

Activation of type-2 cannabinoid receptor inhibits neuroprotective and antiinflammatory actions of glucocorticoid receptor α : when one is better than two

Elisa Bisicchia · Valerio Chiurchiù · Maria Teresa Viscomi ·
Laura Latini · Filomena Fezza · Luca Battistini ·
Mauro Maccarrone · Marco Molinari

Received: 12 September 2012 / Revised: 21 November 2012 / Accepted: 20 December 2012
© Springer Basel 2013

Abstract Endocannabinoids (eCBs) and glucocorticoids (GCs) are two distinct classes of signaling lipids that exert both neuroprotective and immunosuppressive effects; however, the possibility of an actual interaction of their receptors [i.e., type-2 cannabinoid (CB₂) and glucocorticoid receptor α (GR α), respectively] remains unexplored. Here, we demonstrate that the concomitant activation of CB₂ and GR α abolishes the neuroprotective effects induced by each receptor on central neurons and on glial cells in animal models of remote cell death. We also show that the ability of eCBs and GCs, used individually, to inhibit tumour necrosis

factor- α (TNF- α) and interferon- γ (IFN- γ) production from activated human T lymphocytes is lost when CB₂ and GR α are activated simultaneously. In addition, signal transduction pathways triggered by concomitant activation of both receptors led to increased levels of GR β , heat-shock proteins-70 and -90, and p-JNK, as well as to reduced levels of p-STAT6. These effects were reversed only by selectively antagonizing CB₂, but not GR α . Overall, our study demonstrates for the first time the existence of a CB₂-driven negative cross-talk between eCB and GC signaling in both rats and humans, thus paving the way to the possible therapeutic exploitation of CB₂ as a new target for chronic inflammatory and neurodegenerative diseases.

E. Bisicchia and V. Chiurchiù contributed equally to the work.

M. Maccarrone and M. Molinari were equally senior authors.

Electronic supplementary material The online version of this article (doi:10.1007/s00018-012-1253-5) contains supplementary material, which is available to authorized users.

E. Bisicchia · V. Chiurchiù
Department of Biomedical Sciences, University of Teramo,
Piazza A. Moro 45, 64100 Teramo, Italy

E. Bisicchia · V. Chiurchiù · M. T. Viscomi · L. Latini · F. Fezza ·
L. Battistini · M. Maccarrone · M. Molinari
I.R.C.C.S. Santa Lucia Foundation, Via del Fosso di Fiorano 64,
00143 Rome, Italy

F. Fezza
Department of Experimental Medicine and Surgery, University
of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy

M. Maccarrone
Center of Integrated Research, Campus Bio-Medico University
of Rome, Via Alvaro del Portillo 21, 00128 Rome, Italy

M. Molinari (✉)
Fondazione S. Lucia, Via Ardeatina 306, 00179 Rome, Italy
e-mail: m.molinari@hsantalucia.it

Keywords Glucocorticoid receptors · Inflammation ·
Neurodegeneration · Type-2 cannabinoid receptor

Introduction

Endocannabinoids (eCBs) and glucocorticoids (GCs) are two distinct classes of signaling lipids involved in the adaptive responses of the organism to stressful internal and/or environmental challenges. eCBs are local mediators produced on demand, which have been implicated in a wide variety of physiological processes, including nociception, memory and cognition, appetite control, and immunoregulation [1, 2]. The effects of eCBs are mainly mediated through the activation of two G-protein coupled cannabinoid receptors (CB₁ and CB₂), of which CB₁ has a widespread distribution and is mainly active in the whole brain, whereas CB₂ is mainly expressed in the immune and hematopoietic systems [3–5]. The role of CB₂ at peripheral sites is generally antiinflammatory in several chronic inflammatory and neurodegenerative diseases [6–8]. However, recent

studies have also reported CB₂ expression in the brain, in both glial and neuronal cells [9, 10], where our group was the first to report a CB₂-dependent protective role in central neurons undergoing remote cell death [11]. GCs are a class of steroid hormones derived from the hypothalamic–pituitary–adrenal axis (HPA) and crucial for the regulation of basal and stress-related homeostasis [12, 13]. Their anti-inflammatory effects are mediated by a ubiquitous intracellular glucocorticoid receptor (GR), which functions as a hormone-activated transcription factor of target genes [14]. GR presents two splicing variants (GR α and GR β), of which GR α is the classic receptor located in the cytoplasm as part of hetero-oligomeric complexes that mainly contain heat shock proteins (hsp); it is translocated to the nucleus upon binding to GCs [14, 15]. Instead, GR β does not bind to GCs but functions as a modulator of their responses either by acting as a dominant negative inhibitor of GR α -induced transcriptional activity or through a novel, intrinsic and GR α -independent transcriptional activity [16]. GCs and GR α are at the apex of a regulatory network that blocks several inflammatory pathways through genomic or non-genomic mechanisms [13]. Due to their distinct actions in many biological processes, GCs are currently the most widespread anti-inflammatory drugs used for the treatment of several chronic immune-mediated and neuroinflammatory diseases [17, 18]. Although the benefits of GC therapy are derived from short-term vascular changes and limited immunosuppression, prolonged or high-dose GC therapy has multiple side effects, including hypertension, dyslipidemia and “GC-induced psychoses” [19]. Additionally, it leads to GC insensitivity or resistance [20]. A link between GCs and eCBs has been disclosed at stress-relevant synapses, where eCBs have been reported as critical regulators of the stress response through their ability to modulate the sensitivity and activation of the HPA axis, as well as by contributing to the process of stress habituation in a CB₁-dependent manner [21, 22]. In the present study, we thus investigated the novel possibility of a GR α /CB₂ interaction by co-administration of the selective GR α agonist methylprednisolone (MPSS) and the selective CB₂ agonist JWH-015 to an animal model of remote cell death as well as to activated human peripheral T-lymphocytes.

