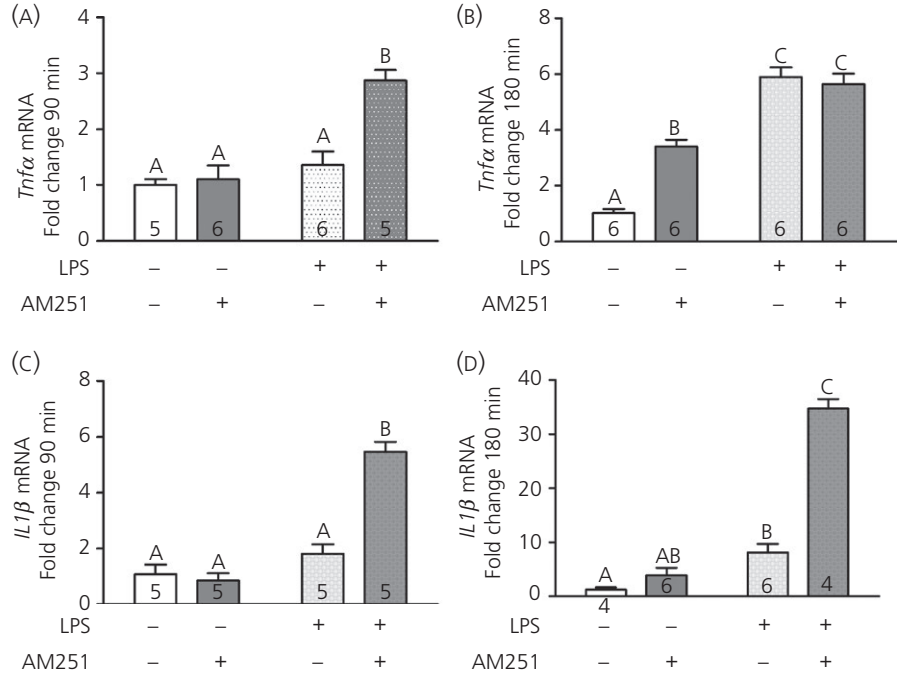

FIGURE 2 AM251 exacerbates medial basal hypothalamus (MBH) inflammatory markers in rats subjected to immune challenge. TNFα and *Il1β* mRNA expression, at 90 minutes (A and C, respectively) and 180 minutes (B and D, respectively) post i.p. administration of lipopolysaccharide (LPS) (5 mg/kg, i.p.) or saline, and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v. Number inside the bar is per group. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

