Regulatory role of the Cannabinoid-2 receptor in stress-induced neuroinflammation in mice

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RUNNING TITLE: Anti-inflammatory effects of cannabinoid-2 receptor
SUMMARY

Background and purpose.

Stress-exposure produces excitotoxicity and neuroinflammation, contributing to the cellular damage observed in stress-related neuropathologies. The endocannabinoid system is present in stress-responsive neural circuits and it is emerging as a homeostatic system. The aim of this study was to elucidate the possible regulatory role of cannabinoid-2 receptor in stress-induced excitotoxicity and neuroinflammation.

Experimental approach

Different genetic and pharmacological approaches were used: 1) Wild type (WT), transgenic over-expressing CB2 receptor (CB2xP) and CB2 receptor knockout (CB2-KO) mice were exposed to immobilization/acoustic stress (2h/day for 4 days), and 2) the CB2 receptor agonist JWH-133 was administered daily (2 mg kg$^{-1}$, i.p.) to WT and CB2 receptor-KO animals.

Key results

Stress-induced HPA axis activation was not modified by CB2 receptor manipulations. JWH-133 treatment or overexpression of CB2 resulted in an increase of control levels of glutamate uptake, which is then reduced by stress exposure back to control levels. JWH-133 prevented the stress-induced increase in the cytokines TNF-α and MCP-1, the nuclear factor kappa B, the enzymes inducible nitric oxide synthase 2 and cyclooxygenase-2 and the cellular oxidative/nitrosative damage (lipid peroxidation) in brain frontal cortex. CB2xP mice displayed anti-inflammatory/neuroprotective actions similar to those observed in JWH-133 pre-treated animals. Conversely, CB2-KO mice experiments indicated that the lack of CB2 receptor exacerbated stress-induced...
neuroinflammatory responses and validated the CB2 receptor-dependent effects of JWH-133.

Conclusions and Implications

These results suggest that pharmacological manipulation of CB2 receptor is a potential therapeutic strategy for the treatment of stress-related pathologies with a neuroinflammatory component, such as depression.

Keywords

CB2 receptor, stress, excitotoxicity, neuroinflammation, brain frontal cortex, JWH-133, CB2xP mice.

INTRODUCTION

The relationship between stress and the immune system has been widely studied during the last decades (Licinio and Wong, 1999; Sorrells et al., 2009), but the precise mechanisms implicated are still a matter of debate, probably due to the complex interactions existing between the periphery and the central nervous system (CNS) (Capuron and Miller, 2011).

Chronic stress exposure and stress-related diseases, such as depression or chronic fatigue syndrome, have been classically associated with an inhibition of the adaptative immunity, with important negative effects on health (Herbert and Cohen, 1993). However, in the last years it has been demonstrated that after stress exposure or during certain episodes of depression an innate inflammatory/immune response is strongly activated (García-Bueno et al., 2008; Farooq et al., 2012).

The inflammatory response allows the organism to cope with diverse threatening challenges, but under pathological and long lasting conditions the maintenance of this
response could become deleterious. Previous studies indicate that long-lasting stress (physical, psychological, or mixed) affects synaptic plasticity, dendritic morphology, and neurogenesis in animals (Kim and Yoon 1998) and induces both clinical and anatomical features of neurotoxic damage in humans (Bremner et al., 1995).

In the last years, much effort has been made to elucidate the precise molecular/cellular events responsible for the brain damage produced by stress exposure. Previous studies have reported stress-induced excitotoxicity due to the massive release of the excitatory amino acid, glutamate, in some brain areas (e.g., frontal cortex –FC-) (Moghaddam, 1993). This over-accumulation induces the release of pro-inflammatory cytokines such as tumour necrosis factor-\textit{alpha} (TNF-\textit{a}) (Madrigal et al., 2002). Stress also activates the inflammatory nuclear transcription factor-\textit{kappa} B (NF-\textit{k}B) pathway in a TNF-\textit{a} dependent mechanism (Bierhaus et al., 2003; Madrigal et al., 2002). NF-\textit{k}B activation elicits the expression and activity of pro-inflammatory enzymatic sources, such as inducible nitric oxide (NO) synthase (NOS-2) and cyclooxygenase-2 (COX-2) (Madrigal et al., 2006). The result of this sequence of events is the accumulation of oxidative/nitrosative mediators, which alters membrane phospholipids and causes cell damage in a process known as lipid peroxidation. This has been observed in the brain after stress exposure (Madrigal et al., 2006).

This potentially deleterious neuroinflammatory response is regulated by different anti-inflammatory mechanisms, also activated at CNS level after stress exposure, such as the one led by the prostaglandin 15d-PGJ\textsubscript{2}, a COX-2-derived lipid mediator and a potent endogenous agonist of the anti-inflammatory transcription factor peroxisome proliferator-activated nuclear receptor \textit{gamma} (PPAR\textsubscript{\gamma}) (García-Bueno et al., 2008).
Currently, the study of alternative/related anti-inflammatory pathways has focused on the endocannabinoid system (ECS). ECS refers to a group of endogenous arachidonate-based lipids (i.e. anandamide and 2-arachidonoylglycerol), known as “endocannabinoids”; their G protein-coupled receptors, namely CB1 and CB2, the two main synthesis enzymes N-Acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and Diacylglycerol lipase (DGL) and finally, the enzymes Fatty acid amide hydrolase (FAAH) and Monoacylglycerol (MGL) lipase that are responsible for their degradation and/or reuptake. ECS is considered as an endogenous homeostatic system activated by different immune challenges, restoring brain balance in different experimental settings (Mechoulam and Shohami, 2007; Bambico et al., 2009; Cabral and Griffin-Thomas, 2009). In particular, it has been recently demonstrated that stress exposure up-regulates CB1 receptor in brain FC and its selective pharmacological activation prevents the stress-induced excitotoxic/neuroinflammatory process (Zoppi et al., 2011).

CB2, the other classical cannabinoid receptor, has been recognized as a major regulator of the immune system in the periphery, due to its high expression in a wide range of immune cells (Arévalo-Martín et al., 2003). However, it is now accepted that the CB2 receptor is also expressed in CNS by microglia, astrocytes and subpopulations of neurons present in brain areas related to HPA stress axis activity (Gong et al., 2006), although the extent of CB2 receptor expression in neurons remains controversial (Atwood and Mackie, 2010). Furthermore, numerous studies show the inducible nature of CB2 receptor in microglia under neuroinflammatory conditions, suggesting that its up-regulation is a common pattern of response against different types of chronic human neurodegenerative/neurologic pathologies (Bisogno and Di Marzo, 2010). Although there is still controversy regarding the role of the CB2 receptor in the brain (Atwood
and Mackie, 2010), specific functions for CB2 receptor in neuropsychiatric pathologies are currently emerging (Onaivi et al., 2012).

