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RESEARCH PAPER

Regulatory role of the cannabinoid CB_2 receptor in stress-induced neuroinflammation in mice

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BACKGROUND AND PURPOSE

Stress exposure produces excitotoxicity and neuroinflammation, contributing to the cellular damage observed in stress-related neuropathologies. The endocannabinoids provide a homeostatic system, present in stress-responsive neural circuits. Here, we have assessed the possible regulatory role of cannabinoid CB_2 receptors in stress-induced excitotoxicity and neuroinflammation.

EXPERIMENTAL APPROACH

We used wild type (WT), transgenic overexpressing CB_2 receptors (CB2xP) and CB_2 receptor knockout (CB2-KO) mice exposed to immobilization and acoustic stress (2 h·day^{-1} for 4 days). The CB_2 receptor agonist JWH-133 was administered daily (2 mg·kg^{-1}, i.p.) to WT and CB2-KO animals. Glutamate uptake was measured in synaptosomes from frontal cortex; Western blots and RT-PCR were used to measure proinflammatory cytokines, enzymes and mediators in homogenates of frontal cortex.

KEY RESULTS

Increased plasma corticosterone induced by stress was not modified by manipulating CB_2 receptors. JWH-133 treatment or overexpression of CB_2 receptors increased control levels of glutamate uptake, which were reduced by stress back to control levels. JWH-133 prevented the stress-induced increase in proinflammatory cytokines (TNF-α and CCL2), in NF-κB, and in NOS-2 and COX-2 and in the consequent cellular oxidative and nitrosative damage (lipid peroxidation). CB2xP mice exhibited anti-inflammatory or neuroprotective actions similar to those in JWH-133 pretreated animals. Conversely, lack of CB_2 receptors (CB2-KO mice) exacerbated stress-induced neuroinflammatory responses and confirmed that effects of JWH-133 were mediated through CB_2 receptors.

CONCLUSIONS AND IMPLICATIONS

Pharmacological manipulation of CB_2 receptors is a potential therapeutic strategy for the treatment of stress-related pathologies with a neuroinflammatory component, such as depression.

Abbreviations

ECS, endocannabinoid system; HPA, hypothalamic–pituitary–adrenal; MDA, malondialdehyde

Introduction

The relationship between stress and the immune system has been extensively studied over the last few decades (Licinio and Wong, 1999; Sorrells *et al.*, 2009), but the precise mechanisms involved are still a matter of debate, probably due to the complex interactions existing between the periphery and the CNS (Capuron and Miller, 2011). Chronic exposure to stress and stress-related diseases, such as depression or chronic fatigue syndrome, have been classically associated with an inhibition of adaptive immunity, with important negative effects on health (Herbert and Cohen, 1993). However, more recently, a marked activation of innate inflammatory and immune responses has been found, after stress exposure or during certain episodes of depression (García-Bueno *et al.*, 2008; Farooq *et al.*, 2012). The inflammatory response allows the organism to cope with a wide range of threats and challenges but, under pathological and chronic conditions, the maintenance of this response could become deleterious. For instance, long-lasting stress (physical, psychological or mixed) affected 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).

Over the past few years, much effort has been made to elucidate the molecular and cellular events responsible for the brain damage produced by exposure to stress. Stress-induced excitotoxicity follows a massive release of the excitatory amino acid, glutamate, in brain areas such as the frontal cortex (Moghaddam, 1993). This over-accumulation induces the release of pro-inflammatory cytokines such as TNF-α (Madrigal *et al.*, 2002). Stress also activates the inflammatory NF-κB pathway through a TNF-α-dependent mechanism (Madrigal *et al.*, 2002; Bierhaus *et al.*, 2003). NF-κB activation elicits the expression and activity of pro-inflammatory enzymes, such as the inducible NOS (NOS-2) and COX-2 (Madrigal *et al.*, 2006). The result of this sequence of events is the accumulation of oxidative and nitrosative mediators, which alter membrane phospholipids and cause cell damage by lipid peroxidation. This has been observed in the brain after exposure to stress (Madrigal *et al.*, 2006). This potentially deleterious neuroinflammatory response is regulated by different anti-inflammatory mechanisms, also activated in the CNS after stress, such as the biosynthesis of 15d-PGJ_2, a COX-2-derived lipid mediator and a potent endogenous agonist of the anti-inflammatory transcription factor PPAR-γ (García-Bueno *et al.*, 2008).

Currently, the study of anti-inflammatory pathways has focused on the endocannabinoid system (ECS). The ECS comprises a group of endogenous arachidonate-based lipids (mainly anandamide and 2-arachidonoylglycerol), known as 'endocannabinoids'; the corresponding GPCRs, CB_1 and CB_2 receptors (nomenclature follows Alexander *et al.*, 2013a), the two main synthetic enzymes, N-acylphosphatidylethanolamine-phospholipase D and diacylglycerol lipase, and, finally, the degradative enzymes, fatty acid amide hydrolase and monoacylglycerol lipase. The ECS is considered to be 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, exposure to stress up-regulated CB_1 receptors in the frontal cortex and selective pharmacological activation of these receptors prevented the stress-induced excitotoxic and neuroinflammatory processes (Zoppi *et al.*, 2011).

The other cannabinoid receptor type, the CB_2 receptor, has been recognized as a major regulator of the immune system in the periphery, as it is highly expressed in a wide range of immune cells (Arévalo-Martín *et al.*, 2003). However, it is now accepted that CB_2 receptors are also expressed in CNS, by microglia, astrocytes and subpopulations of neurons present in brain areas related to the hypothalamic–pituitary–adrenal (HPA) axis (Gong *et al.*, 2006), although the extent of CB_2 receptor expression in neurons remains controversial (Atwood and Mackie, 2010). Furthermore, CB_2 receptors were inducible in microglia under neuroinflammatory conditions, suggesting that such up-regulation could be a common pattern of response against different types of chronic human neurodegenerative and neurological pathologies (Bisogno and Di Marzo, 2010). Although there is still controversy regarding the role of the CB_2 receptor in the brain (Atwood and Mackie, 2010), specific functions for CB_2 receptors in neuropsychiatric conditions are currently emerging (Onaivi *et al.*, 2012).

Taking into account all these findings, the aim of the present study was to explore the effect of genetic (overexpression or knockout) or pharmacological (with the selective agonist JWH-133) manipulations of CB_2 receptors, on stress-induced excitotoxicity and neuroinflammation in mice.

Methods

Animals

All animal care and experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense, in accordance with European legislation (2003/65/EC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 75 animals were used in the experiments described here. Transgenic mice overexpressing CB_2 receptors (CB2xP) and CB_2 receptor knockout mice (CB2-KO) were kindly supplied by Dr. Jorge Manzanares (Instituto de Neurociencias, Universidad Miguel Hernández-CSIC Alicante, Spain), and their corresponding wild-type (WT) littermates (ICR –Swiss strain) by Harlan Iberica (Madrid, Spain). The mice were housed individually with standard temperature and humidity conditions in a 12 h light/dark cycle (lights on at 0800 h) 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