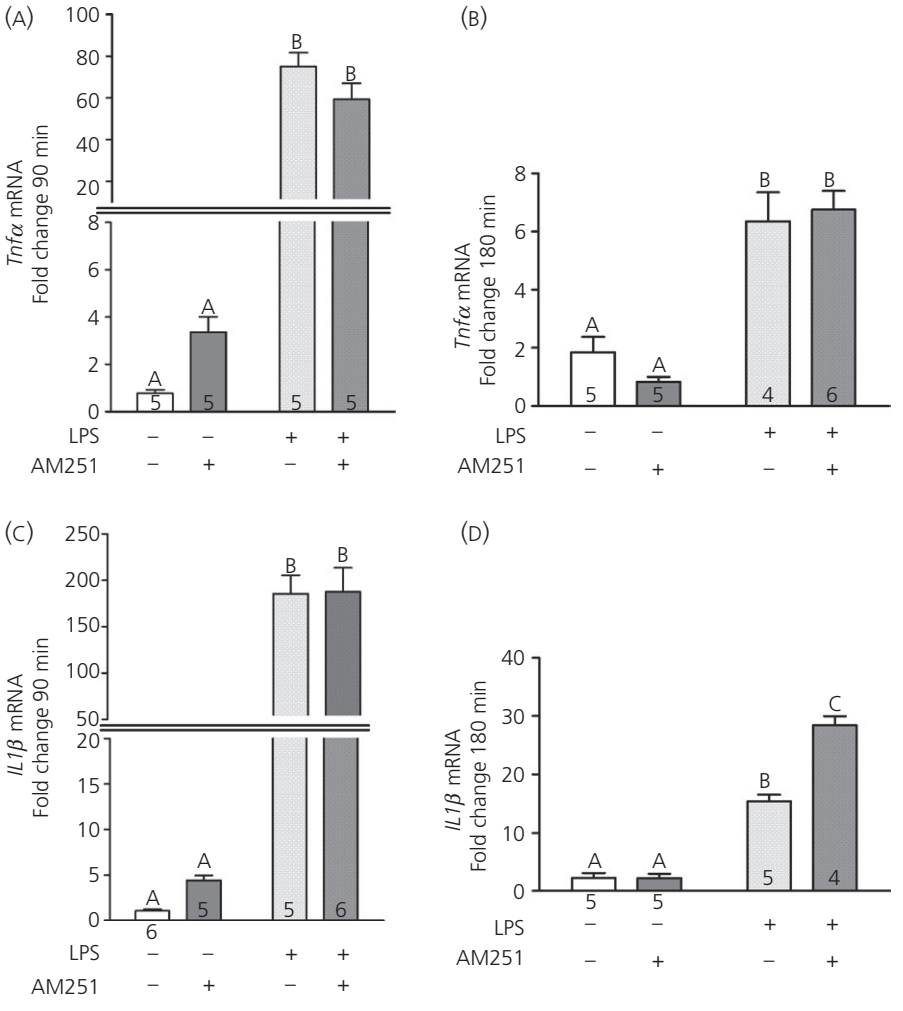

FIGURE 3 Effect of AM251 on adenohypophyseal inflammatory markers in rats subjected to immune challenge. *Tnfα* and *Il1β* mRNA expression, at 90 minutes (A and C, respectively) and 180 minutes (B and D, respectively) post i.p. administration of LPS (5 mg kg^{-1}, i.p.) or saline, and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v. Number inside the bar is per group. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

In the adenohypophysis, *Tnfα* mRNA expression at 90 minutes (Figure 3A) showed no interaction of the factors LPS/AM251 ($F_{1,16}$=3.24, P=.0906) and a nonsignificant effect of the factor AM251 ($F_{1,16}$=1.67, P=.2147), although a significant main effect of LPS was found ($F_{1,16}$=110.5, P<.0001). The same effects were found at 180 minutes (Figure 3B), with no interaction of factors ($F_{1,16}$=1.28, P=.2748), no main effect of AM251 ($F_{1,16}$=0.25, P=.6230) and a significant main effect of LPS ($F_{1,16}$=69.89, P<.0001). A post-hoc evaluation at 90 and 180 minutes showed that LPS induced a significant increase of TNFα in the presence or absence of AM251. Moreover, *Il1β* mRNA expression at 90 minutes (Figure 3C) showed no interaction of the factors LPS/AM251 ($F_{1,16}$=0.001, P=.9670), a nonsignificant main effect of AM251 ($F_{1,16}$=0.02, P=.8770) and a significant main effect of LPS ($F_{1,18}$=119.16, P<.0001). A post-hoc test showed a significant increase of the cytokine with LPS and LPS plus AM251. However, at 180 minutes (Figure 3D), a significant interaction of LPS and AM251 was found ($F_{1,15}$=40.85, P<.0001). Post-hoc analysis showed that AM251 increased the effect of LPS significantly.

Taken together, these results suggest that hypothalamic cytokine production in response to immune challenge is delayed compared to the adenohypophyseal response and that CB1 blockade enhances the expression of pro-inflammatory mediators in both tissues in the presence of LPS.

3.3 | Participation of hypothalamic CB1 on reproductive hormones altered by immune challenge

We next studied the possible participation of CB1 receptors on the pathway that leads to the inhibition of reproductive axis triggered by immune challenge. At 90 minutes, no significant changes were observed in *Gnrh*, *Kiss1* and *Rfrp3* mRNA expression in MBH from rats of each group (Figure S1). However, statistical analysis at 180 minutes showed a significant interaction of factors LPS and AM251 ($F_{1,19}$=6.81, P=.0172) for *Kiss1* mRNA expression. Post-hoc analysis showed that the significant decrease found in *Kiss1* mRNA as a result of LPS was prevented by AM251 (Figure 4A), suggesting that kisspeptinergic neurotransmission is inhibited during an immune challenge and that the effect is mediated by CB1. *Rfrp3* mRNA expression analysis at 180 minutes showed a significant interaction of factors ($F_{1,15}$=17.43, P=.0008). Post-hoc analysis showed a significant increase of *Rfrp3* mRNA expression exclusively with LPS, although a tendency to an increase with AM251 alone was observed compared to control. The administration of AM251 prevented the effect of LPS (Figure 4B), suggesting that the RFRP inhibitory pathway controlling GnRH release is activated during an immune challenge and involves CB1 receptors activation. *Gnrh* mRNA expression analysis showed a nonsignificant interaction of factors LPS/AM251 ($F_{1,18}$=3.13, P=.0938) and no main effect of AM251 ($F_{1,18}$=0.82, P=.3785), although there was a significant inhibitory effect of LPS ($F_{1,18}$=10.38, P=.0047). Post-hoc analysis showed that the decrease found in *Gnrh* mRNA was partially prevented by AM251 (Figure 4C). The same pattern was found for GnRH peptide release: nonsignificant interaction of factors LPS/AM251 ($F_{1,16}$=4.20, P=.0572), no significant main effect of AM251 ($F_{1,16}$=1.66, P=.2160) and a significant main effect of LPS ($F_{1,16}$=13.47, P=.0021). Post-hoc analysis showed that the significant decrease found in GnRH was partially prevented in the presence of AM251 (Figure 4D). Finally, because PGE2, mainly from astrocytes, is known to trigger GnRH release from hypothalamic neurones,[35-37] PGE2 content in MBH was measured after 180 minutes of immune challenge (Figure 4E). Statistical analysis showed a significant interaction of factors LPS/AM251 ($F_{1,16}$=17.23, P=.0008). Post-hoc analysis showed a significant decrease of PGE2 exclusively in the presence of LPS without AM251. This inhibitory effect was prevented by AM251, suggesting that CB1 participates in PGE2 release during an immune challenge.