Taking into account all this background, the aim of the present study was to explore the effect of genetic (over-expression and knockout) or pharmacological (selective agonist JWH-133) manipulations of CB2 receptor on stress-induced excitotoxicity and neuroinflammation.

**EXPERIMENTAL PROCEDURES**

**Animals**

Transgenic mice over-expressing CB2 receptor (CB2xP), CB2 knockout mice (CB2KO) and their correspondent wild-type (WT) littermates (ICR –Swiss- strain) were used in all the experiments. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense in accordance with European legislation (2003/65/EC). The mice were housed individually with standard temperature and humidity conditions and in a 12-hour light/dark cycle (lights on at 8:00 am) with free access to food and water. All the animals were maintained under constant conditions for 4 days before the exposure to stress.

**Stress protocol**

Mice were exposed to sub-chronic immobilization and acoustic stress (2h from 13:00h to 15:00h for 4 days) as previously described (Kiank et al., 2003). Stressed animals were sacrificed immediately after the last stress session (whilst still in the restrainer) using sodium pentobarbital (320 mg kg$^{-1}$ i.p.). Control animals were not submitted to stress but were handled at 13:00 h for a few seconds and food and water were removed. Blood for plasma determinations was collected by cardiac puncture and
anticoagulated in the presence of trisodium citrate (3.15% (wt/vol), 1 vol citrate per 9 vol blood). After decapitation, the brain was removed from the skull, and after careful removal of the meninges and blood vessels, the prefrontal cortical areas from both brain hemispheres were excised and frozen at −80°C until assayed. Mouse brain FC was chosen because of its relatively high levels of CB2 receptor (Gong et al., 2006) and its susceptibility to excitotoxic/neuroinflammatory processes elicited by stress (García-Bueno et al, 2008). The brain FC is an important neural substrate for the regulation of the Hypothalamic/Pituitary/Adrenal (HPA) axis response to stress (Radley et al., 2006).

**Preparation of cytosolic and nuclear extracts**

A modified procedure based on the method of Schreiber et al. (1989) was used. Briefly, tissues were homogenized with 300 μL of buffer (see SI for details). After 15 min, Nonidet P-40 (Roche) was added to a 0.5% concentration level. The tubes were gently vortexed for 15 sec, and nuclei were collected by centrifugation at 8000g for 5 min. Supernatants were taken as a cytosolic fraction. The pellets were resuspended in 100 μL of buffer supplemented with 20% glycerol and 0.4 mol L⁻¹ KCl and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000g for 5 min, and aliquots of the supernatant were stored at −80°C. All steps of the fractionation were carried out at 4°C.

**Western Blot Analysis:**

To determine the expression levels of the astroglial excitatory amino-acid transporter-2 (EAAT-2), NOS-2 and COX-2, brain prefrontal cortices were used. In the case of the NF-κB subunit p65 the analysis was carried out in nuclear extracts from FC samples, and for the inhibitory protein of NF-κB IκBα, cytosolic extracts were used.
(see previous point). After determining and adjusting protein levels, homogenates of prefrontal cortex tissue, once centrifuged (12000 g, 20 min at 4°C) were mixed with Laemmli sample buffer with beta mercaptoethanol (50 μL per mL of Laemmli) and 20 μL (2 μg/μL) were loaded into an electrophoresis gel. Once separated by molecular weight, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Spain) with a semi-dry transfer system (Bio-rad) and were incubated with specific antibodies (see SI for details):

After washing with 10 mM Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 90 min at room temperature. Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and were quantified by densitometry (NIH ImageJ® software). All densitometries are expressed in arbitrary units of optical density (O.D). In all Western blot analysis, the house keeping gene β-actin was used as loading control except for the case of NF-κB in which the loading control was the nuclear factor SP1 (blots shown in the respective figures).

**mRNA analysis**

Total cytoplasmic RNA was prepared from samples of FC using TRIZOL® reagent (Invitrogen); aliquots were converted to cDNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by RT-PCR using the following cycling conditions: 35 cycles of denaturation at 95°C for 10 s, annealing at 58–61°C for 15 s depending on the specific set of primers, and extension at 72°C for 20 s. Reactions were carried out in the presence of SYBR green (1:10000 dilution of stock solution from Molecular Probes, Eugene, OR, USA), carried out in a 20- L reaction in a Rotor-
Gene (Corbett Research, Mortlake, NSW, Australia). Relative mRNA concentrations were obtained by comparing the take-off point of the different samples using the software provided in the unit. It establishes an inverse correlation between the number of cycles before take-off and the concentration of mRNA, while assigning arbitrary units to the results obtained. Tubulin primer levels were used to normalize data. See SI for information about the primers used.

**Preparation of Synaptosomes**

After decapitation, half of the forebrain was dissected on ice. All subsequent steps were performed at 4°C. Cortical tissue was immediately homogenized in 25 vols of 0.32M sucrose in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 200g for 10 min, and the supernatant was then collected and centrifuged at 20 000g for 20 min. The pellet was resuspended in 0.32M sucrose and centrifuged at 20 000g for 20 min. The crude synaptosomal pellet was finally resuspended in 1ml of 0.32M sucrose.

**[3H]Glutamate Uptake by Synaptosomes**

Synaptosomes were isolated and sodium-dependent glutamate uptake was measured according to the procedure:

In brief, 25-ml aliquots of synaptosomes were added to 250 ml of incubation buffer containing L-[3H]glutamic acid 0.125 mM (1mCi/ml; Amersham Biosciences Europe GmbH, Freiburg, Germany) and incubated for 3 min at 37°C in a shaking bath. The reaction was terminated using 1ml of ice-cold choline buffer (an incubation buffer
in which an equimolar concentration of choline chloride was substituted for NaCl), and the samples were centrifuged at 10,000g for 2 min to recover the synaptosomes. The \(^3\)H-bound radioactivity was measured using a liquid scintillation counter.

**Plasma corticosterone levels**

Plasma was obtained from blood samples by centrifuging the sample at 1,000g for 15 min immediately after stress. All plasma samples were stored at -20ºC before assay. Corticosterone was measured by the radioimmunoassay (RIA) kit Coat-a-Count\(^\circledR\) (Siemens, Los Angeles, CA, USA) in a \(\gamma\) counter.