Male adult (8-12 weeks old) mice were exposed to sub-chronic immobilization and acoustic stress (2 h from 1300 to 1500 h for 4 days), as previously described (Kiank *et al.*, 2006). Stressed animals were given a lethal injection of sodium pentobarbital (320 $mg \cdot kg^{-1}$ i.p.) immediately after the last stress session (while still in the restrainer). Control animals were not submitted to stress but were handled at 1300 h for a

few seconds, food and water were removed for 2h and then killed as described above. Blood for plasma determinations was collected by cardiac puncture and anticoagulated with trisodium citrate [3.15% (w/v), 1 volume citrate per 9 volumes blood]. The mice were decapitated, the brain was removed from the skull and, after careful removal of the meninges and blood vessels, the frontal cortical areas from both brain hemispheres were excised and frozen at –80°C until assayed. The frontal cortex we used included the prefrontal cortex, cingulate cortex and motor cortex (M1 and M2); the cortical areas that we isolated were from Bregma 3mm to 0.5mm, approximately. The more lateral and ventral cortical structures (primary somatosensory cortex, granular insular cortex) were excluded. This brain area was chosen because of its relatively high levels of CB_2 receptors (Gong *et al.*, 2006) and its susceptibility to excitotoxic and neuroinflammatory processes elicited by stress (García-Bueno *et al.*, 2008). The frontal cortex is also an important neural substrate for the regulation of the responses of the HPA axis 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 (30 mg) were homogenized with 300 µL of buffer (composition, in mM; HEPES, 10; EDTA, 1; EGTA, 1; KCl, 10; DTT, 1; phenylmethylsulfonyl fluoride, 0.5; NaF, 5; $NaVO_4$, 1; sucrose, 0.5; and Na_2MoO_4, 10, with aprotinin, 0.1 µg L^{-1}; leupeptin, 1 µg L^{-1}; and Nα-p-tosyl-L-lysine-chloromethyl ketone, 1 µg mL^{-1}; pH 7.9). After 15 min, Nonidet P-40 (Roche®, Mannheim, Germany) was added to a final concentration of 0.5%. The tubes were gently vortexed for 15 s, and nuclei (in the pellet) were collected by centrifugation at 8000× *g* 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 13 000× *g* 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 (SLC1A2; EAAT-2; nomenclature follows Alexander *et al.*, 2013b), NOS-2 and COX-2, homogenates of the frontal cortex were used. In the case of the NF-κB subunit p65, the analysis was carried out in nuclear extracts of the homogenates and, for the inhibitory protein of NF-κB IκBα, cytosolic extracts were used (see above). After determining and adjusting protein levels, homogenates of frontal cortex were centrifuged (12 000× *g*, 20 min at 4°C) and the supernatant mixed with Laemmli sample buffer with β-mercaptoethanol (50 µL·mL^{-1} of Laemmli; Bio-Rad®, Hercules, CA, USA) and 20 µL of the mixture (containing 2 µg protein·μL^{-1}) was loaded into an electrophoresis gel. After separation, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Madrid, Spain) with a semi-dry transfer system (Bio-Rad) and were incubated with the specific antibodies shown below.

All antibodies were supplied by Santa Cruz Biotechnology (CA, USA). A rabbit polyclonal antibody against EAAT-2, raised against an epitope corresponding to amino acids 1-85 mapping at the N-terminus of EAAT-2 of human origin in a dilution of 1:1000 in 5% BSA in TBS-Tween (sc-15317); a rabbit polyclonal antibody against IκBα (epitope mapping at the C-terminus of IκBα of human origin) in a dilution of 1:1000 in 5% skimmed milk in BSA;a rabbit polyclonal antibody against NF-κB p65 (epitope mapping within the N-terminus of NF-κB p65 of human origin) in a dilution of 1:500 in BSA 2% (sc-109); a rabbit polyclonal antibody against NOS-2 raised against a peptide mapping at the amino terminus of NOS-2 of human origin in a dilution of 1:1000 in TBS-Tween (sc-651); e) a goat polyclonal antibody against COX-2 raised against a peptide mapping at the C-terminus of COX-2 of human origin in a dilution of 1:750 in 5% BSA in TBS-Tween (sc-1745).

After washing with 10 mM Tris-buffered saline containing 0.1% Tween-20 (Bio-Rad), the membranes were incubated with the respective HRP-conjugated secondary antibodies for 90 min at room temperature. Blots were imaged using an Odyssey® Fc System (Li-Cor Biosciences®, Lincoln, NE, USA) and were quantified by densitometry (NIH ImageJ® software; National Institutes of Health, Bethesda, MD, USA). All densitometries are expressed in arbitrary units of optical density. In all Western blot analyses, the housekeeping gene β-actin was used as loading control except for analysis 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 frontal cortex using TRIzol® reagent (Invitrogen®, Carlsbad, CA, USA); aliquots were converted to cDNA using random hexamer primers. Quantitative changes in mRNA levels were estimated by reverse transcription-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 (1:10 000 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 Supporting Information Appendix S1 for details about the primers used.

Preparation of synaptosomes

Synaptosomes were prepared from anterior cortical structures of the right hemisphere of the forebrain, discarding other brain areas such as the olfactory bulb or basal ganglia. The tissues were dissected on ice and all subsequent steps were performed at 4°C. Tissue was immediately homogenized in 25 volumes of 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 200× *g* for 10 min, and the supernatant was then collected and centrifuged at 20 000× *g* for 20 min. The pellet was resuspended in 0.32 M sucrose and centrifuged at 20 000× *g* for 20 min. The crude synaptosomal pellet was finally resuspended in 1 mL of 0.32 M sucrose.

[^{3}H]Glutamate uptake by synaptosomes

Sodium-dependent glutamate uptake by synaptosomes (see above) was measured, as follows. In brief, 25 µL of aliquots of synaptosomes were added to 250 µL of incubation buffer (5mM Tris, 10mM HEPES, 2.5mM KCl, 1.4M NaCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM KH_2PO_4, and 10mM dextrose, pH 7.4.) containing L-[^{3}H]glutamic acid 0.125 mM (1 mCi·mL^{-1}; Amersham Biosciences Europe GmbH, Freiburg, Germany) and incubated for 3 min at 37°C in a shaking bath. The reaction was terminated using 1 mL of ice-cold choline buffer (incubation buffer in which an equimolar concentration of choline chloride was substituted for NaCl), and the samples were centrifuged at 10 000× *g* for 2 min to recover the synaptosomes. The bound ^{3}H-radioactivity was measured using a liquid scintillation counter.

Plasma corticosterone levels

Plasma was obtained from blood samples by centrifuging the sample at 1000× *g* for 15 min immediately after stress. All plasma samples were stored at –20°C before assay. Corticosterone was measured by the RIA kit Coat-a-Count® (Siemens, Los Angeles, CA, USA) in a γ counter. The values obtained in the control animals (84±12 ng mL^{-1}) matched the kit manufacturer's expected values in adult mice at the time of blood collection (approximately 15:00h).

Levels of nitrite (NO_2^-)

The stable metabolite of the free radical NO, NO_2^- was assayed by the Griess method (Green *et al.*, 1982), measuring the optical density at 540nm in a microplate reader (Synergy 2; BioTek, Winooski, VT, USA).

Lipid peroxidation

Lipid peroxidation was measured using the thiobarbituric acid test for malondialdehyde (MDA) following the method described by Das and Ratty (1987) with some modifications. Frontal cortex was sonicated in 10 volumes of 50 mM phosphate buffer (pH 7.4) and deproteinized with 40% trichloroacetic acid and 5 mol·L^{-1} HCl, followed by the addition of 2% (w/v) thiobarbituric acid in 0.5 mol·L^{-1} NaOH. The reaction mixture was heated in a water bath at 90°C for 15 min and centrifuged at 12 000× *g* for 10 min. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer (Beckman® Coulter, Brea, CA, USA).

PGE_2 levels in frontal cortex

PGE_2 levels were measured by enzyme immunoassay (EIA) using reagents in kit form (PGE_2 EIA Kit-Monoclonal, Cayman Chemical®, Tallin, Estonia). Samples of frontal cortex (20 mg) were sonicated in 300 µL of homogenization buffer (0.1 M phosphate buffer, pH 7.4, 1 mM EDTA and 10 µM indomethacin) and purified by incubation with ethanol (4 x sample volume) for 5 min at 4°C and then centrifugation at 3000× *g* for 10 min. Extracts 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. PGE_2 was eluted with ethyl acetate. Samples were then evaporated to dryness under nitrogen and resuspended in EIA buffer. Levels of PGE_2 were measured at 405 nm following the manufacturer's instructions. The sensitivity of the assay for PGE_2 was 15 pg ml^{-1}; intra- and interassay coefficients of variation were 6.6% and 15.5%, respectively, at 62.5 pg ml^{-1}.