Subsequently, to assess the scope of hypothalamic CB1 participation in the regulation of the hormones associated with reproduction plasma LH and testosterone levels were measured. Two-way ANOVA showed a nonsignificant interaction of factors ($F_{1,16}$=1.52, P=.235), although there was a main effect of LPS ($F_{1,16}$=12.81, P=.0025), and AM251 ($F_{1,16}$=11.98, P=.0032). Post-hoc analysis showed that LH plasma levels significantly decreased 180 minutes after LPS, although an absence of inhibition was observed when, additionally, AM251 was administered (Figure 5A). Moreover, at the end of the reproductive axis, plasmatic free testosterone levels were measured (Figure 5B). Statistical analysis showed a nonsignificant interaction of factors LPS/AM251 ($F_{1,15}$=2.15, P=.162). However, a main effect of LPS ($F_{1,15}$=10.86, P=.0049) with a nonsignificant effect of AM251 ($F_{1,15}$=0.91, P=.356) was observed. Post-hoc analysis showed that LPS was sufficient to significantly decrease testosterone levels. No changes were found in the presence of AM251. Altogether, our results show for the first time the participation of hypothalamic CB1 receptors activation in the mechanisms underlying central reproductive inhibition induced by an immune challenge in an in vivo model.

3.4 | Interaction between CB1 and the vanilloid system in the hypothalamus

To further understand the underlying mechanisms of the effects aforementioned, the expression of hypothalamic *Cb1* and *Trpv1* mRNA were determined at 180 minutes post LPS. Two-way ANOVA analysis for *Trpv1* mRNA expression (Figure 6A) showed no interaction of factors LPS/AM251 ($F_{1,19}$=1.83, P=.192). A significant main effect was observed for AM251 ($F_{1,19}$=8.51, P=.0088) but not for LPS ($F_{1,19}$=2.50, P=.1301). Post-hoc analysis showed a significant increase of *Trpv1* mRNA expression in the presence of LPS with AM251 exclusively, suggesting that the effects observed at the reproductive level may also be explained by the involvement of the vanilloid system when the CB1 receptors are blocked. Regarding *Cb1*mRNA expression, statistical analysis showed no significant interaction of factors LPS/AM251 ($F_{1,16}$=0.02, P=.9005) and no main effect of LPS ($F_{1,16}$=2.35, P=.1445), although there was a significant main effect for AM251 ($F_{1,16}$=215.19, P<.0001). Post-hoc analysis showed that the presence of AM251 was sufficient to induce a significant increase in *Cb1* mRNA expression (Figure 6B).