**Nitrites (NO\(_2^-\)) levels**

As the stable metabolites of the free radical nitric oxide (NO\(_-\)), NO\(_2^-\) were measured by using the Griess method (Green et al. 1982). Briefly, in an acidic solution with 1% sulphanilamide and 0.1% NEDA, nitrites convert into a pink compound that is photometrically calculated at 540 nm in a microplate reader (Synergy 2; BioTek, USA) (Salter et al. 1996).

**Lipid peroxidation**

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde following the method described by Das and Ratty (1987) with some modifications. Cerebral cortex was sonicated in 10 volumes of 50 mmol/L phosphate buffer and deproteinized with 40% trichloroacetic acid and 5 mol/L HCl, followed by the addition of 2% (wt/vol) thiobarbituric acid in 0.5 mol/L NaOH. The reaction mixture was heated in a water bath at 90ºC for 15 min and centrifuged at 12000g for 10
min. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer (Beckman).

**Brain prostaglandin E$_2$ (PGE$_2$) levels**

PGE$_2$ brain levels were measured by enzyme immunoassay (EIA) using reagents in kit form (Prostaglandin E$_2$ EIA Kit-Monoclonal; (Cayman Chemical®, Tallin, ES). Samples were sonicated in 300μl homogenization buffer (0.1M phosphate buffer, pH7.4, 1mM EDTA and 10μM indomethacin) and purified by incubation in ethanol at 4x sample volume for 5 min at 4° C and then centrifugation at 3,000 x g for 10 min. They were acidified with glacial acetic acid to pH 3.5 and PGE$_2$ was extracted using SPE (C-18) columns (Amersham Biosciences, Buckinghamshire, UK) rinsed with methanol and water. After the application of samples, columns were washed with water and hexane and PGE$_2$ were eluted with ethyl acetate. Samples were then evaporated to dryness under nitrogen and re-suspended in EIA buffer. Levels of PGE$_2$ were measured at 405 nm following manufacturer’s instructions.

**Pharmacological tools**

To selectively activate the CB2 receptor JWH-133 (6aR,10aR)-6,6,9-trimethyl-3-(2-methylpentan-2-yl)-6a,7,10a-tetrahydrobenzo[c]chromene (JWH) (Tocris Bioscience®, Bristol, UK) was administered to some groups of WT and CB2-KO mice at the onset of each session of the stress (1300 h). JWH is a potent CB2 receptor agonist ($K_i$ = 3.4 nM), 200-fold selective over CB1 receptors (Pertwee, 1997, Huffman et al., 1999). The dose (2 mg kg$^{-1}$) was intra-peritoneally administered (i.p.) and it was chosen based on previous *in vivo* studies of neuroprotection in mice (Zarruk et al. 2012). JWH was dissolved in DMSO:Tween:PBS (1:1:18) and the total volume injected into each
animal was 200μl. Control and stressed animals (CWT and SWT, respectively) were injected with vehicle to mirror the stress produced by the injection and the possible effects of the vehicle used on neuroinflammatory parameters.

Drug/molecular target nomenclature (e.g. receptors) conforms to BJP’s Guide to Receptors and Channels (Alexander et al., 2011).

Protein assay

Protein levels were measured using a method based on the principle of protein-dye binding (Bradford, 1976).

Chemicals and statistical analyses

Unless otherwise stated, the chemicals were from Sigma Spain, Madrid. Data in text and figures are expressed as mean ± SEM. First, to determine whether the different CB2 genetic and pharmacological approaches used restore the glutamate reuptake mechanisms to the level of control, a one way ANOVA followed by the Dunnett’s post hoc test (all groups against control) were made. For multiple comparisons, a two-way ANOVA followed by the Bonferroni post hoc test were made, considering as first factor the presence or absence of stress and, as second one, the presence or absence of pharmacological or genetic manipulations of CB2 (CB2 receptor over-expression or deletion, respectively). Specifically, to validate whether JWH anti-inflammatory actions are dependent on CB2 receptor activation, a two-way ANOVA followed by the Bonferroni post hoc test were made, considering as first factor the presence or absence of pharmacological treatment, and as second one, the genotype of the mice (WT or CB2 KO). A p value <0.05 was considered statistically significant.

RESULTS
1. CB2 receptor-dependent effects on stress-induced changes in synaptosomal glutamate uptake and on the expression of glutamate transporters.

The ECS has been shown to confer neuroprotection inhibiting glutamatergic excitotoxicity by a CB1-related mechanism (Zoppi et al., 2011). We decided to explore the possible effects of CB2 receptor on glutamate transport in brain cortical synaptosomes from WT, WT+JWH, CB2xP and CB2KO mice under control and stress conditions.

JWH treatment or overexpression of CB2 resulted in an increase in control levels of glutamate uptake (Fig 1A-B). As it has been previously described (García-Bueno et al., 2007), stress exposure lead to a marked decrease in glutamate uptake compared to CWT group (Fig 1A-C). JWH treatment or overexpression of CB2 did not prevent this stress-induced impairment in glutamate uptake function, but SJWH and SCB2xP levels were not significantly different than the CWT group (JWH F (1, 21) = 7.946, p=0.014; CB2xP (F1, 21) = 9.87, p =0.0003).

The lack of CB2 receptor did not modify the glutamate uptake in cortical synaptosomes compared to WT mice in both control and stress conditions, but the levels of SCB2KO were significantly lower than the CWT group (Fig 1C) (CB2KO F (1, 21) = 4.02, p = 0.0236).

We also analysed if the effects found in control conditions were due to expressional changes in the main brain glutamate transporter EAAT-2, but there were no changes at protein level (data not shown).

3. CB2 receptor effects on HPA axis activity. Plasma corticosterone levels
Corticosterone is the main stress hormone in rodents, widely known as a classical regulator of the inflammatory/immune response in brain and periphery (Madrigal et al., 2006). We analysed whether CB2 receptor manipulations modify HPA axis activity in basal and stress conditions. Thus, corticosterone quantification by RIA revealed an expected increase in plasma corticosterone in all groups of stressed animals compared to their respective controls (Fig 2). Therefore, neither the genetic nor the pharmacological modulation of CB2 receptor modified plasma corticosterone levels after stress at the time of blood extraction (approximately 15:00h) (Fig 2). (stress (F1,19) = 120.24, p < 0.0001; JWH-treatment (F1:19) = 1.93, p = 0.184; stress x JWH-treatment (F1, 19) = 2.54, p=0.1305); (stress (F1, 19) = 36.9, p <0.0001; CB2xP genotype (F1, 19) = 1.15, p = 0.2963; stress x CB2xP genotype (F1, 19) = 0.62, p = 0.4416); (stress (F1, 17) = 105.99; CB2KO genotype (F1, 17) = 1.14, p = 0.3046; stress x CB2KO genotype (F1, 17) = 1.25, p = 0.2833).