Pharmacological tools

The selective CB_2 receptor agonist, JWH-133 (6aR, 10aR)-6,6,9-trimethyl-3-(2-methylpentan-2-yl)-6a,7,10,10a-tetrahydrobenzo[c]chromene (Tocris Bioscience®, Bristol, UK) was given (i.p.) to groups of WT and CB2-KO mice at the onset of each session of the stress (1300 h). JWH-133 is a potent CB_2 receptor agonist (K_i = 3.4 nM), 200-fold selective over CB_1 receptors (Pertwee, 1997; Huffman *et al.*, 1999). The dose (2 mg·kg^{-1}) was chosen ,based on previous *in vivo* studies of neuroprotection in mice (Zarruk *et al.*, 2012). The compound was dissolved in DMSO : Tween : PBS (1:1:18), and the total volume injected into each animal was 200 µL. Control (un-stressed) and stressed WT animals, injected with vehicle, were used to allow for the stress produced by the injection and the possible effects of the vehicle used on neuroinflammatory parameters.

Protein assay

Protein levels were measured using Bradford's method (Bradford, 1976).

Data analysis

Data in text and figures are expressed as mean ± SEM. Results from the assays of glutamate uptake by synaptosomes were analysed with one-way ANOVA followed by Dunnett's *post hoc* test (all groups against control). For multiple comparisons, a two-way ANOVA followed by the Bonferroni *post hoc* test was used, considering as the first factor the presence or absence of stress and, as the second, the presence or absence of pharmacological or genetic manipulations of the CB_2 receptor (receptor overexpression or deletion). To confirm that the anti-inflammatory actions of JWH-133 were dependent on activation of CB_2 receptors, a two-way ANOVA followed by the Bonferroni *post hoc* test was used, 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.

Materials

Unless otherwise stated, all chemicals were obtained from Sigma, Madrid, Spain.

Results

CB_2 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 by inhibiting glutamatergic excitotoxicity, through a CB_1 receptor-related mechanism (Zoppi *et al.*, 2011). We explored the effects of CB_2 receptors on glutamate transport in frontal

cortical synaptosomes from WT, WT + JWH-133, CB2xP and CB2-KO mice under control and stress conditions.

Treatment of WT mice with JWH-133 (JWH group) or overexpression of CB_2 receptors (CB2xP group) increased the control levels of glutamate uptake (Figure 1A,B). As previously described (García-Bueno *et al.*, 2007), exposure to stress markedly decreased glutamate uptake, compared with the control, unstressed, WT group (CWT) (Figure 1A–C). Stress also decreased glutamate uptake in the JWH or CB2xP groups, but only down to the levels observed in the CWT group [JWH $F(1, 21) = 7.95$, $P = 0.014$; CB2xP $F(1, 21) = 9.87$, $P = 0.0003$].

The absence of CB_2 receptors (CB2KO group) did not modify the glutamate uptake by synaptosomes, compared with WT mice, in either control or stress conditions, with stress decreasing uptake in the CB2-KO mice, as it did in the WT mice (Figure 1C) [CB2-KO $F(1, 21) = 4.02$, $P = 0.0236$].

We also measured the levels of the major brain glutamate transporter, EAAT-2, in unstressed, WT and CB2xP and CB2-KO mice and found no changes at protein level (data not shown).

CB_2 receptor effects on HPA axis activity: plasma corticosterone levels

Corticosterone is the main stress hormone in rodents and is widely known as a classical regulator of the inflammatory and immune response in brain and periphery (Madrigal *et al.*, 2006). In our model, exposure to stress increased plasma corticosterone equally in all groups of animals, compared with their respective controls (Figure 2) [stress $F(1, 19) = 120.24$, $P < 0.0001$; JWH treatment $F(1, 19) = 1.93$, $P = 0.184$; stress × JWH treatment $F(1, 19) = 2.54$, $P = 0.1305$]; [stress $F(1, 19) = 36.9$, $P < 0.0001$; CB2xP genotype $F(1, 19) = 1.15$, $P = 0.2963$; stress × CB2xP genotype $F(1, 19) = 0.62$, $P = 0.4416$]; [stress $F(1, 17) = 105.99$; CB2-KO genotype $F(1, 17) = 1.14$, $P = 0.3046$; stress × CB2-KO genotype $F(1, 17) = 1.25$, $P = 0.2833$].

Anti-inflammatory effects elicited by activation of CB_2 receptors: mechanisms involved

The ECS has been proposed as an endogenous protective system against excessive inflammatory and immune responses in several CNS pathologies (Wolf *et al.*, 2008). We therefore assayed a number of proinflammatory factors in our model.

Pro-inflammatory cytokines and chemokines in brain. The pro-inflammatory cytokine TNF-α and the chemokine CCL2 (Madrigal *et al.*, 2006; Conductier *et al.*, 2010), are the first pro-inflammatory mediators to be activated in the brain after exposure to stress. We found, by PCR, a consistent increase in mRNA for TNF-α in samples of the frontal cortex from WT mice after stress, an effect that was blocked by JWH and in CB2xP mice (Figure 3A,B) [stress $F(1, 14) = 53.64$, $P < 0.0001$; JWH treatment $F(1, 14) = 84.45$, $P < 0.0001$; stress × JWH treatment $F(1, 14) = 25.93$, $P = 0.0003$]; [stress $F(1, 14) = 108.89$, $P < 0.0001$; CB2xP genotype $F(1, 14) = 6.05$, $P = 0.032$; stress × CB2xP genotype $F(1, 14) = 16.8$, $P = 0.0018$]. However, in CB2-KO mice, stress increased the mRNA for TNF-α mRNA (Figure 3C) [stress $F(1, 14) = 33.43$, $P = 0.0002$; CB2-KO genotype $F(1, 14) = 11.49$, $P = 0.0069$; stress × CB2-KO genotype $F(1, 14) = 0.53$, $P = 0.4826$].

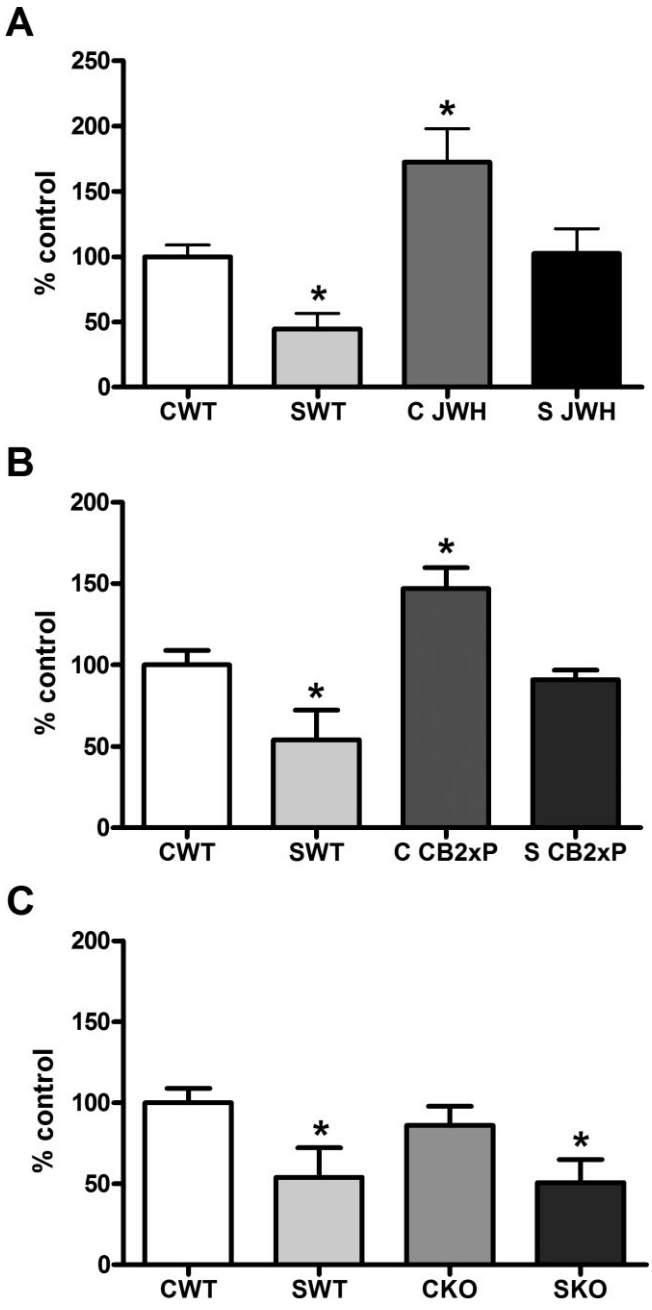

Figure 1

CB_2 receptor effects on glutamate uptake mechanisms. (A) Glutamate uptake in frontal cortical synaptosomes from WT mice, with (SWT) or without stress (CWT). The effects of treatment of WT mice with JWH-133, before (C JWH) or after stress (S JWH) is also shown. In (B), the corresponding data from CB2xP mice before (CCB2xP) and after stress (S CB2xP) are shown. In (C), data from CB2-KO mice before (CKO) and after stress (SKO) are shown. The data shown are the means ± SEM of six mice in each group. *$P < 0.05$ versus CWT; one-way ANOVA with Dunnett's multiple comparison post test.