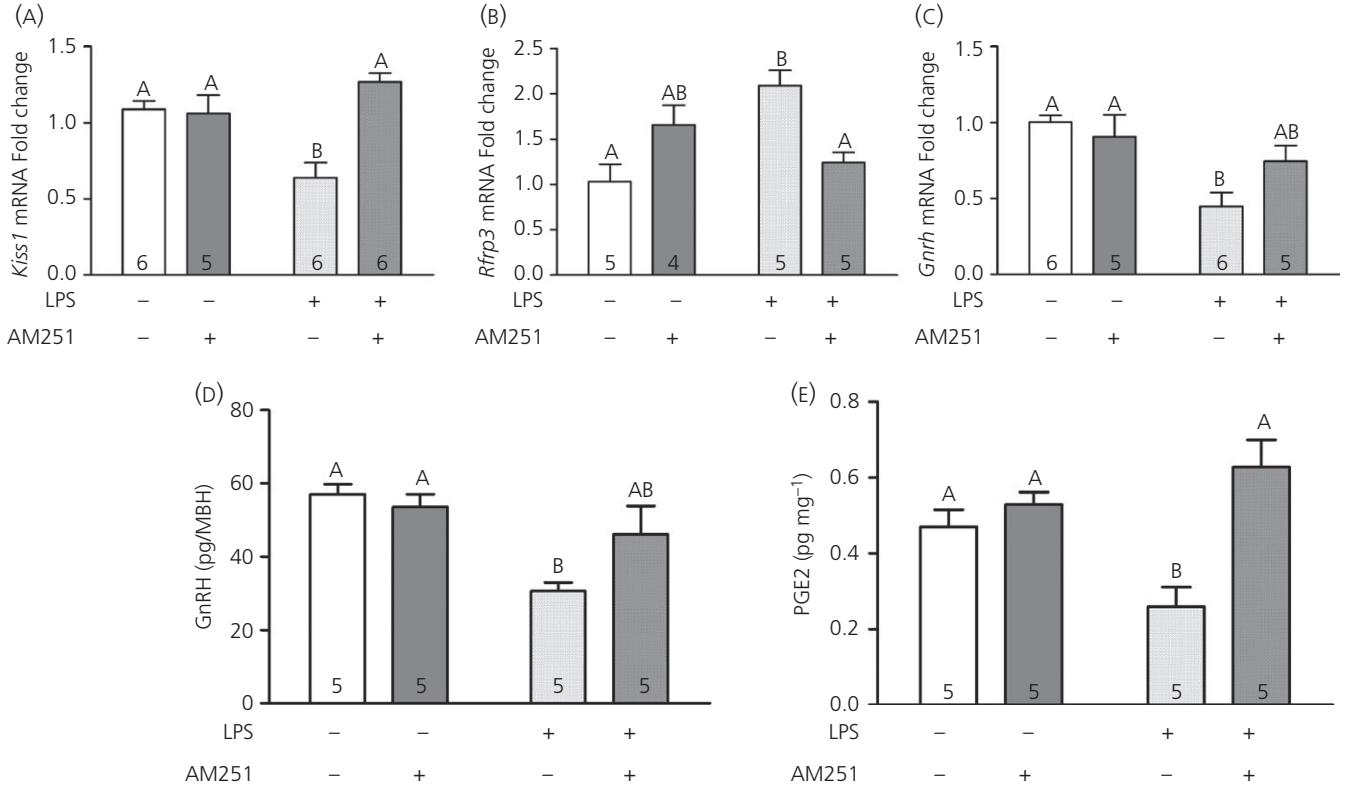

FIGURE 4 AM251 prevented the disruption in reproductive sexual parameters in rats subjected to immune challenge. Medial basal hypothalamus (MBH) (A) *Kiss1*, (B) *Rfrp3* and (C) *Gnrh* mRNA expression and (D) gonadotrophin-releasing hormone (GnRH) and (E) PGE2 content in rats subjected to lipopolysaccharide (LPS) (5 mg kg^{-1}, i.p.) or saline and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v., 180 minutes post i.p. administration. Number inside the bar is per group. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

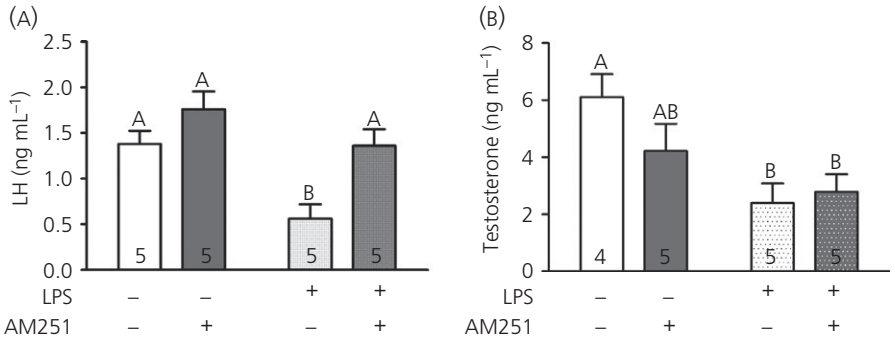

FIGURE 5 AM251 effects on plasma sexual markers from rats subjected to immune challenge. (A) Luteinising hormone (LH) levels in rats subjected to lipopolysaccharide (LPS) (5 mg/kg, i.p.) or saline, and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v., 180 minutes post i.p. administration. Number inside the bar is per group. (B) Plasma testosterone levels 90 minutes post i.p. administration. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