4.- Anti-inflammatory effects elicited by CB2 activation. Mechanisms involved

ECS has been proposed as an endogenous protective system against excessive inflammatory/immune responses in multiple CNS pathologies (Wolf et al., 2008). Our following studies were aimed at clarifying the potential role of CB2 receptor as a regulator of the stress-induced inflammatory.

4.1: Brain pro-inflammatory cytokines and chemokines

Pro-inflammatory cytokines and chemoattractant chemokines, such as TNF-α or MCP-1 (Madrigal et al., 2006; Conductier et al., 2010), are the first pro-inflammatory
mediators activated in the brain after stress exposure. PCR studies showed a consistent increase in TNF-α in WT mouse FC after stress, an effect that is blocked by JWH and in CB2xP mice (Fig. 3A-B). (stress (F1,14) = 53.64, p < 0.0001; JWH-treatment (F1:14) = 84.45, p < 0.0001; stress x JWH-treatment (F1, 14) = 25.93, p=0.0003); (stress (F1, 14) = 108.89, p < 0.0001; CB2xP genotype (F1, 14) = 6.05, p = 0.032; stress x CB2xP genotype (F1, 14) = 16.8, p = 0.0018); Conversely, stressed CB2 KO animals presented a significant increase in TNF-α mRNA (Fig. 3C). (stress (F1, 14) = 33.43, p = 0.0002; CB2KO genotype (F1, 14) = 11.49, p = 0.0069; stress x CB2KO genotype (F1, 14) = 0.53, p = 0.4826).

A slightly different profile was found for MCP-1: stress also increased MCP-1 levels in the FC of WT mice but only the genetic over-expression of CB2 decreased them (Fig 3D-E). (stress (F1,18) = 22.53, p = 0.0003; JWH-treatment (F1:18) = 1.45, p =0.247; stress x JWH-treatment (F1, 18) = 0.13, p=0.7204); (stress (F1, 18) = 9.97, p = 0.007; CB2xP genotype (F1, 18) = 5.58, p = 0.0331; stress x CB2xP genotype (F1, 18) = 1.16, p = 0.30). Stressed CB2 KO mice presented higher levels of MCP-1 mRNA compared with SWT group (Fig. 3F) (stress (F1, 17) = 19.5, p = 0.0006; CB2KO genotype (F1, 17) = 11.36, p = 0.0046; stress x CB2KO genotype (F1, 17) = 1.35, p = 0.2655).

4.2: NF-κB

The release of TNF-α after stress accounts for NF-κB activation (Madrigal et al., 2002), and we also tested the possible involvement of CB2 receptor in the level of NF-κB expression.
Stress exposure induced a decrease in the expression of IκBα in cytosolic extracts from WT mice, this was reversed in CB2xP mice an in those treated with JWH administration and (Fig. 3G-H) (stress (F1,13) = 9.84, p=0.01; JWH-treatment (F1:13) = 86.39, p<0.0001; stress x JWH-treatment (F1, 13) = 68, p<0.0001); (stress (F1, 27) = 24.8, p<0.0001; CB2xP genotype (F1, 27) = 14.96, p = 0.0007; stress x CB2xP genotype (F1, 27) = 24.8, p<0.0001). Levels of the pro-inflammatory NF-κB subunit p65 in nuclear extracts from the different groups studied (Fig 3J-K) mirrored the expression of IκBα (stress (F1,30) = 4.73, p = 0.0386; JWH-treatment (F1:30) = 10.7, p =0.0029; stress x JWH-treatment (F1, 30) = 6.96, p=0.0137); (stress (F1, 60) = 4.01, p=0.05; CB2xP genotype (F1, 60) = 5.94, p = 0.018; stress x CB2xP genotype (F1, 60) = 9.04, p=0.0039).

IκBα protein levels in control CB2 KO animals are lower than in the CWT ones, under stress conditions, CB2 KO mice presented similar levels to those from the SWT group (Fig. 3I) (stress (F1, 22) = 7.72, p = 0.013; CB2KO genotype (F1, 22) = 7.22, p = 0.0156; stress x CB2KO genotype (F1, 22) = 13.7, p = 0.0018). In addition, p65 protein expression in nuclear extracts is increased in CB2 KO mice in both control and stress conditions (Fig 3L), suggesting a state of chronic NF-κB activation in these animals (stress (F1, 23) = 6.03, p = 0.024; CB2KO genotype (F1, 23) = 127.75, p < 0.0001; stress x CB2KO genotype (F1, 23) = 2.04, p = 0.1705).

4.3: Pro-inflammatory enzymes (NOS-2 and COX-2)

NF-κB regulates the expression of genes involved in the accumulation of oxidative/nitrosative and inflammatory mediators after stress exposure (Madrigal et al., 2006). Among others, two main pro-inflammatory enzymes NF-κB dependent are
inducible NO synthase (NOS-2) and cyclooxygenase-2 (COX-2). Their major products (NO and PGE₂, respectively) are potent oxidant/pro-inflammatory molecules that have been widely connected to damage and even cellular death in multiple CNS pathologies, including stress-related ones (García-Bueno et al., 2008). We decided to explore CB2 receptor effects on the expression and activity of these enzymes.

NOS-2 and COX-2 expression increased in the FC after stress exposure in WT mice (Fig. 4A, D). In addition, JWH and the over expression of CB2 receptor completely blocked NOS-2 up-regulation produced by stress exposure (Fig. 4A-B). As in the case of other pro-inflammatory mediators, CB2KO mice, both in control and stress conditions showed a consistent NOS-2 up-regulation compared with their WT control groups, and a higher increase after stress (Fig. 4C) (stress (F1,19) = 6.07, p = 0.0255; JWH-treatment (F1:19) = 8.08, p =0.0118; stress x JWH-treatment (F1, 19) = 1.76, p=0.2027); (stress (F1, 16) = 4.5, p=0.0495; CB2xP genotype (F1, 16) = 18.51, p = 0.0013; stress x CB2xP genotype (F1, 16) = 44.25, p<0.0001); (stress (F1, 21) = 4.56, p = 0.047; CB2KO genotype (F1, 21) = 10.77, p < 0.0041; stress x CB2KO genotype (F1, 21) = 0.91, p = 0.35).