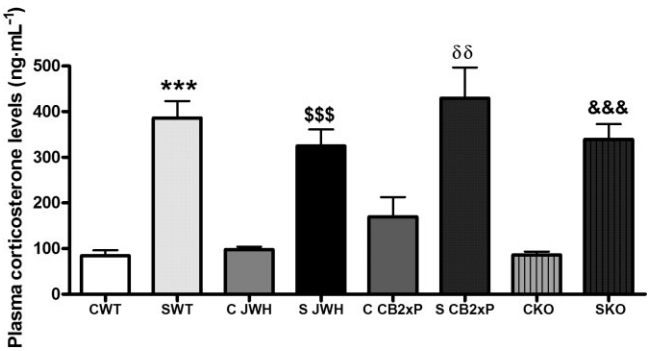

Figure 2

CB_2 receptor effects on plasma corticosterone levels. Corticosterone levels in plasma (ng·mL^{-1}) at the time of blood sampling (1500 h) are shown for all experimental groups and conditions (indicated as in Figure 1). Exposure to stress increased plasma corticosterone in all groups, relative to their corresponding control levels. The data represent the mean ± SEM of six mice. ***$P < 0.001$ versus CWT; $^{\$\$\$}P < 0.001$ versus C JWH; $^{\delta\delta}P < 0.01$ versus C CB2xP; $^{\&\&\&}P < 0.001$ versus CKO; two-way ANOVA with Bonferroni post test.

A slightly different profile was found for the chemokine CCL2. Exposure to stress increased mRNA for CCL2 in the frontal cortex of WT mice and this was blocked only by the overexpression of CB_2 receptors, not by JWH-133 treatment (Figure 3D,E) [stress $F(1, 18) = 22.53$, $P = 0.0003$; JWH treatment $F(1, 18) = 1.45$, $P = 0.247$; stress × JWH treatment $F(1, 18) = 0.13$, $P = 0.7204$]; [stress $F(1, 18) = 9.97$, $P = 0.007$; CB2xP genotype $F(1, 18) = 5.58$, $P = 0.0331$; stress × CB2xP genotype $F(1, 18) = 1.16$, $P = 0.30$]. Stressed CB2-KO mice presented higher levels of CCL2 mRNA, compared with SWT group (Figure 3F) [stress $F(1, 17) = 19.5$, $P = 0.0006$; CB2-KO genotype $F(1, 17) = 11.36$, $P = 0.0046$; stress × CB2-KO genotype $F(1, 17) = 1.35$, $P = 0.2655$].

NF-κB. The release of TNF-α after stress activates the NF-κB transcription factor (Madrigal *et al.*, 2002) in the cortex. In our present model, we found that exposure to stress decreased the expression of IκBα in cytosolic extracts of frontal cortex from WT mice. This effect was reversed in CB2xP mice and in WT mice treated with JWH-133 (Figure 3G,H) [stress $F(1, 13) = 9.84$, $P = 0.01$; JWH treatment $F(1, 13) = 86.39$, $P < 0.0001$; stress × JWH treatment $F(1, 13) = 68$, $P < 0.0001$]; [stress $F(1, 27) = 24.8$, $P < 0.0001$; CB2xP genotype $F(1, 27) = 14.96$, $P = 0.0007$; stress × CB2xP genotype $F(1, 27) = 24.8$, $P < 0.0001$]. Levels of the pro-inflammatory NF-κB subunit p65 in nuclear extracts of frontal cortex from different groups studied (Figure 3J,K) mirrored the expression of IκBα, with increased levels in stressed WT mice and blockade in the JWH and CB2xP groups [stress $F(1, 30) = 4.73$, $P = 0.0386$; JWH treatment $F(1, 30) = 10.7$, $P = 0.0029$; stress × JWH treatment $F(1, 30) = 6.96$, $P = 0.0137$]; [stress $F(1, 60) = 4.01$, $P = 0.05$; CB2xP genotype $F(1, 60) = 5.94$, $P = 0.018$; stress × CB2xP genotype $F(1, 60) = 9.04$, $P = 0.0039$].

In CB2-KO mice, the basal IκBα protein levels in frontal cortex were lower than in the WT mice but, after stress, CB2-KO mice presented similar levels to those from the WT group (Figure 3I) [stress $F(1, 22) = 7.72$, $P = 0.013$; CB2-KO genotype $F(1, 22) = 7.22$, $P = 0.0156$; stress × CB2-KO genotype $F(1, 22) = 13.7$, $P = 0.0018$]. In addition, p65 protein expression in nuclear extracts was increased in CB2-KO mice in both control and stress conditions (Figure 3L), suggesting a state of chronic NF-κB activation in these animals [stress $F(1, 23) = 6.03$, $P = 0.024$; CB2-KO genotype $F(1, 23) = 127.75$, $P < 0.0001$; stress × CB2-KO genotype $F(1, 23) = 2.04$, $P = 0.1705$].

Pro-inflammatory enzymes (NOS-2 and COX-2). NF-κB regulates the expression of genes involved in the production of oxidative, nitrosative and inflammatory mediators after stress exposure (Madrigal *et al.*, 2006). Two major pro-inflammatory, NF-κB dependent, enzymes are NOS-2 and COX-2. Their products (NO and PGE_2, respectively) are potent oxidant and pro-inflammatory molecules that are associated with damage and even cell death in many CNS pathologies, including those related to stress (García-Bueno *et al.*, 2008).

We found that NOS-2 and COX-2 expression was increased in the frontal cortex, following stress in WT mice (Figure 4A,D) and treatment of the WT with JWH-133 or the overexpression of CB_2 receptors completely blocked this NOS-2 up-regulation (Figure 4A,B). As seen with other pro-inflammatory mediators, the CB2-KO mice, both in control and stress conditions, showed a consistent NOS-2 up-regulation, compared with the WT control groups, and a higher increase after stress (Figure 4C) [stress $F(1, 19) = 6.07$, $P = 0.0255$; JWH treatment $F(1, 19) = 8.08$, $P = 0.0118$; stress × JWH treatment $F(1, 19) = 1.76$, $P = 0.2027$]; [stress $F(1, 16) = 4.5$, $P = 0.0495$; CB2xP genotype $F(1, 16) = 18.51$, $P = 0.0013$; stress × CB2xP genotype $F(1, 16) = 44.25$, $P < 0.0001$]; [stress $F(1, 21) = 4.56$, $P = 0.047$; CB2-KO genotype $F(1, 21) = 10.77$, $P < 0.0041$; stress × CB2-KO genotype $F(1, 21) = 0.91$, $P = 0.35$].