Materials and methods

Animals

Experiments were performed using 100 adult male Wistar rats (body weight 200–250 g; Harlan San Pietro al Natisolone, Udine, Italy): 60 were lesioned, and 40 were used as controls (Table 1). Animals were group-housed in standard cages and maintained under a 12-h light–dark

Table 1 Lesion and treatments in the different experimental groups

Group codes	<i>n</i>	Drug treatments for 7 days (i.p./once a day)
CTRL-saline	10	DMSO 5 % in saline
CTRL-MPSS	10	50 mg/kg
CTRL-JWH-015	10	3 mg/kg
CTRL-MPSS + JWH-015	10	MPSS: as above + JWH-015: as above
HCb-saline	15	DMSO 5 % in saline
HCb-MPSS	15	50 mg/kg
HCb-JWH-015	15	3 mg/kg
HCb-MPSS + JWH-015	15	MPSS: as above + JWH-015: as above

CTRL, Control; HCb, hemicerebellectomy; MPSS, methylprednisolone; i.p., intraperitoneal

cycle in an air-conditioned facility. The Italian Ministry of Health approved the experimental protocol in agreement with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. For surgical procedures, the rats were deeply anesthetized by intraperitoneal injections of xylazine (Rompun, 10 mg/ml; Bayer) and tiletamine and zolepam (Zoletil 100, 50 mg/ml; Virbac, Italy), and then they were positioned in a stereotaxic apparatus. The skin of the skull was incised, and the occipital bone was drilled and removed. Subsequently, the dura mater was incised in order to expose the cerebellum, and hemicerebellectomy (HCb) was induced by removal of the right cerebellar hemisphere, as described previously [23]. Then, the wound was sutured, and the animals were returned to their cages. For the unlesioned group, surgery was interrupted after the dura lesion was made, and after suturing, the animals were returned to their cages.

Peripheral blood cell preparation and stimulation

Peripheral blood lymphocytes were isolated, after venous puncture, from healthy donors and were separated by density gradient over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), as previously reported [5]. Informed consent was obtained from all the subjects, and the experiments conformed to the principles set out in the WMA Declaration of Helsinki. Briefly, heparinized blood was diluted with one volume of Dulbecco’s phosphate-buffered saline (PBS) and gently layered over the Ficoll. Following centrifugation at 660g for 30 min, cells at the interface of the gradient were collected and washed twice with PBS, and then they were re-suspended in complete RPMI 1640 medium supplemented with 5 % heat-inactivated human

serum. For the identification of CD4 and CD8 T lymphocytes, cells were stained with anti-CD3, anti-CD4 and anti-CD8. PBMCs were pre-treated with MPSS and JWH-015, alone or in combination, for 30 min and then were challenged with phorbol-myristate-13-acetate (PMA) and ionomycin (Iono) for 5 h in the presence of Brefeldin A in order to prevent exocytosis of cytokine-containing vesicles.

Drugs and treatments

For the in vivo studies the following GR agonist was used: MPSS (Solumedrol®, Pharmacia, Nerviano, Italy; 50 mg/kg, i.p./daily for 7 days). Further, the following CB₂ receptor agonist and antagonist, respectively, were used: JWH-015 (3 mg/kg, i.p./daily for 7 days; Cayman Chemicals, Ann Arbor, MI, USA) and SR144528 (SR2; 3 mg/kg, i.p./daily for 7 days; Cayman Chemicals). All animals, either single or double treated, received only one intraperitoneal injection per day. In fact, to avoid stressful conditions due to multiple injections, in double-treated animals, each time, the needle was maintained in the peritoneum and the two solutions were injected separately by changing the syringe. Treatments in the different experimental groups are listed in Table 1.

For the in vitro studies the following GR agonist and antagonist were used: MPSS and RU-486 (Sigma Aldrich). Further, the following CB₂ receptor agonists were used: JWH-015 (Cayman Chemicals) and GP 1a (Tocris Bioscience, Bristol, UK). SR144528 (SR2) was used as CB₂ receptor antagonist. PBMC detailed treatments are reported in Table 2.

Antibodies

The following primary antibodies were used: rabbit anti-GR α (1:500; Abcam, UK), rabbit anti-GR β (1:500; Abcam UK), rabbit anti-pSTAT6 (1:500; Abcam, UK), mouse anti-GFAP (MAB360; 1:500; Millipore, USA), mouse anti-CD11b (OX-42, 1:500; Abcam, UK), mouse anti-actin (1:5,000; Sigma Aldrich, Italy), mouse anti-pJNK (1:1,000; Cayman Chemical, USA), rabbit anti-hsp70 (1:200; Santa Cruz Biotechnology, USA), mouse anti-hsp90 (1:1,000; Santa Cruz Biotechnology, USA) and goat anti-cytochrome-*c* (cyt-*c*) (1:400; Santa Cruz Biotechnology, USA). Secondary Cy3-, Cy2- and Cy5-conjugated antibodies (1:200) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:5,000, or anti-mouse, 1:5,000) were purchased from Cell Signaling Technology (Boston, MA, USA).