The interaction between CB2 receptor and COX-2 is more complex, as suggested by our results. PGE₂ cortical levels, a presumed COX-2 product in the brain, are increased after stress exposure in WT mice (Fig. 4G). In addition, the pharmacological activation and over-expression of CB2 receptor reduced PGE₂ levels compared with those in the SWT animals (Fig. 4G-H). However, COX-2 up-regulation produced by stress exposure was only prevented in CB2xP mice (Fig 4E). As it can be observed in Fig 5I, CB2 SKO animals presented significant differences in PGE₂ content compared with the SWT group but no significant changes on COX-2 protein levels were found (Fig 4F). For COX-2: (stress (F1,16) = 3.9, p = 0.07; JWH-treatment (F1,16) =
0.13, \( p = 0.722 \); stress x JWH-treatment (F1, 16) = 0.5, \( p = 0.49 \); (stress (F1, 18) = 4.22, \( p = 0.05 \); CB2xP genotype (F1, 18) = 6.28, \( p = 0.0233 \); stress x CB2xP genotype (F1, 18) = 5.94, \( p = 0.0278 \); (stress (F1, 43) = 5.21, \( p = 0.028 \); CB2KO genotype (F1, 43) = 2.12, \( p = 0.1534 \); stress x CB2KO genotype (F1, 43) = 0.86, \( p = 0.36 \)). For PGE2: (stress (F1,17) = 4.5, \( p = 0.05 \); JWH-treatment (F1:17) = 12.4, \( p = 0.0034 \); stress x JWH-treatment (F1, 17) = 7.71, \( p = 0.015 \); (stress (F1, 16) = 4.96, \( p = 0.042 \); CB2xP genotype (F1, 16) = 26.03, \( p = 0.0002 \); stress x CB2xP genotype (F1, 16) = 4.74, \( p = 0.0485 \); (stress (F1, 28) = 5.65, \( p = 0.025 \); CB2KO genotype (F1, 28) = 14.13, \( p = 0.0009 \); stress x CB2KO genotype (F1, 28) = 0.02, \( p = 0.90 \)).

4.4: Lipid peroxidation

As a final index of stress-induced cellular damage that could be affected by CB2 receptor-selective modulation, we measured the accumulation of the lipid peroxidation marker malondialdehyde (MDA) and of the nitric oxide stable metabolites nitrites (NO2−) in the brain FC of the different mice groups. Stress exposure caused a smaller accumulation of MDA in CB2xP and JWH treated mice (Figs. 5A-B) (stress (F1, 23) = 4.7, \( p = 0.042 \); JWH-treatment (F1, 23) = 30.08, \( p < 0.0001 \); stress x JWH-treatment (F1, 23) = 15.32, \( p = 0.0099 \); (stress (F1, 25) = 4.81, \( p = 0.039 \); CB2xP genotype (F1, 25) = 4.33, \( p = 0.0492 \); stress x CB2xP genotype (F1, 25) = 4.97, \( p = 0.036 \)). Conversely, CB2KO mice presented higher levels of MDA after stress than the SWT group (Fig. 5C); (stress (F1, 19) = 15.61, \( p = 0.011 \); CB2KO genotype (F1, 19) = 5.1, \( p = 0.038 \); stress x CB2KO genotype (F1, 19) = 0.71, \( p = 0.41 \)).

The results for NO2− (Figs 5D-F) followed a similar pattern in all groups of mice studied, with the exception of CB2xP mice which presented a significant over-
accumulation of NO\textsubscript{2}^{-}, possibility related to a compensatory mechanism, which may be worth to further explore (Fig. 5E) (stress (F1, 18) = 9.79, p = 0.007; JWH-treatment (F1, 18) = 12.86, p=0.0027; stress x JWH-treatment (F1, 18) = 7.28, p=0.0165); (stress (F1, 19) = 10.91, p=0.0045; CB2xP genotype (F1, 19) = 14.43, p = 0.0016; stress x CB2xP genotype (F1, 19) = 8.98, p=0.0085); (stress (F1, 29) = 11.25, p = 0.0025; CB2KO genotype (F1, 29) = 14.07, p = 0.0009; stress x CB2KO genotype (F1, 29) = 0.15, p = 0.70).

4.5 Validation of JWH CB2-Dependent Anti-inflammatory Effects

Finally, to elucidate whether JWH actions are mediated by CB2 receptor, we examined its anti-inflammatory effects on some representative inflammatory/oxidative parameters (I\textsubscript{K\beta} and NOS-2 expression and MDA and NO\textsubscript{2}^{-} levels) in CB2 KO mice submitted to stress exposure. As can be observed in Figure 6 (A-D), none of JWH anti-inflammatory/antioxidant effects were observed in CB2KO mice, suggesting the direct involvement of CB2 receptor in our model. For I\textsubscript{K\beta} WB data (JWH treatment (F1, 19) = 15.85, p=0.0026; CB2KO genotype (F1, 19) = 71.10, p<0.0001; JWH treatment x CB2KO genotype (F1, 19) = 30.83, p = 0.002). For NOS-2 (JWH treatment (F1, 19) = 9.50, p=0.0095; CB2KO genotype (F1, 19) = 69.17, p=0.001; JWH treatment x CB2KO genotype (F1, 19) = 4.78, p = 0.049). For NO\textsubscript{2}^{-} (JWH treatment (F1, 19) = 9.81, p= 0.0069; CB2KO genotype (F1, 19) = 29.63, p< 0.0001; JWH treatment x CB2KO genotype (F1, 19) = 0.63, p = 0.4407). For MDA (JWH treatment (F1, 19) = 11.12, p= 0.0049; CB2KO genotype (F1, 19) = 25.21, p= 0.0002; JWH treatment x CB2KO genotype (F1, 19) = 0.43, p = 0.5216).
DISCUSSION AND CONCLUSIONS

Our results indicate a general anti-inflammatory role for CB2 receptor in the FC of mice exposed to sub-chronic restraint/acoustic stress. JWH-133 treatment or overexpression of CB2 resulted in an increase in control levels of glutamate uptake, which is then reduced by stress exposure back to control levels. These effects are not due to changes in the general response to stress, since different manipulations on CB2 receptor do not modify plasma corticosterone levels in our model.