The interaction between CB_2 receptors and COX-2 was more complex. In the frontal cortex, levels of PGE_2, a major product of COX-2 in the brain, were increased after stress exposure in WT mice (Figure 4G). In addition, the pharmacological activation and overexpression of CB_2 receptors reduced PGE_2 levels, compared with those in the stressed WT animals (Figure 4G,H). However, COX-2 up-regulation produced by stress was only blocked in CB2xP mice (Figure 4E). As it can be observed in Figure 4I, CB2-KO animals exhibited higher levels of PGE_2, with and without stress, compared with the corresponding WT groups, but no comparable changes of COX-2 protein were found (Figure 4F). For COX-2, [stress $F(1, 16) = 3.9$, $P = 0.07$; JWH treatment $F(1, 16) = 0.13$, $P = 0.722$; stress × JWH treatment $F(1, 16) = 0.5$, $P = 0.49$]; [stress $F(1, 18) = 4.22$, $P = 0.05$; CB2xP genotype $F(1, 18) = 6.28$, $P = 0.0233$; stress × CB2xP genotype $F(1, 18) = 5.94$, $P = 0.0278$]; [stress $F(1, 43) = 5.21$, $P = 0.028$; CB2-KO genotype $F(1, 43) = 2.12$, $P = 0.1534$; stress × CB2-KO genotype $F(1, 43) = 0.86$, $P = 0.36$]. For PGE_2, [stress $F(1, 17) = 4.5$, $P = 0.05$; JWH treatment $F(1, 17) = 12.4$, $P = 0.0034$; stress × JWH treatment $F(1, 17) = 7.71$, $P = 0.015$]; [stress $F(1, 16) = 4.96$, $P = 0.042$; CB2xP genotype $F(1, 16) = 26.03$, $P = 0.0002$; stress × CB2xP genotype $F(1, 16) = 4.74$, $P = 0.0485$]; [stress $F(1, 28) = 5.65$, $P = 0.025$; CB2-KO genotype $F(1, 28) = 14.13$, $P = 0.0009$; stress × CB2-KO genotype $F(1, 28) = 0.02$, $P = 0.90$].

Lipid peroxidation. As a final index of stress-induced cellular damage, we measured the accumulation of the lipid

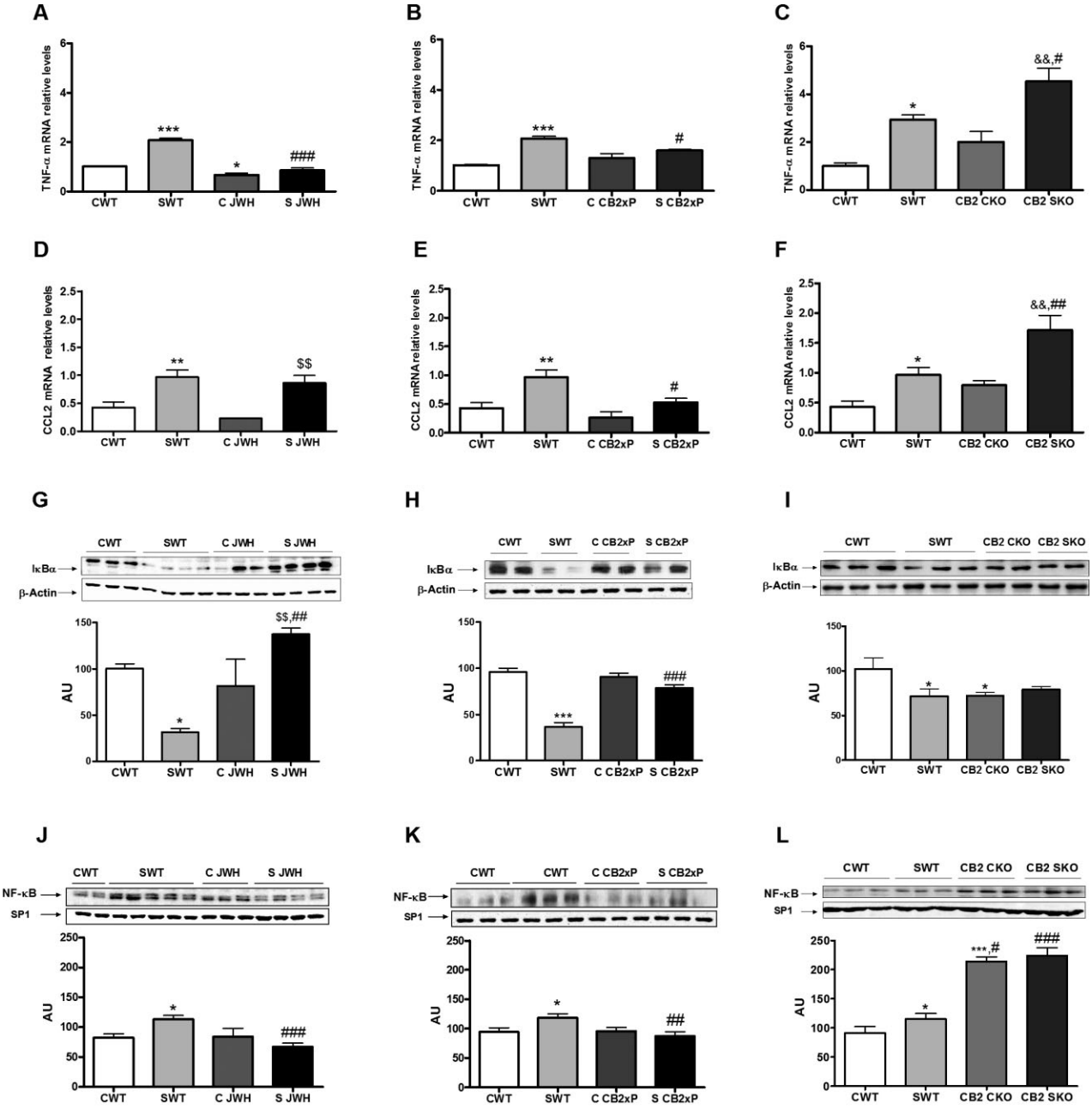

Figure 3

Anti-inflammatory effects of CB_2 receptors in the frontal cortex. Pro-inflammatory cytokines, chemokines and NF-κB in homogenates of the frontal cortex. Quantitative PCR (qPCR) analysis of mRNA for TNF-α. (A) Data are shown for untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (B), results from CB2xP mice, with and without stress and in (C) for CB2-KO mice, with and without stress. Data are normalized by tubulin and are representative of three experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus CWT; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus SWT; $^{\&\&}P < 0.01$ versus CKO; two-way ANOVA with Bonferroni post test. In (D), data for mRNA for CCL2 from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (E), results from CB2xP mice, with and without stress and in (F) for CB2-KO mice, with and without stress. Data are normalized by tubulin and are representative of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus CWT; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ versus SWT; $^{\$\$}P < 0.01$ versus C JWH; $^{\&\&}P < 0.01$ versus CKO; two-way ANOVA with Bonferroni post test. (G) Western blot and densitometric analysis of the NF-κB inhibitory protein IκBα in cytosolic extracts of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (H), results from CB2xP mice, with and without stress and in (I) for CB2-KO mice, with and without stress. Data are normalized by β-actin (lower band) and are representative of three experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus CWT; $^{\#\#}P < 0.01$ versus SWT; $^{\$\$}P < 0.05$ versus C JWH; two-way ANOVA with Bonferroni post test. (J) Western blot and densitometric analysis of the NF-κB pro-inflammatory subunit p65 in nuclear extracts of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (K), results from CB2xP mice, with and without stress and in (L) for CB2-KO mice, with and without stress. Data are normalized by β-actin (lower band) and are representative of three experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ versus CWT; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus SWT ; two-way ANOVA with Bonferroni post test. AU, arbitrary units.