4 | DISCUSSION

The HPG axis not only participates in the regulation of development, reproduction and ageing, but also performs a complex cross-talk with the immune response.[38] In the present study, we found evidence that hypothalamic ECS might participate in the regulatory mechanisms induced during the activation of the immune system by maintaining inflammatory parameters to fight against infections without damaging host tissue but in detriment of the endocrine sexual tone. This mechanism appears to be crucial for survival, providing energy to the immune system and allowing the recovery from infection.

Conflicting reports on the effects of eCB during inflammation abound in the literature, with some studies arguing that eCB display an anti-inflammatory response in the CNS,[39-41] whereas others suggest that the eCB rather enhance the pro-inflammatory effects of LPS.[28,42] Roche et al.[43] showed the complexity of eCB mechanisms underlying the modulation of inflammatory parameters, finding that both CB1 agonist and antagonist/inverse agonist separately were

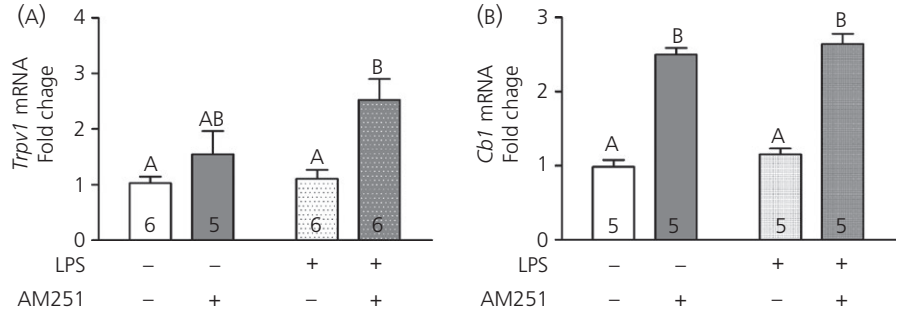

FIGURE 6 AM251 increases *Trpv1* and *Cb1* mRNA expression. Rats were treated with lipopolysaccharide (LPS) (5 mg kg^{-1}, i.p.) or saline, and to AM251 (5 μL, 100 ng μL^{-1}) or its vehicle via i.c.v., and 180 minutes post i.p. administration (A) *Trpv1* and (B) *Cb1* mRNA expression were determined in the medial basal hypothalamus (MBH). Number inside the bar is per group. Different letters indicate $P<.05$. The analysis was made by means of two-way ANOVA followed by Tukey's test

able to suppress central and peripheral cytokine responses during an immune challenge. The results from our experimental conditions show an anti-inflammatory role of central CB1 activation on LPS-induced inflammation. Although CB2 is highly expressed in glial cells and it also participates in the inflammatory response, the present study focused on the CB1 receptor, which participates in neuronal signalling. For this reason, we used the antagonist/inverse agonist AM251,[44] which exhibits approximately 306-fold selectivity for CB1 over CB2, with a K_i value of 0.23-7.49 nmol L^{-1} for CB1.[45] Inflammation could be modulated by CB1 receptors located in several central areas, whereas GnRHergic neuronal control is mainly restricted to the hypothalamus. Therefore, i.c.v. administration ensures that AM251 access from the lateral ventricles to the hypothalamic nuclei, where the neurones controlling the reproductive axis are located. The concentration of AM251 used for i.c.v. administration was defined based on previous studies[23,42] that had guaranteed CB1 inhibition.