Neurological evaluation

The Neurologic Severity Score (NSS) was used to evaluate neurological conditions in rats [11]. NSS is a composite of motor, sensory, reflex and balance tests in which, for each test, one point is awarded for the inability to perform or for the lack of a tested reflex, and zero points are awarded for success. An NSS of 18 indicates severe injury, whereas a score of zero signifies healthy, uninjured rats. We also calculated the Δ NSS as the difference between the NSS at 24 h after damage and the NSS at successive time points. Δ NSS reflects the progression of recovery. The NSS was evaluated at 24, 72 h, and 5 and 7 days after damage by an investigator who was blind to the experimental groups.

Table 2 Treatments in the different experimental groups

Group codes	<i>n</i>	Drugs treatment for 7 days
CTRL	6	1 μ g/ml Brefeldin A for 4 h
PMA/Iono	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin for 5 h
PMA/Iono-MPSS	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + MPSS 10 μ M for 5 h
PMA/Iono-JWH-015	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + JWH-015 20 nM for 5 h
PMA/Iono-MPSS + JWH-015	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + MPSS 10 μ M + JWH-015 20 nM for 5 h
PMA/Iono-MPSS + JWH-015 + RU486	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + MPSS 10 μ M + JWH-015 20 nM + RU486 10 μ M for 5 h
PMA/Iono-MPSS + JWH015 + SR144528	6	1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + MPSS 10 μ M + JWH-015 20 nM + SR144528 1 μ M for 5 h
PMA/Iono-GP 1a		1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + GP 1a 1 nM for 5 h
PMA/Iono-MPSS + GP 1a		1 μ g/ml Brefeldin A for 4 h + 1 μ g/ml PMA and Ionomycin + MPSS 10 μ M + GP 1a 1 nM for 5 h

CTRL, control; PMA, phorbol-myristate-13-acetate; Iono, ionomycin; MPSS, methylprednisolone

Histology and immunohistochemistry

Animals were perfused transcardially, 7 days after surgery, with 250 ml of saline, followed by 250 ml of 4 % paraformaldehyde under anesthesia that was induced by intraperitoneal injections of Rompun (xylazine, 20 mg/ml, 0.5 ml/kg body weight) and Zoletil (tiletamine and zolazepam, 100 mg/ml, 0.5 ml/kg body weight). Each brain was removed immediately, post fixed in the same paraformaldehyde solution for 2 h, and after three washes in PBS was transferred to 30 % sucrose solution at 4 °C until it sank. Each brainstem was cut into four series of 40- μ m-thick transverse sections using a cryostat, and slices were collected in PBS. For each animal, a series of sections involving pontine nuclei (Pn) were processed for immunohistochemical studies. Following incubation with a solution of primary antibodies, the sections were incubated for 2 h at room temperature with specific secondary antibodies. Sections were examined under a confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) equipped with four laser lines: violet diode emitting at 405 nm (for DAPI), argon emitting at 488 nm, and helium/neon emitting at 543 and 633 nm.

Qualitative and quantitative analyses

Qualitative and quantitative observations were limited to the Pn of the experimental side that was projecting to the lesioned hemispheric side. Using the Stereo Investigator System (MicroBrightField Europe e.K., Magdeburg, Germany), an optical fractionator, stereological design, was applied to obtain unbiased estimates of total Nissl-stained cells. A stack of MAC 5,000 controller modules (Ludl Electronic Products Ltd., Hawthorne, NY, USA) was configured to interface an Olympus BX 50 microscope with a motorized stage and a HV-C20 Hitachi color digital camera with a Pentium II PC workstation. A three-dimensional optical dissector counting probe (x, y, z dimension of $30 \times 30 \times 10 \mu\text{m}$, respectively) was applied. Five sections for each specimen were analyzed, and Pn was outlined using the $4\times$ objective, while the $100\times$ oil immersion objective was used for marking the neuronal cells. The total Pn cell number was estimated according to the formula:

$$N = \sum Q \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf},$$

where $\sum Q$ represents the total number of neurons counted in all optically sampled fields of the Pn, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction.

To assess cyt-c expression within the Pn, quantitative analyses were performed off-line on confocal images acquired through the $20\times$ objective at the 0.07 zoom factor.

Quantitative data were obtained by adopting a different sampling strategy. Three digital square frames ($200 \times 200 \mu\text{m}$) were placed at a regular distance to sample the entire medio-lateral extent of the Pn. All quantitative analyses were conducted blinded to the animal's experimental group identity.

Cell lysate preparation and Western blot analyses

Rats ($n = 5/\text{group}$) were deeply anesthetized with intraperitoneal injections of Rompun (xylazine, 20 mg/ml, 0.5 ml/kg body weight) and Zoletil (tiletamine and zolazepam, 100 mg/ml, 0.5 ml/kg body weight), and they were killed by decapitation. The brains were dissected, and the Pn were isolated. Both Pn and PBMC were homogenized, and proteins were extracted in RIPA buffer (PBS supplemented with saline, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS and 0.5 M phenylmethylsulfonyl fluoride, 10 lg/ml leupeptin) for 30 min on ice, and then they were centrifuged for 10 min at 4 °C (14,000 rpm). Supernatants were collected, and the protein content was quantified by Bradford's colorimetric assay (Bio-Rad, Milan, Italy). Each protein sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were saturated with 5 % dried nonfat milk and incubated overnight with specific primary antibodies. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL; Amersham Biosciences).