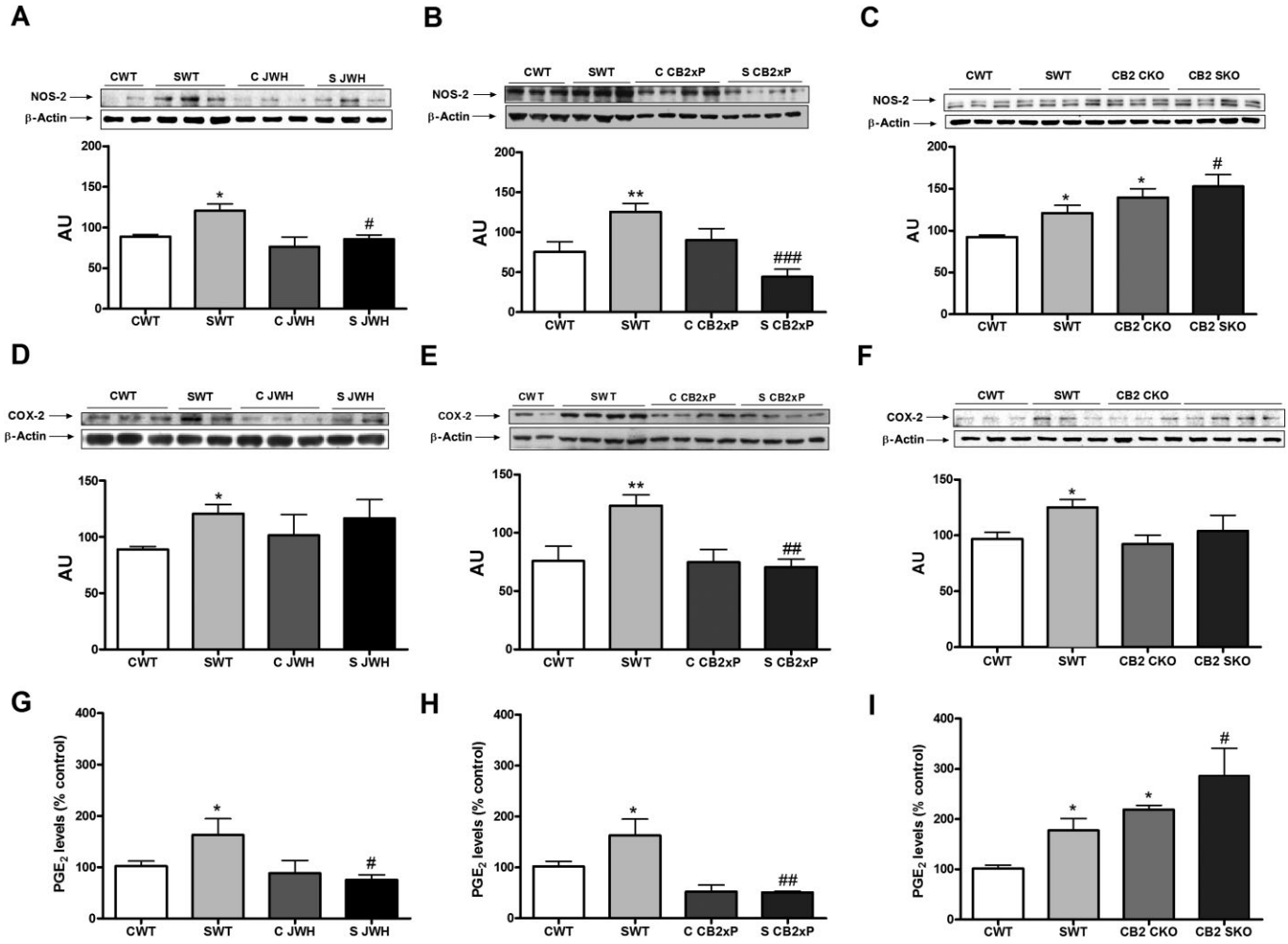

Figure 4

Anti-inflammatory effects of CB_2 receptors in the frontal cortex; pro-inflammatory enzymes. (A) Western blot and densitometric analysis of NOS-2 in homogenates of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (B), results from CB2xP mice, with and without stress and in (C) for CB2-KO mice, with and without stress. Data are normalized by β-actin (lower band) and are representative of three experiments. *$P < 0.05$, **$P < 0.01$ versus CWT; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus SWT; two-way ANOVA with Bonferroni post test. (D) Western blot and densitometric analysis of COX-2 in homogenates of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (E), results from CB2xP mice, with and without stress and in (F) for CB2-KO mice, with and without stress. Data are normalized by β-actin (lower band) and are representative of three experiments. *$P < 0.05$, **$P < 0.01$ versus CWT; $^{\#\#}P < 0.01$ versus SWT; two-wayANOVA with B onferroni post test. (G) Levels of PGE_2 in homogenates of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (H), results from CB2xP mice, with and without stress and in (I) for CB2-KO mice, with and without stress. The data represent the mean ± SEM of six mice. *$P < 0.05$ versus CWT; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus SWT; two-way ANOVA with Bonferroni post test. AU, arbitrary units.

peroxidation marker MDA and of the NO stable metabolite of NO, nitrites (NO_2^-), in the frontal cortex of the experimental groups. Stress exposure caused a smaller accumulation of MDA in CB2xP and JWH-treated WT mice (Figure 5A,B) [stress $F(1, 23) = 4.7$, $P = 0.042$; JWH treatment $F(1, 23) = 30.08$, $P < 0.0001$; stress × JWH treatment $F(1, 23) = 15.32$, $P = 0.0009$]; [stress $F(1, 25) = 4.81$, $P = 0.039$; CB2xP genotype $F(1, 25) = 4.33$, $P = 0.0492$; stress × CB2xP genotype $F(1, 25) = 4.97$, $P = 0.036$]. Conversely, CB2-KO mice presented higher levels of MDA after stress than the WT group (Figure 5C) [stress $F(1, 19) = 15.61$, $P = 0.011$; CB2-KO genotype $F(1, 19) = 5.1$, $P = 0.038$; stress × CB2-KO genotype $F(1, 19) = 0.71$, $P = 0.41$].

The results for NO_2^- (Figure 5D–F) followed a similar pattern in all groups of mice studied, with the exception of the CB2xP mice where there was a significant over-production of NO_2^-, possibly related to a compensatory mechanism, which may be worth further exploration (Figure 5E) [stress $F(1, 18) = 9.79$, $P = 0.007$; JWH treatment $F(1, 18) = 12.86$, $P = 0.0027$; stress × JWH treatment $F(1, 18) = 7.28$, $P = 0.0165$]; [stress $F(1, 19) = 10.91$, $P = 0.0045$; CB2xP genotype $F(1, 19) = 14.43$, $P = 0.0016$; stress × CB2xP geno-