Studies regarding GnRH modulation by ECS have shown different results when comparing male and female rats, and have been attributed to oestrogens. For example, it has been reported that the hormones controlling reproduction in females are stimulated by THC, whereas the effect is inhibitory in males.[46] Our previous report showed that AEA inhibited GnRH release in male rats but not in ovariectomised (OVX) rats.[23] Because of these discrepancies observed according to the sex of the animal, our present experimental design was performed in male rats only. A concentration of LPS of 5 mg kg^{-1} (for i.p. administration) was chosen because this dose is able to interfere with the reproductive physiology without causing an irreversible state occurring in septic shock, as demonstrated by previous studies reporting 80% of survival in rats, 7 days after LPS administration at 5 mg kg^{-1}.[46,47] The activation of the immune system as a result of LPS challenge alters the activity of the hypothalamus in several ways. First, the immune system response modifies the activity of the hypothalamic-pituitary-adrenal axis, promoting the release of corticosterone, which in turn modulates the TNFα response.[48] Second, in response to LPS the hypothalamus plays multiple roles, by modulating inflammatory pain,[49] thermoregulation,[50] hypotension,[51] reproduction[52] and the inflammatory response.[53] Previous reports have shown that LPS administration induces eCB synthesis.[54] Given that eCS participate in the immune response, it is not surprising to find that hypothalamic CB1 modulate the systemic inflammatory response. Our results suggest that the hypothalamic activation of the CB1 pathway might be needed to prevent an excessive immune response. It was reported previously that TRPV1 activation is associated with a pro-inflammatory status[55] and that the activation of CB1 suppresses TRPV1 expression in a pro-inflammatory context.[56] Taken together, the effects observed using AM251 in the present study may lead to two main hypotheses underlying reproduction and immune modulation. Blockade/inverse agonism of CB1 may not only evoke changes by a deficiency of its own response, but also could lead to an overexpression of other receptors of the ECS to compensate the lack of signal factors downstream of the CB1 pathway. Our results show that, in the absence of CB1 signalling, *Trpv1* mRNA expression is increased, which may indicate a stronger TRPV1 pathway activation, leading to a pro-inflammatory state.

It has been suggested that a basal endogenous tone of eCB in the hypothalamus controls corticosterone levels under physiological conditions.[57] Previous studies have shown retrograde inhibitory effects of the corticosterone-induced release of AEA and 2-AG[58] from hypothalamic corticotrophin-releasing hormone neurones to presynaptic neurones.[59,60] In the present study, AM251 altered this process, allowing a sustained hormone increase. However, considering that AM251 may also act as an inverse agonist, a direct effect on corticosterone release cannot be ruled out. LPS is a stressor factor per se, and this might explain why the effect of AM251 was overshadowed in the presence of this toxin. A similar stimulatory effect of AM251 was shown for *TNFα* mRNA expression on MBH at 180 minutes post LPS administration that could be explained by the same two hypotheses: an endogenous tone of eCB or an inverse agonist effect. Although corticosterone may participate in the mechanisms leading to an immune response in the presence of LPS, which indirectly might alter the HPG axis, its influence does not appear to be altered by a hypothalamic CB1 blockade during immune challenge because, in the presence of LPS, AM251 did not change corticosterone levels but changed immunological and sexual parameters in the MBH. These results are in accordance with a study by Rorato et al.,[61] who found no changes in LPS-increased plasma corticosterone when CB1 receptors were blocked.

Pro-inflammatory cytokines in CNS are either produced locally[62] or arrive from peripheral sources crossing the blood-brain barrier

(where present), or via fenestrated capillaries,[63] Our results show that LPS triggers a systemic inflammation at 90 minutes. However, this condition does not appear to be present in the MBH, which reacts to LPS at 180 minutes, as the *Tnf*α and *Il1*β mRNA expression levels indicate. These results are in agreement with Kakizaki et al.,[64] who found a delay in the hypothalamic cytokine response to i.p. LPS. This delay may probably occur because the CNS is a protected and isolated structure from the periphery.