Although, previous results suggested that the excitotoxic process in stress conditions was regulated by CB1 (Zoppi et al., 2011), other authors have demonstrated a role for CB1 and CB2 receptors in the regulation of AMPA excitotoxicity in in vivo and in vitro models of multiple sclerosis through the up-regulation of EAAT-2 (Docagne et al., 2007; Loría et al., 2010). In our conditions we did not find changes on EAAT-2 protein expression. Probably, alternative approaches such as the determination of glutamate levels in the tissue will help to elucidate whether the effects of CB2 receptor manipulations on glutamate uptake are due to inflammatory-related actions on EAAT-2 activity or to a direct effect of CB2 receptor in neurons.

According to our results, CB2 receptor is not directly implicated in the mechanism controlling the production of plasma corticosterone in this protocol of stress. In agreement with this, the increase in plasma corticosterone levels elicited by systemic endotoxin administration did not change after the pharmacological modulation of CB2 receptor (Roche et al., 2010). However, the expression of CB2 receptor in stress-responsive neural circuits (e.g. hippocampus, amygdala and hypothalamus) indirectly suggests that CB2 receptor activation could regulate the neuroendocrine response (García-Gutiérrez et al., 2010). In fact, CB2xP mice submitted to 30-min of
restraint stress presented lower levels of pro-opiomelanocortin mRNA in the *arcuate nuclei* than their WT counterparts, as well as a complete blockage of the stress-induced increase in the corticotropin-releasing factor mRNA in the paraventricular nucleus of the hypothalamus (García-Gutiérrez et al., 2011). Thus, more detailed neuroendocrine studies regarding the time course of synthesis and release of corticosterone and other stress hormones in the sub-chronic stress protocol used here are needed to completely discard a role of CB2 receptor in the regulation of HPA axis activation.

Classically, because of its high level of expression in diverse types of immune cells and organs (Klein et al., 2003), CB2 receptor mediated antiinflammatory effects have been described in periphery. However, CB2 agonists anti-inflammatory effects in CNS have also been shown in traumatic brain injury, spinal cord injury, stroke and EAE (Mechoulam and Shohami, 2007; Adhikary 2011; Castillo et al., 2010; Zarruk et al., 2012; Arévalo-Martín et al., 2003). In this vein, some authors have demonstrated that CB2 receptor is expressed by glia and neurons in the brain (Gong et al., 2006; Aracil-Fernández et al., 2012), although some controversy still exists, particularly in the case of neurons (Atwood and Mackie, 2010). In this way, we demonstrate here that CB2 receptor activation regulates stress-induced neuroinflammation at multiple levels in the FC. This anti-inflammatory profile is especially relevant considering that neuroinflammatory processes have been proposed to underlie the pathophysiology of several stress-related neuropsychiatric disorders (Madrigal et al., 2006; Wager-Smith and Markou, 2011).

The regulatory effect of CB2 receptor on the levels of pro-inflammatory cytokines (TNF-α) in the brain has been extensively documented (Jean-Gilles et al., 2010). However, the inhibitory role of CB2 receptor activation on MCP-1 *in vivo* has remained partly unknown. Only recent studies reported an inhibitory effect of CB2
receptor activation on MCP-1 mRNA levels in animal models of stroke and multiple sclerosis (Zarruk et al., 2012; Palazuelos et al., 2008). These results are especially relevant considering that MCP-1 is implicated in inflammatory cell migration into inflamed tissues (CNS included) and nociception, processes that have been related to CB2 receptor (Miller and Stella, 2008; Adhikary et al., 2011; Racz et al., 2008).

In agreement with our results, other authors have demonstrated that CB2 receptor activation inhibits the activity of the major inflammatory mediator NF-κB, mainly in some immune cells in vitro exposed to inflammatory/immune stimuli, such as macrophages and microglia (Jeon et al., 1996; Correa et al., 2010). However, to avoid the oversimplification of the effects of CB2 receptor on TNF-α and NF-κB, it is necessary to remark that some studies proved the beneficial effects of both inflammatory mediators in neuronal survival (Marchetti et al., 2004). Thus, their precise role in inflammation is still unclear.

The inhibitory effects of CB2 receptor activation on NOS-2 have been deeply studied in in vivo animal models of neuropathology, such as Huntington’s disease or stroke (Palazuelos et al., 2009; Zarruk et al., 2012), but CB2 receptor-cyclooxygenase interactions may be more complex. Our results suggest possible effects of JWH-133 on the catalytic activity or protein stability of COX-2, on the activity of COX-1 isoform or on tissue-specific prostaglandin E2 synthases, which is still unknown. Indeed, COX-2 inhibition afforded by CB2 receptor activation has been described in different in vivo and in vitro neuropathological experimental settings (Castillo et al., 2010; Martín-Moreno et al., 2012).

As a result of this over-accumulation of consecutive pro-inflammatory mediators, oxidative/nitrosative cellular damage is produced after stress exposure. Our results suggest that a potential therapeutic use for CB2 receptor activation its
antioxidant profile. Similarly, CB2 receptor antioxidant effects have been also found in CB2xP in an experimental model of Parkinson’s disease (Ternianov et al., 2012). The antioxidant effects produced by the pharmacological activation of CB2 receptor has been extensively reviewed in several neurologic/neurodegenerative diseases (Fernández-Ruiz, 2009).

CB2xP mice and the pre-treatment with JWH in WT mice are different experimental models, because CB2xP mice over-express CB2 receptor in glia but also in neurons, and present endocrine and/or peripheral alterations, such as hyperglycemia (Romero-Zerbo et al., 2012). These alterations could limit their use to obtain determinate conclusions. In the conditions of the present study, the anti-inflammatory profile of both experimental groups is very similar, with the exception of COX-2 protein levels in which the treatment with JWH-133 is not capable to reduce them, while CB2xP mice presented a clear decrease of COX-2 protein. CB2xP mice present an up-regulation of CB2 receptor on FC neurons, an effect that could affect their characteristic constitutive expression of COX-2 in this brain area (Yamagata et al., 1993).

Although JWH presents higher affinity for CB2 than for CB1 receptor (Huffman et al., 1999), this compound is CB2 receptor-selective but not CB2 receptor-specific. We decided to elucidate whether JWH anti-inflammatory effects were exclusively CB2 receptor mediated. Our results suggest that, at least in our model, JWH lacks its anti-inflammatory profile in CB2KO animals. However, it cannot be excluded that this compound elicits CB2 receptor-independent effects using different doses or routes of administration or in other models of neuropathology.

In contrast to JWH anti-inflammatory effects, CB2KO mice presented an enhanced neuroinflammatory response after stress exposure in the FC. Deletion of CB2
receptor also induces schizophrenia and depression-like behaviours in mice (Ortega-Alvaro et al., 2011), but further investigation is needed to elucidate whether the excessive neuroinflammation present in CB2KO is directly related to the pathophysiology of these major psychiatric diseases or just an epiphenomenon.