Mitochondrial and cytosolic fractions

Pn were isolated as reported previously [24] and homogenized in buffer A (50 mM Tris-HCl, pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF) plus protease inhibitor cocktail by 30 strokes with a glass Pyrex microhomogenizer. The homogenate was centrifuged at $1,000\times g$ for 10 min, and the resulting supernatant was centrifuged at $10,000\times g$ for 20 min to obtain the mitochondria-containing pellet and the supernatant. Mitochondria were washed three times with buffer B (10 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM EGTA) by centrifugation for 10 min at $10,000\times g$. The supernatant was centrifuged at $100,000\times g$ for 1 h to generate the cytosolic fraction.

Flow cytometric analysis

PBMCs were washed twice and stained at the cell surface with anti-CD3-PC7, anti-CD4-ECD and anti-CD8-v450. Cells were washed and then fixed with 4 % formaldehyde for 10 min on ice and then were stained intracellularly with anti-TNF- α -PE and anti-IFN γ -APC in 0.5 % saponin at room temperature. Intracellular cytokine production by

T lymphocytes was analyzed by flow cytometry (FACS-Cyan ADP, Beckman Coulter) by gating at both CD4 and CD8 subsets and reporting the percentage of the T cell populations positive for the indicated cytokines. Cells were analyzed using the Flowjo software (TreeStar, Ashland, OR, USA).

Co-immunoprecipitation

PBMCs were lysed in lysis buffer [10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 % SDS, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM Na₃VO₄, 5 mM NaF, 1 % protease inhibitor cocktails (Sigma) and 20 mM Na-molybdate]. The lysate was centrifuged for 5 min at 4 °C (800×g), the supernatant was recovered and centrifuged for 30 min at 4 °C (10,000×g), and the protein content was quantified by Bradford's colorimetric assay (Bio-Rad).

The protein A-agarose (Roche) was washed three times in lysis buffer and incubated for 45 min at 4 °C with 300 μl of cell lysate (2 mg protein) and 300 μl of 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1 % SDS, 50 mM NaCl, 10 % glycerol, 1 % protease inhibitor cocktails (Sigma) and 20 mM Na-molybdate. After centrifugation for 3 min at 4 °C (10,000×g), 300 μl of a buffer containing 6 μg of anti-GR α was added to the supernatant and incubated overnight at 4 °C. Beads were finally washed three times with lysis buffer, and co-immunoprecipitated proteins were released by boiling in SB (2 % SDS, 20 % glycerol, 100 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol).

Human phospho-kinase array kit

PBMCs were challenged with PMA/iono and/or single- or double-treated with MPSS or JWH-015 and subsequently screened for 46 different kinases by means of a phospho-kinase array kit (R&D Systems, Oxford, UK), according to manufacturer's instructions. Briefly, capture and control antibodies were spotted in duplicate on nitrocellulose membranes, and cellular extracts were diluted and incubated overnight with the human phospho-kinase array. The array was washed to remove unbound proteins followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied, and a signal was produced at each capture spot corresponding to the amount of each phosphorylated protein bound.

Statistical analysis

Statistical analysis was performed through GraphPad Prism 5.0 (GraphPAD Software for Science, San Diego, CA). Parametric statistical analysis (mean \pm SD) was performed

using standard methods. Significant differences were calculated using one- or two-way ANOVA, followed by Bonferroni post hoc multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

Effect of MPSS and JWH-015, alone or in combination, on neuroprotection after focal brain damage

Previous studies from our group clearly demonstrated that HCb induces degeneration in precerebellar nuclei, namely inferior olive (IO) and Pn, contralateral to the cerebellar lesion, that can be reduced by selective stimulation of either GR α or CB₂ receptors [11, 23]. These data suggested a possible synergic neuroprotective effect of a combined GR α and CB₂ stimulation. To test this hypothesis, we analyzed neuronal degeneration, cyt-c release, functional recovery and glial reaction in Pn after HCb upon different treatments (Table 1). In keeping with previous results, a single treatment with MPSS or JWH-015 protected Pn neurons from HCb-induced degeneration by increasing neuronal survival (Fig. 1a), and cyt-c release was reduced in neurons when lesioned animals were treated with either MPSS or JWH-015 (Fig. 1b–c). In addition, functional recovery, measured as NSS, was significantly diminished in MPSS and JWH-015-treated groups at 7 days after lesion, with JWH-015 being more effective in inducing a functional recovery already after 72 h following HCb lesion (Fig. 1d). Concerning HCb-induced glial response (Fig. 2a), we observed a significant decrease in the number of both GFAP astrocytes (Fig. 2b) and OX-42 microglial cells (Fig. 2c) in both the HCb-JWH-015 and HCb-MPSS groups. Then we combined MPSS and JWH-015 in order to ascertain a possible synergic effect in neuroprotection. To this aim, HCb groups were treated singularly or concomitantly with MPSS and/or JWH-015 (Table 1), and neuronal and glial responses were assessed.