However, the systemic effects of the central CB1 blockade via i.c.v. administration appear before hypothalamic inflammation because AM251 enhanced TNFα plasma levels 90 minutes post-LPS. The time of response of the hypothalamus to immune challenge appears to be crucial to understanding the mechanisms underlying the inhibition of GnRH release because LPS have a disruptive effect in this organ only at 180 minutes when mRNA cytokine expression is increased. This may indicate that hypothalamic synthesis of cytokines is needed to evoke a disruptive effect in the release of reproductive hormones. Our group have previously reported that LPS and TNFα increased anandamide synthase activity in both in vivo and in vitro models, and that treatment with AM251 prevented the inhibitory effect induced by TNFα on GnRH release from MBH, suggesting that cytokines can disrupt the GnRH release by promoting eCB synthesis.[29] The results reported in the present study show that LPS-induced inflammation inhibits the release of GnRH at least partially via hypothalamic CB1 activation in vivo. Furthermore, multiple targets modulating GnRH release are sensitive to AM251, such as RFRP and kisspeptinergic neurones. Previous studies have reported decreased expression of hypothalamic *Kiss1* mRNA levels and increased hypothalamic *Rfrp3* mRNA levels in rats treated with LPS, with consequently reduced levels of GnRH and LH. However, these effects were prevented with subsequent i.c.v. administration of kisspeptin.[31,65] Furthermore, in acute immobilisation stress experiments performed in rats, AM251 prevented the reduced expression of *kiss1* mRNA, suggesting that kisspeptinergic neurones might be influenced by CB1 activation.[66] For the first time, the present study shows the response of these neurones to the modulation of CB1 under LPS-induced immune challenge. Kisspeptinergic neurones responded by decreasing the mRNA expression of this neurotransmitter at 180 minutes post-LPS, and this effect was blocked by i.c.v. administration of AM251. Concerning *Rfrp3* mRNA levels, although a tendency to increase of *Rfrp3* expression was observed in rats treated with AM251 solely, this treatment did not affect GnRH release, probably because it was not accompanied by changes in *Kiss1*. However, LPS increased *Rfrp3* mRNA expression at 180 minutes post-LPS with AM251 preventing this change, suggesting that eCB modulate the activity of different neurones involved in the mechanisms underlying GnRH release. We hypothesise that CB1 receptors may be located in kisspeptinergic and RFRP neurones, leading to a direct influence of eCB. In this scenario, receptors should be found not only at the presynaptic level, but also in somas because the presence of AM251 modulated the levels of *Rfrp3* and *Kiss1* mRNA. However, an indirect influence mediated by another neurone carrying CB1 should not be discarded. Additionally, the LH plasma level responded similarly to hypothalamic stimulatory neuropeptides because they were reduced by LPS after 180 minutes, an effect that was prevented by AM251. Based on the results reported in the present study (Figure 7), we have summarised the hypothetical mechanisms of action triggered by CB1 in the presence of LPS. Because GnRH and LH are modulated by multiple factors, further studies are needed to fully characterise the mechanisms mediating hypothalamic-adenohypophyseal response in the context of systemic inflammation.

The disruptive effect of immune challenge on testosterone release started to be observed before the GnRH inhibition occurs. This indicates a direct effect of LPS on gonads and bypassing hypothalamic signals, similar to that reported previously.[67] In this context, it would be difficult to find a proper modulation via hypothalamic CB1 on testosterone release, as seen in rats treated i.c.v. with AM251. A long-term experiment should be designed to assess possible modulatory effects mediated by hypothalamic CB1 to control testosterone release in rats exposed longer times to an immune challenge, probably 72 hours post LPS when the effects of the immune challenge relieves,[67] allowing to observe the hormonal modulation of testosterone in the presence of AM251 acting on hypothalamic CB1.

Inhibitory effects of IL1β on GnRH and LH release from the hypothalamus and AH, respectively, have been demonstrated previously.[52] Some studies demonstrated a direct inhibition of this cytokine on GnRH neurones,[68] whereas others suggest an indirect effect mainly via PGE2 inhibition[69] because it has been shown that PGE2 stimulates GnRH secretion.[35-37] Accordingly, our findings show decreased levels of hypothalamic PGE2 in response to LPS, which were reversed on blocking CB1. A possible explanation for this may involve the PGE2 biosynthetic pathway because metabolic products of AEA degradation can be substrates of PGE2 synthesis. Conversely, it has been proposed that CB1 participates in the feedback loop existing between GnRHergic neurones, GABAergic neurones and astrocytes in female OVX mice. This suggests that GnRHergic neurones could inhibit their own response directly via the release of eCB to presynaptic GABAergic neurones, therefore preventing GABA release to GnRHergic neurones,[25] and/or indirectly by stimulating astrocyte production of prostaglandins, which in turn would induce CB1 signalling in presynaptic GABAergic neurones.[70] Further studies are warranted to fully understand the mechanisms mediating PGE2 response in the pathophysiological condition of our model.

In summary, the results reported in the present study indicate that hypothalamic CB1 participates in the mechanisms induced by immune challenge in male rats, favouring the inflammatory response in detriment of sexual hormone release.