This study presents some limitations: first, although it is not a main initial goal of this work, in order to find out if CB2 receptor plays a role in the regulation of HPA axis activation more detailed neuroendocrine studies will be needed, including a time course of the synthesis and release of the main stress hormones. Second, the study of different brain areas involved in the stress response would draw a more comprehensive picture of the regulatory role of CB2 receptor. Third, studies carried out in extended stress exposure models as inductors of depressive-like behaviours, (e.g. 2 chronic mild stress, chronic unpredictable stress) would strengthen translational conclusions.

In conclusion, we have found evidence of an anti-inflammatory profile for CB2 receptor activation. These effects are not related to alterations in plasma corticosterone levels. CB1 receptor activation elicits a consistent neuroprotective response in the same stress paradigm (Zoppi et al., 2011), but it is known that direct CB2 receptor activation produces less undesired effects (e.g. psychoactive effects) (Mechoulam and Parker, 2013). Our previous and current findings open the possibility for the use of CB1/CB2 receptors dual agonists or endocannabinoids reuptake inhibitors for the management of neurologic/neurodegenerative and neuropsychiatric diseases.

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LIST OF REFERENCES


neuroprotection against behavioral and neurochemical alterations induced by intracaudate administration of 6-hydroxydopamine. Neurobiol Aging 33(2): 421.e1-16.


FIGURE LEGENDS

Figure 1.- CB2 receptor effects on glutamate uptake mechanisms.

(A) Glutamate uptake in forebrain synaptosomes of control (CWT) (100±8.83; 100±9.3; 100±9.76), stressed during 4d (SWT) (44.71±11.7; 53.93±18.3; 50.7±17.5), control+JWH-133 (C JWH) (172.2±25.8) and stressed+JWH-133 (S JWH) (102.4±18) wild type mice; (B) control CB2xP (C CB2xP) (146.8±12.96) and stressed CB2xP (S CB2xP) (90.94±5.8) mice and (C) control (CKO) (86.04±11.7) and stressed CB2 KO (SKO) (50.77±14.35) mice. The data represents the mean± SEM of six mice. *p<0.05 vs CWT. One way ANOVA (ow-ANOVA) following Dunnett’s multiple comparison post-test).

Figure 2. Plasma corticosterone levels in all control and stressed groups of mice.

Corticosterone plasma levels (ng ml⁻¹) at the time of blood extraction (15:00h) of control (CWT) (84.58±12.05), stressed during 4d (SWT) (386±36.95), control+JWH-133 (C JWH) (97.89±6.69), stressed+JWH-133 (S JWH) (324.7±36.48) mice, control CB2xP (C CB2xP) (170±42.65), stressed CB2xP (S CB2xP) (429.6±67.33) mice, and control (CKO) (85.96±7.57) and stressed CB2 KO (SKO) (339.2±33.51) mice. The data represents the mean± SEM of six mice. ***p<0.001 vs CWT; $$$p<0.001 vs C JWH; δδ p<0.01 vs C CB2xP; &&& p<0.001 vs CKO. (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).
Figure 3.- CB2 receptor anti-inflammatory effects on brain I. Pro-inflammatory cytokines, chemokines and NF-κB.

A) Q-PCR analysis of the pro-inflammatory cytokine TNF-α mRNA in the FC of control (CWT) (1.017±0.021; 1.02±0.018, 1.1±0.05) stressed during 4d (SWT) (2.1±0.08; 2.075±0.091; 2.85±0.18), control+JWH-133 (C JWH) (0.67±0.066) and stressed+JWH-133 (S JWH) (0.86±0.01) wild type mice; (B) control CB2xP (C CB2xP) (1.3±0.19) and stressed CB2xP (S CB2xP) (1.6±0.04) mice and (C) control (CKO) (2.01±0.37) and stressed CB2 KO (SKO) (4.4±0.58) mice. Data is normalized by tubulin and is representative of 3 experiments. *p<0.05 vs CWT; ***p<0.001 vs CWT; #p<0.05 vs SWT; ###p<0.001 vs SWT; &&p<0.01 vs CKO. Two-way ANOVA (tw-ANOVA) following Bonferroni post-test.

Q-PCR analysis of the chemokine MCP-1 in FC homogenates (D) of control (CWT) (0.43±0.1; 0.48±0.085; 0.45±0.092), stressed during 4d (SWT) (0.97±0.127; 0.953±0.143; 0.985±0.105), control+JWH-133 (C JWH) (0.23±0.002) and stressed+JWH-133 (S JWH) (0.862±0.135) wild type mice; (E) control CB2xP (C CB2xP) (0.263±0.1) and stressed CB2xP (S CB2xP) (0.528±0.07) mice and (F) control (CKO) (0.92±0.04) and stressed CB2 KO (SKO) (1.65±0.38) mice. Data is normalized by tubulin and is representative of 3 experiments. *p<0.05 vs CWT; **p<0.01 vs CWT; #p<0.05 vs SWT; ###p<0.001 vs SWT; ssp<0.01 vs C JWH, &&p<0.01 vs CKO. Two-way ANOVA (tw-ANOVA) following Bonferroni post-test.

(G) Western blot and densitometric analysis of the NF-κB inhibitory protein IκBα in FC cytosolic extracts of control (CWT) (100.2±5.2; 96±4.1; 102±12), stressed during 4d (SWT) (31.4±3.98; 36.7±4.6; 67±9.1), control+JWH-133 (C JWH) (81.5±50.21) and stressed+JWH-133 (S JWH) (137.5±7) wild type mice; (H) control CB2xP (C CB2xP) (90.72±4.1) and stressed CB2xP (S CB2xP) (78.73±3.3) mice and
control (CKO) (72.3±3.8) and stressed CB2 KO (SKO) (79.2±3.54) mice. Data are normalized by β-actin (lower band) and are representative of 3 experiments. *p<0.05 vs CWT; **p<0.01 vs CWT; ***p<0.001 vs CWT; ###p<0.001 vs SWT; $p<0.05$ vs C JWH. Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).