Unexpectedly, simultaneous activation of both GR α and CB₂ by MPSS and JWH-015 failed to provide any neuroprotection after HCb (Fig. 1). Instead, the double treatment (HCb-MPSS + JWH-015) increased neuronal cell death and cyt-c release with respect to each single treatment of HCb groups (Fig. 1a–c). The lack of neuroprotection in double-treated animals was paralleled by the lack of any inhibitory effect on glial activation, inasmuch as both astrocytes and microglial cells remained highly activated in the HCb-MPSS + JWH-015 group (Fig. 2b–c). Furthermore, HCb double-treated groups showed a recovery curve almost identical to that of the HCb-saline group, thus significantly counteracting the positive effects observed in each single-treated group (Fig. 1d).

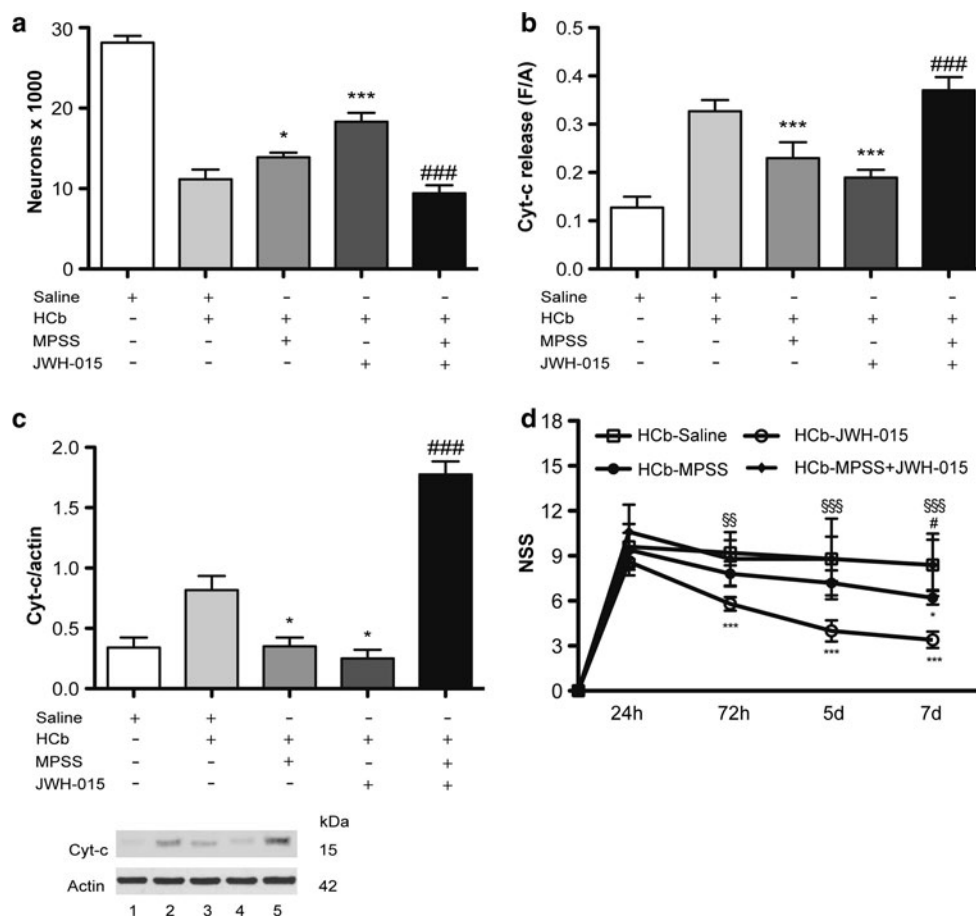


Fig. 1 Combined stimulation of $GR\alpha$ and CB_2 impairs neuron survival and functional recovery after focal brain injury. **a** Histograms of stereological neuronal count in pontine nuclei of control animals (saline) or HCb animals treated with saline or with MPSS, JWH-015, or a combination of MPSS and JWH-015. One-way ANOVA ($F = 239.40$; $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 10$ animals per group). * $p < 0.05$ versus HCB-saline; *** $p < 0.001$ versus HCB-saline; ### $p < 0.001$ versus HCB-MSS and HCB-JWH-015. **b** Histograms of densitometric values of cyt-c release, expressed as mean fluorescence of individual cells, normalized to total cellular surface (F/A; $n = 250$ cells/group) in pontine nuclei of control animals (saline) or HCb animals treated with saline, MPSS, JWH-015 or a combination of MPSS and JWH-015. One-way ANOVA ($F = 62.57$; $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 10$ animals per group). *** $p < 0.001$ versus HCB-saline; ### $p < 0.001$ versus HCB-MSS and HCB-JWH-015. **c** Representative immunoblotting and densitometry of the cytosolic

levels of cyt-c in pontine nuclei of control animals (saline) or HCb animals treated with saline, MPSS, JWH-015 or a combination of MPSS and JWH-015, normalized to the loading control (Actin). One-way ANOVA ($F = 94.66$; $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 5$ animals per group). * $p < 0.05$ versus HCB-saline; ### $p < 0.001$ versus HCB-MSS and HCB-JWH-015. **d** Time course of neurological recovery (NSS) in HCB-saline and HCB animals treated with saline, MPSS, JWH-015 or a combination of MPSS and JWH-015. Two-way ANOVA (time \times treatment) followed by Bonferroni multiple comparison tests showed an overall significant effect for time ($F = 222.2$; $p < 0.0001$) and for treatment ($F = 58.51$; $p < 0.0001$), and also interaction (time \times treatment) was significant ($F = 16.77$; $p < 0.0001$). Data are expressed as mean \pm SD ($n = 10$ per group). * $p < 0.05$ versus HCB-saline at 7 day; *** $p < 0.001$ versus HCB-saline at 72 h, 5 and 7 day; # $p < 0.05$ versus HCB-MPSS; §§ $p < 0.001$ versus HCB-JWH-015 at 72 h; §§§ $p < 0.001$ versus HCB-JWH-015 at 5 and 7 day