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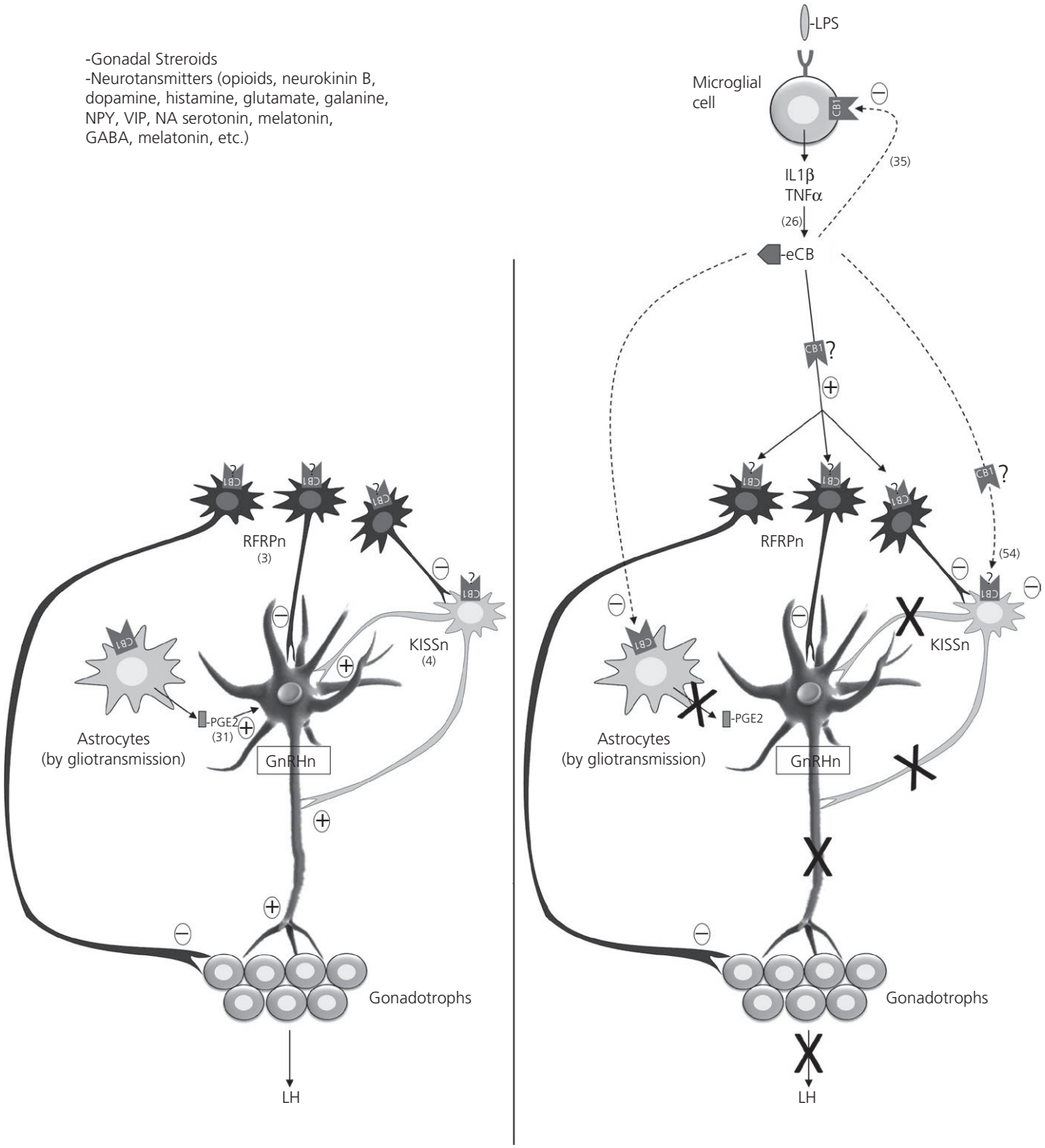

FIGURE 7 Hypothetic hypothalamic CB1 participation during immune challenge. The schema contains the hypothalamic mediators of gonadotrophin-releasing hormone (GnRH) secretion analysed in the present study. However, multiple factors not included in the study participate in GnRH secretion, such as gonadal steroids and other neurotransmitters (top left). Kisspeptin and PGE2 stimulate GnRH production and secretion. Nevertheless, RFamide-related peptides (RFRP) neurones negatively modulate GnRH and kisspeptin production, as well as luteinising hormone (LH) production by gonadotrophs (left). In the presence of lipopolysaccharides (LPS) (right), microglial cells and astrocytes produce tumour necrosis factor (TNF)α and interleukin (IL)1β, which in turn induce endocannabinoids (eCB) production. eCB not only modulate the production of cytokines by the microglia, diminishing cytokine production, but also PGE2 production and *Kiss1* synthesis, increasing *Rfrp3* in this condition. The effects of eCB on these neurones can be direct if they express CB1, or indirect if another interneurone expressing CB1 innervated them. Further studies are needed to confirm this hypothesis. Numbers indicate the reference for each mechanism described previously. NPY, neuropepetide Y; VIP, vasoactive intestinal peptide; NA, noradrenaline

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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