(J) Western blot and densitometric analysis of the NF-κB pro-inflammatory subunit p65 in FC nuclear extracts of control (CWT) (82.2±6.45; 94.27; 90.7±11.5), stressed during 4d (SWT) (113±6.5;118±7.1; 115±9.8), control+JWH-133 (C JWH) (84±14) and stressed+JWH-133 (S JWH) (67±5.95) wild type mice; (K) control CB2xP (C CB2xP) (96±6.3) and stressed CB2xP (S CB2xP) (87.5±7.3) mice and (L) control (CKO) (214±7.2) and stressed CB2 KO (SKO) (224±13) mice. Data are normalized by β-actin (lower band) and are representative of 3 experiments. *p<0.05 vs CWT; ***p<0.001 vs CWT; $#p<0.01$ vs SWT; ###p<0.001 vs SWT (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).

Figure 4.- CB2 receptor anti-inflammatory effects on brain III. Pro-inflammatory enzymes.

A) Western blot and densitometric analysis of the pro-inflammatory enzyme NOS-2 in FC homogenates of control (CWT) (89±2.6; 75.5±12; 92.2±2.5), stressed during 4d (SWT) (120.5±8; 126±10; 121±9), control+JWH-133 (C JWH) (76±12) and stressed+JWH-133 (S JWH) (86±5) wild type mice; (B) control CB2xP (C CB2xP) (90±14.5) and stressed CB2xP (S CB2xP) (44±9.5) mice and (C) control (CKO) (140±9.5) and stressed CB2 KO (SKO) (153±14) mice. Data are normalized by β-actin (lower band) and are representative of 3 experiments. *p<0.05 vs CWT; **p<0.01 vs
CWT; *p<0.05 vs SWT; **p<0.01 vs SWT (Two-way ANOVA (tw-ANOVA) following Bonferri post-test). AU: arbitrary units.

(D) Western blot and densitometric analysis of the pro-inflammatory enzyme COX-2 in FC homogenates of control (CWT) (89±2.6; 75±12; 97±5), stressed during 4d (SWT) (121±8;123±9;125±7), control+JWH-133 (C JWH) (102±18) and stressed+JWH-133 (S JWH) (117±16) wild type mice; (E) control CB2xP (C CB2xP) (75±10) and stressed CB2xP (S CB2xP) (71±7) mice and (F) control (CKO) (92±8) and stressed CB2 KO (SKO) (104±14) mice. Data are normalized by β-actin (lower band) and are representative of 3 experiments. *p<0.05 vs CWT; **p<0.01 vs CWT; ##p<0.001 vs SWT (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test). AU: arbitrary units.

(G) Pro-inflammatory PGE₂ levels (index of COX-2 activity) in FC homogenates of control (CWT) (100±10; 100±9; 100±6.5) stressed during 4d (SWT) (162±32;160±36;177±24), control+JWH-133 (C JWH) (88±25) and stressed+JWH-133 (S JWH) (76±9) wild type mice; (H) control CB2xP (C CB2xP) (52.4±13) and stressed CB2xP (S CB2xP) (51±10) mice and (I) control (CKO) (219± 9) and stressed CB2 KO (SKO) (287±54) mice. The data represent the mean± SEM of six mice. *p<0.05 vs CWT; *p<0.05 vs SWT; ###p<0.001 vs SWT (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).

Figure 5.- CB2 receptor neuroprotective effects against stress-induced oxidative/nitrosative cellular damage.

MDA levels in FC homogenates of (A) control (CWT) (100±17; 100±19; 100±3), stressed during 4d (SWT) (171±17; 168±23; 125.3±7.52) , control+JWH-133
(C JWH) (67±11) and stressed+JWH-133 (S JWH) (32±3) wild type mice; (B) control CB2xP (C CB2xP) (94±13) and stressed CB2xP (S CB2xP) (93±12) mice and (C) control (CKO) (110.5±17) and stressed CB2 KO (SKO) (154.1±13) mice. Data represents the mean± SEM of six mice. *p<0.05 vs CWT; **p<0.01 vs CWT; #p<0.05 vs SWT; ##p<0.01 vs SWT; ###p<0.001 vs SWT; &p<0.05 vs CKO (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).

NO stable metabolite Nitrites (NO₂⁻) levels in FC homogenates of (D) control (CWT) (0.04±0.008; 0.035±0.0078; 0.039±0.007) stressed during 4d (SWT) (0.07±0.002; 0.068±0.0025, 0.055±0.003) control+JWH-133 (0.035±0.004) (C JWH) (0.035±0.004) and stressed+JWH-133 (S JWH) (0.037±0.0044) wild type mice; (E) control CB2xP (C CB2xP) (0.082±0.007) and stressed CB2xP (S CB2xP) (0.046±0.0027) mice and (F) control (CKO) (0.057±0.005) and stressed CB2 KO (SKO) (0.071±0.003) mice. Data represents the mean± SEM of six mice. *p<0.05 vs CWT; **p<0.01 vs CWT; ***p<0.001 vs CWT; #p<0.05 vs SWT; ##p<0.01 vs SWT; ###p<0.001 vs SWT; &p<0.05 vs C CB2xP (Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).

Figure 6.- Validation of CB2 receptor -mediated JWH effects.

Western blot and densitometric analysis of the NF-κB inhibitory protein IκBα (A) and the pro-inflammatory enzyme iNOS (B) in FC samples of stressed during 4d (SWT+VEH) (IκBα=100±4; iNOS=100±5), stressed+JWH-133 wild type mice (SWT+JWH) (IκBα=150±10; iNOS=77±6.7) and stressed CB2 KO mice with (SKO+JW) (IκBα=77±4; iNOS=125±5) or without JWH (SKO+VEH) (IκBα=85±5; iNOS=128±7.8) daily pre-treatment. Data are normalized by β-actin (lower band) and
are representative of 3 experiments. * p<0.05 vs SWT+VEH; ** ** p<0.001 vs SWT+VEH; 

$$ p<0.01 \text{ vs SWT+JW. Two-way ANOVA (tw-ANOVA) following Bonferroni post-}$$

PO stable metabolite Nitrites (NO$_2^-$) (C) and MDA (D) levels in FC homogenates of stressed during 4d (SWT+VEH) (NO$_2^-$=100±10; MDA=100±6), stressed+JWH (SWT+ JWH) (NO$_2^-$=72±7; MDA=66±14) wild type mice and stressed CB2 KO mice with (SKO+JW) (NO$_2^-$=116±3; MDA=114±27) or without JWH-133 (SKO+VEH) (NO$_2^-$=133±3; MDA=137±8) daily pre-treatment. * p<0.05 vs SWT+VEH; 

$$ p<0.01 \text{ vs SWT+JW; } $$$ p<0.001 \text{ vs SWT+JW. Two-way ANOVA (tw-ANOVA) following Bonferroni post-test).}$$

Statement of conflicts of interest

None