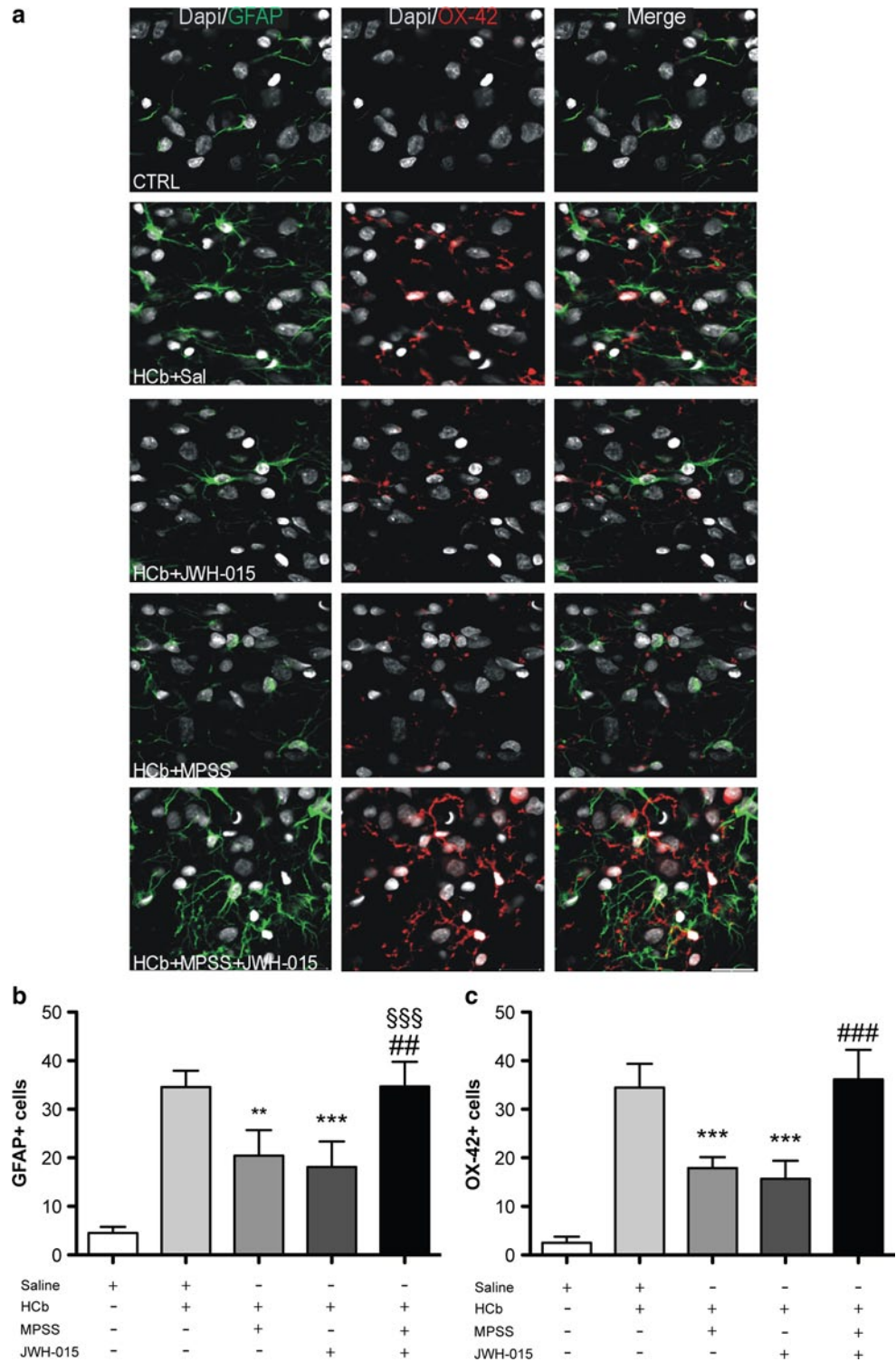
No significant differences were observed in all the above mentioned parameters among unlesioned animals treated with saline (CTRL-saline), with MPSS (CTRL-MPSS), with JWH-015 (CTRL-JWH-015) or double treated (CTRL-MPSS + JWH-015) (Supplementary Table 1).

Overall, neuroprotection was entirely lost when MPSS and JWH-015 were administered together after HCB, suggesting a functional interaction of a negative nature between $GR\alpha$ and CB_2 receptors.