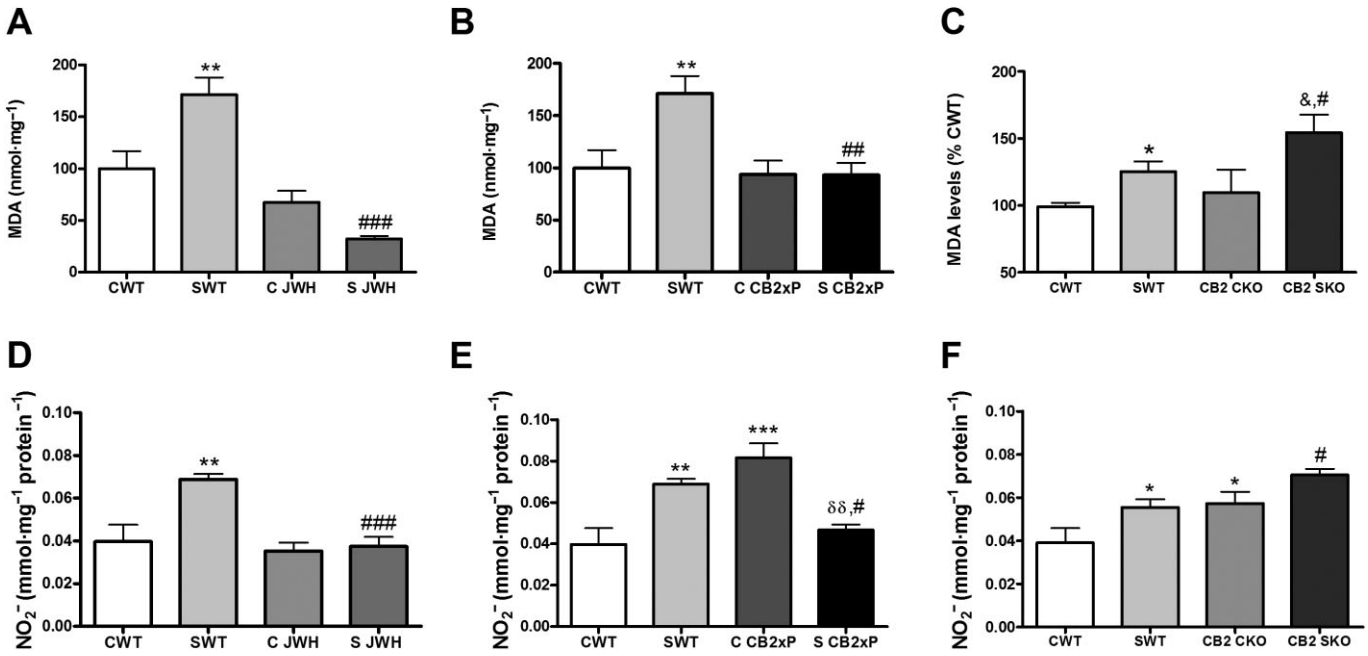

Figure 5

Neuroprotective effects mediated by CB_2 receptors against stress-induced oxidative and nitrosative cellular damage. In (A), MDA levels in homogenates of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (B), results from CB2xP mice, with and without stress and in (C) for CB2-KO mice, with and without stress. Data represent the mean ± SEM of six mice. *$P < 0.05$, **$P < 0.01$ versus CWT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus SWT; $^{\&}P < 0.05$ versus CKO; two-way ANOVA with Bonferroni post test. In (D), nitrite (NO_2^-) levels in homogenates of the frontal cortex from untreated WT mice and WT mice treated with JWH-133, both with and without stress. In (E), results from CB2xP mice, with and without stress and in (F) for CB2-KO mice, with and without stress. Data represent the mean ± SEM of six mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus CWT; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus SWT; $^{\delta\delta}P < 0.01$ versus C CB2xP; two-way ANOVA with Bonferroni post test.

type $F(1, 19) = 8.98$, $P = 0.0085$]; [stress $F(1, 29) = 11.25$, $P = 0.0025$; CB2-KO genotype $F(1, 29) = 14.07$, $P = 0.0009$; stress × CB2-KO genotype $F(1, 29) = 0.15$, $P = 0.70$].

Confirmation of the mediation of the effects of JWH-133 by CB_2 receptors. Finally, to confirm that the actions of JWH-133 were mediated by CB_2 receptors, we examined its effects on some representative inflammatory or oxidative parameters (IκBα and NOS-2 expression, and MDA and NO_2^- levels) in CB2-KO mice, exposed to stress. As shown in Figure 6A–D, treatment with JWH-133 did not alter any of the responses to stress in CB2-KO mice, suggesting the direct involvement of CB_2 receptors in our model. For IκBα Western blot data [JWH treatment $F(1, 19) = 15.85$, $P = 0.0026$; CB2-KO genotype $F(1, 19) = 71.10$, $P < 0.0001$; JWH treatment × CB2-KO genotype $F(1, 19) = 30.83$, $P = 0.002$]. For NOS-2 [JWH treatment $F(1, 19) = 9.50$, $P = 0.0095$; CB2-KO genotype $F(1, 19) = 69.17$, $P = 0.001$; JWH treatment × CB2-KO genotype $F(1, 19) = 4.78$, $P = 0.049$]. For NO_2^- [JWH treatment $F(1, 19) = 9.81$, $P = 0.0069$; CB2-KO genotype $F(1, 19) = 29.63$, $P < 0.0001$; JWH treatment × CB2-KO genotype $F(1, 19) = 0.63$, $P = 0.4407$]. For MDA [JWH treatment $F(1, 19) = 11.12$, $P = 0.0049$; CB2-KO genotype $F(1, 19) = 25.21$, $P = 0.0002$; JWH treatment × CB2-KO genotype $F(1, 19) = 0.43$, $P = 0.5216$].

Discussion and conclusions

Our results indicate a general anti-inflammatory role for CB_2 receptors in the frontal cortex of mice exposed to sub-chronic restraint and acoustic stress. JWH-133 treatment or overexpression of CB_2 receptors resulted in an increase in control levels of glutamate uptake, which was then reduced by stress, back to control levels. These effects are not due to changes in the general response to stress, as the different manipulations of the CB_2 receptors did not modify plasma corticosterone levels, in our model.

Although previous results suggested that the excitotoxic process in stress conditions was regulated by CB_1 receptors (Zoppi *et al.*, 2011), other authors have demonstrated a role for CB_1 and CB_2 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 model, we did not find changes in EAAT-2 protein expression. Other approaches such as the determination of glutamate levels in the tissue will help to show whether the effects of CB_2 receptor manipulations on glutamate uptake are due to inflammation-related actions on EAAT-2 activity or to a direct effect of the CB_2 receptor in neurons.