Effect of MPSS and JWH-015, alone or in combination, on cytokine release from human T cells

To provide further evidence of the interaction between $GR\alpha$ and CB_2 , we extended the investigation to an ex vivo human model of inflammation, i.e. activated human peripheral lymphocytes (Table 2). When challenged with $1 \mu\text{g/ml}$ phorbol-12-myristate-13-acetate (PMA) and $1 \mu\text{g/ml}$ Iono for 5 h, CD4+ and CD8+ T cells produce high levels of

Fig. 2 Combined pharmacological activation of $GR\alpha$ and CB_2 impairs astrocytic and microglial responses. **a** Double-labeled and merged confocal images of Glial fibrillary acidic protein (GFAP; green) and OX-42 (red) plus DAPI-counterstaining (gray) in pontine nuclei of control animals (saline), HCb animals treated with saline or with MPSS, JWH-015 or a combination of MPSS and JWH-015, scale bar = 20 μ m. Histograms of the number of GFAP (**b**) and OX-42-positive cells (**c**) of control animals (saline), HCb animals treated with saline or with MPSS, JWH-015 or a combination of MPSS and JWH-015. One-way ANOVA (GFAP: $F = 34.19$, $p < 0.0001$; OX-42: $F = 48.44$, $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 10$ animals per group). ** $p < 0.01$ versus HCb-saline; *** $p < 0.001$ versus HCb-saline; ## $p < 0.01$ versus HCb-MPSS; §§§ $p < 0.001$ versus HCb-JWH-015; #### $p < 0.001$ versus HCb-MPSS and HCb-JWH-015



inflammatory cytokines [5], such as $TNF-\alpha$ and $IFN-\gamma$ (Fig. 3a). As shown in Fig. 3b, 56 and 32 % of $CD4+$ T cells were positive for $TNF-\alpha$ and $IFN-\gamma$, respectively, whereas 44 and 38 % of $CD8+$ T cells were respectively positive for these cytokines. Of note, administration of MPSS and JWH-015, alone or in combination, in cells not activated

by PMA/Iono, did not result in any cytokine production by both lymphocytes populations (Supplementary Fig. 1). We found that MPSS and JWH-015, when administered alone, induced a ~2-fold $TNF-\alpha$ suppression and a ~3-fold $IFN-\gamma$ inhibition in both $CD4+$ and $CD8+$ activated subpopulations. However, treatment with both receptor agonists

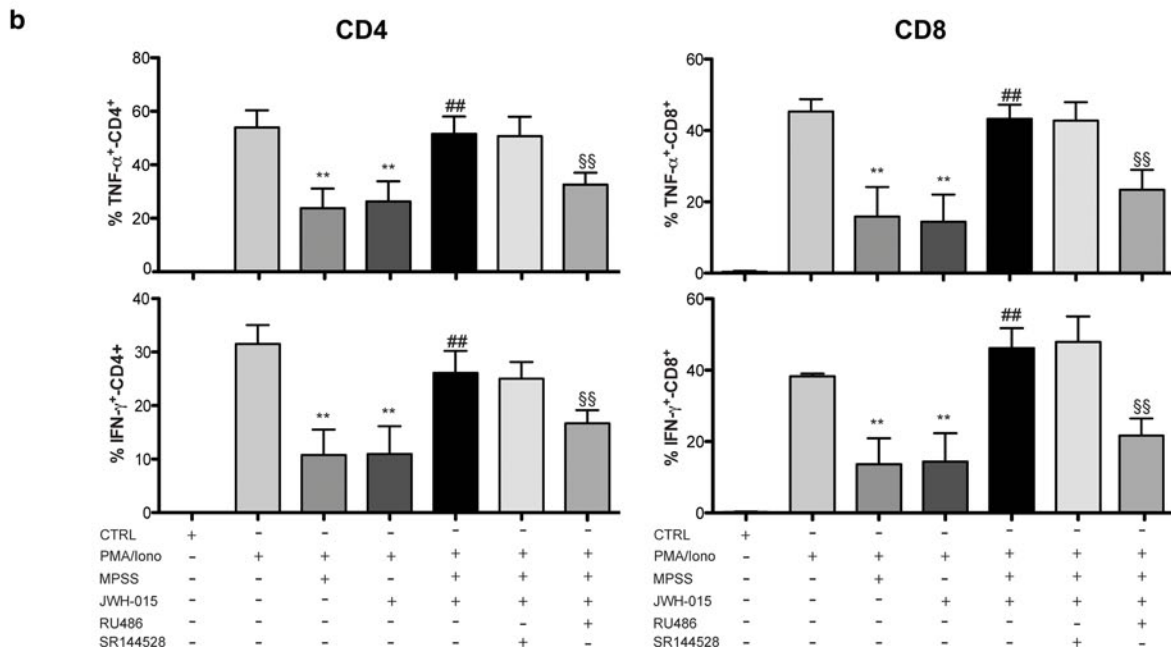
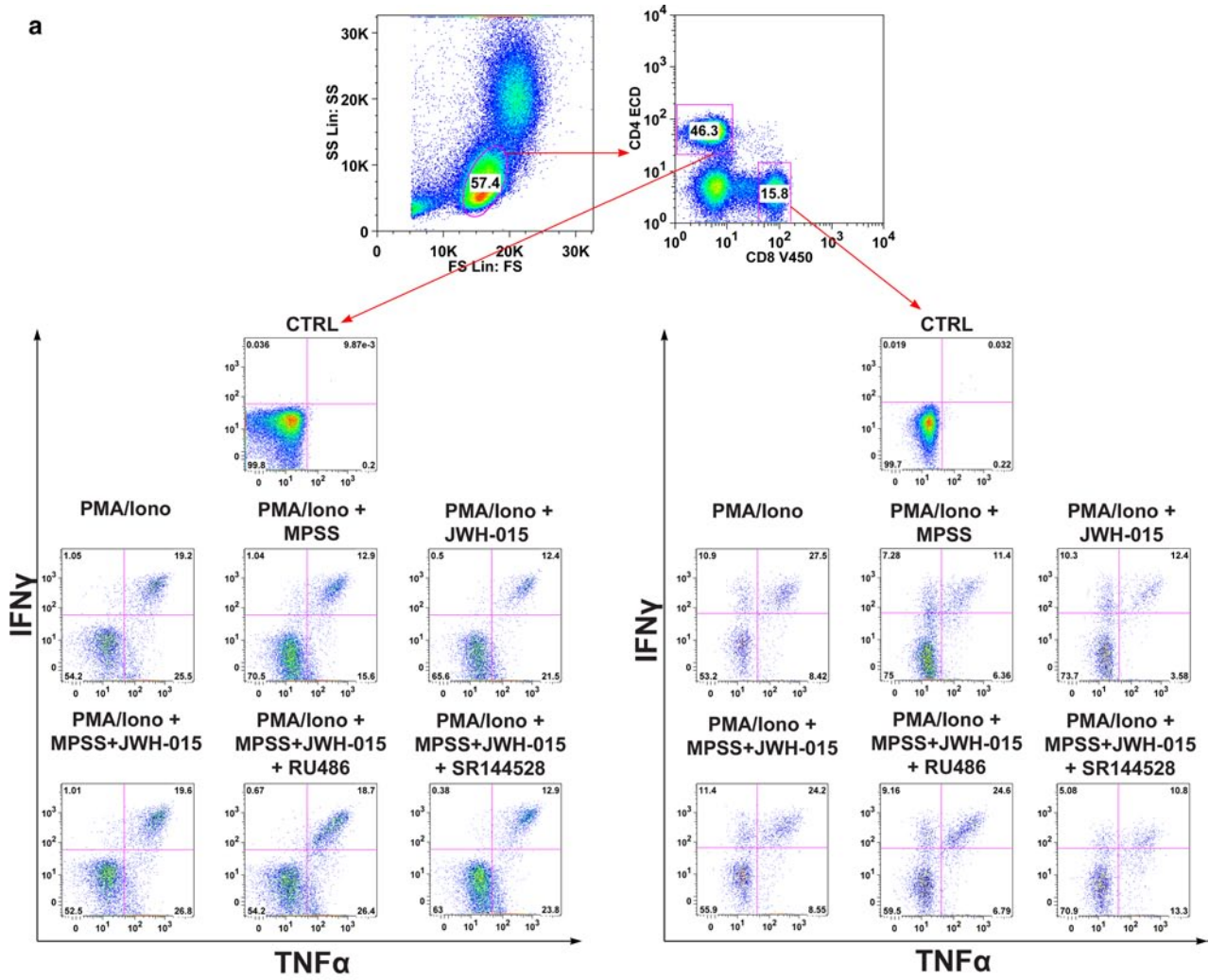