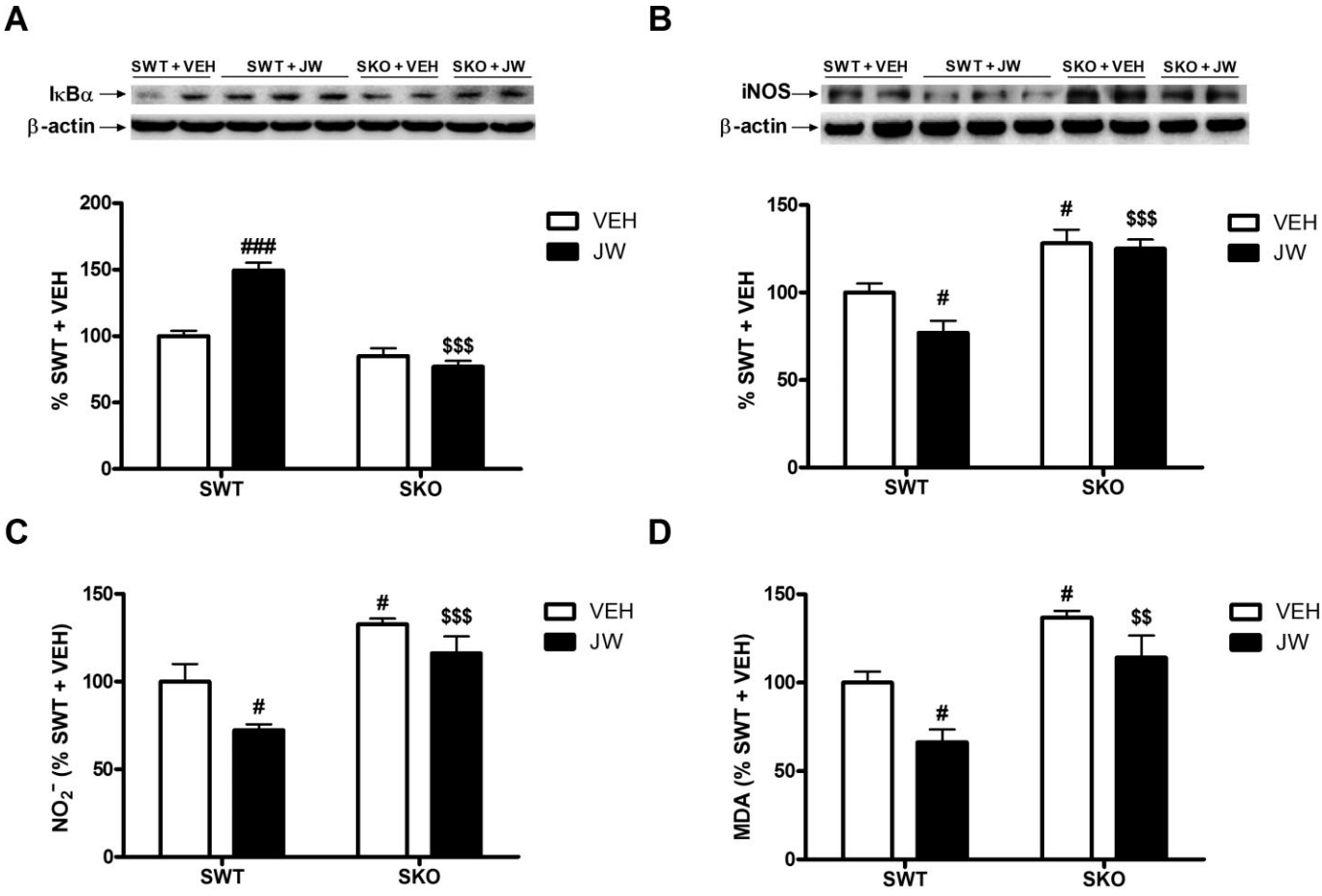

Figure 6

Confirmation that effects of JWH-133 were mediated by CB_2 receptors. Western blot and densitometric analysis of the NF-κB inhibitory protein IκBα (A) in extracts of the frontal cortex from stressed WT mice and CB2-KO mice, both with and without JWH-133. In (B), the corresponding data for NOS-2. Data are normalized by β-actin (lower band) and are representative of three experiments. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus SWT + VEH; $^{\$\$\$}P < 0.001$ versus SWT + JW; two-way ANOVA with Bonferroni post test. In (C), results for nitrite levels in homogenates of the frontal cortex from stressed WT mice and CB2-KO mice, both with and without JWH-133. In (D) the corresponding data for MDA, with and without JWH-133. $^{\#}P < 0.05$ versus SWT + VEH; $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ versus SWT + JW; two-way ANOVA with Bonferroni post test.

According to our results, the CB_2 receptors were not directly involved in the mechanism(s) controlling the production of plasma corticosterone, using this experimental protocol of stress. In agreement with this, increased plasma corticosterone elicited by systemic endotoxin administration did not change after the pharmacological modulation of CB_2 receptors (Roche *et al.*, 2006). However, the expression of these receptors in stress-responsive neural circuits , such as the hippocampus, amygdala and hypothalamus, indirectly suggests that CB_2 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 block of the stress-induced increase in the mRNA for corticotropin-releasing factor in the paraventricular nucleus of the hypothalamus (García-Gutiérrez and Manzanares, 2011). Thus, more detailed neuroendocrine studies regarding the time course of synthesis and release of corticosterone and other stress hormones in the subchronic stress protocol used here are needed to exclude a role of CB_2 receptors in the regulation of the activity of the HPA axis.

Classically, because of its high level of expression in diverse types of immune cells and organs (Klein *et al.*, 2003), CB_2 receptors mediating anti-inflammatory effects have been described in the periphery. However, anti-inflammatory effects of CB_2 receptor agonists in the CNS have been shown in traumatic brain injury, spinal cord injury, stroke and EAE (Arévalo-Martín *et al.*, 2003; Mechoulam and Shohami, 2007; Castillo *et al.*, 2010; Adhikary *et al.*, 2011; Zarruk *et al.*, 2012). Consistent with these findings, CB_2 receptors are 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). Here, we have demonstrated that CB_2 receptor activation regulated stress-induced neuroinflammation at several levels in the frontal cortex. This anti-inflammatory profile is especially relevant considering that neuroinflammatory processes have been proposed to underlie the patho-

physiology of several stress-related neuropsychiatric disorders (Madrigal *et al.*, 2006; Wager-Smith and Markou, 2011).

The regulation by CB_2 receptors of the levels of pro-inflammatory cytokines (TNF-α) in the brain has been extensively documented (Jean-Gilles *et al.*, 2010). However, less is known about the inhibitory role of CB_2 receptors on production of CCL2 *in vivo* and only recently an inhibitory effect of CB_2 receptor activation on CCL2 mRNA levels in animal models of stroke and multiple sclerosis has been reported (Palazuelos *et al.*, 2008; Zarruk *et al.*, 2012). These results are especially relevant considering that CCL2 is implicated in inflammatory cell migration into inflamed tissues (CNS included) and nociception – processes that have been related to CB_2 receptors (Miller and Stella, 2008; Racz *et al.*, 2008; Adhikary *et al.*, 2011)

Our results are in agreement with other authors who demonstrated that CB_2 receptor activation inhibited the activity of the major inflammatory mediator NF-κB, in immune cells (macrophages and microglia) *in vitro,* exposed to inflammatory and immune stimuli (Jeon *et al.*, 1996; Correa *et al.*, 2010). However, to avoid the oversimplification of the effects of CB_2 receptors on TNF-α and NF-κB, it should be noted that some studies have shown 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 CB_2 receptor activation on NOS-2 have been studied in animal models of neuropathology *in vivo*, such as Huntington's disease or stroke (Palazuelos *et al.*, 2009; Zarruk *et al.*, 2012), but the interactions between CB_2 receptors and COX 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 PGE_2 synthases, all of which remain to be assessed. Indeed, COX-2 inhibition following activation of CB_2 receptors 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 the over-production of consecutive pro-inflammatory mediators, oxidative and nitrosative cellular damage is produced after stress exposure. Our results suggest that any potential therapeutic use of CB_2 receptor activation would utilise its antioxidant profile. Similarly, antioxidant effects mediated by CB_2 receptors have also been found in CB2xP mice in an experimental model of Parkinson's disease (Ternianov *et al.*, 2012). The antioxidant effects produced by the pharmacological activation of CB_2 receptors have been extensively reviewed for several neurological or neurodegenerative diseases (Fernández-Ruiz, 2009).

CB2xP mice and pretreatment with JWH-133 in WT mice generate different experimental models, because CB2xP mice overexpress CB_2 receptors not only in glia but also in neurons, and present endocrine and/or peripheral alterations, such as hyperglycaemia (Romero-Zerbo *et al.*, 2012). Such alterations could limit the conclusions drawn from the use of CB2xP mice. Under the conditions of the present study, the anti-inflammatory profile of both experimental groups was very similar, with the exception of COX-2 protein levels, which were resistant to the effects of treatment with JWH-133, whereas CB2xP mice presented a clear decrease of COX-2 protein. CB2xP mice present an up-regulation of CB_2 receptors on frontal cortex neurons, an effect that could affect the characteristic constitutive expression of COX-2 in this brain area (Yamagata *et al.*, 1993).

Although JWH-133 exhibits a higher affinity for CB_2 than for CB_1 receptors (Huffman *et al.*, 1999), this compound is only selective not specific for CB_2 receptors. This is why we tested JWH-133 in CB2-KO animals. Although the anti-inflammatory effects of JWH-133 were absent in CB2-KO animals, we exclude the possibility that this compound elicits CB_2 receptor-independent effects, using different doses or routes of administration, or in other models of neuropathology.

In contrast to the anti-inflammatory effects of the CB_2 receptor agonist JWH-133, the CB2-KO mice presented an enhanced neuroinflammatory response in the frontal cortex, after stress exposure. Deletion of the CB_2 receptors also induced 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 CB2-KO mice is directly related to the pathophysiology of these major psychiatric diseases or is merely an epiphenomenon.

This study has some limitations. First, although it was not the main goal of this work, assessment of the role(s) of CB_2 receptors in the regulation of HPA axis activation requires more detailed neuroendocrine studies, 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 CB_2 receptors. Third, studies carried out in extended stress exposure models, which induce depressive-like behaviours (e.g. two chronic mild stresses, chronic unpredictable stress) would strengthen translational conclusions.

In conclusion, we have found evidence of anti-inflammatory effects of CB_2 receptor activation, which were not related to alterations in plasma corticosterone levels. Activation of CB_1 receptors elicits a consistent neuroprotective response in the same stress paradigm (Zoppi *et al.*, 2011), but direct activation of CB_2 receptors produces less undesired central effects (e.g. psychoactive effects) (Mechoulam and Parker, 2013). Our previous and current findings open possibilities for the use of dual agonists of CB_1 and CB_2 receptors or of endocannabinoid reuptake inhibitors, for the management of neurological and neurodegenerative and neuropsychiatric diseases.

Acknowledgements

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Appendix S1 Details of experimental procedures.