Fig. 3 Combined pharmacological activation of GR α and CB $_2$ impairs cytokine production from peripheral lymphocytes. **a** Flow cytometric progressive gating and representative *dot plots* showing TNF- α and IFN- γ production from unstimulated (CTRL) or PMA/Iono-activated CD4+ and CD8+ T lymphocytes treated or not with MPSS, JWH-015 or a combination of them, or GR α antagonist RU486 or with CB $_2$ antagonist SR144528. **b** Histograms of TNF- α and IFN- γ production from CD4+ (*left*) and CD8+ T lymphocytes (*right*), according to the different experimental conditions. One-way ANOVA ($F = 10.06$; $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 6$). ** $p < 0.01$ versus PMA/Iono; ## $p < 0.01$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015; §§ $p < 0.01$ versus PMA/Iono-MPSS + JWH-015

(PMA/Iono-MPSS + JWH-015) significantly dampened the antiinflammatory activity of GR α and CB $_2$ receptors alone. In fact, the combination of MPSS and JWH-015 showed a level of TNF- α and IFN- γ production comparable to that of PMA/Iono-stimulated T cells, corroborating the hypothesis of a functional interaction between eCBs and GCs receptors. These data were also confirmed by using another potent and selective CB $_2$ agonist, GP 1a. In fact, single treatment with GP 1a displayed a strong and significant inhibition of both TNF- α and IFN- γ production, which was comparable to that exerted by JWH-015. More importantly, combination of both MPSS and GP 1a had the same effects of the MPSS and JWH-015 one. Combination of MPSS and GP 1a treatments significantly blunted the immunosuppressive effect of single stimulation of GR α or CB $_2$, restoring the production of both cytokines (Supplementary Fig. 2).

In order to gain more insights into the contribution of each receptor to such an interaction, activated CD4+ and CD8+ T cells were pre-treated with the selective GR α antagonist RU-486 or the selective CB $_2$ antagonist SR144528 before treating them with a combination of MPSS and JWH-015. Interestingly, in double-treated CD4+ and CD8+ T cells pre-treatment with RU-486 did not affect TNF- α and IFN- γ production, whereas pre-treatment with SR144528 significantly reduced the levels of both cytokines. Taken together, these data point to a predominant role of CB $_2$ in controlling the cross-talk between GR α and CB $_2$.

Mechanistic insights into the GR α /CB $_2$ interaction

Several clinically oriented investigations report a correlation between increased levels of GR β , the dominant negative inhibitor of GR α , with the development of tissue-specific insensitivity to GCs in various disorders, most of them associated with dysregulation of immune function [20, 25]. Thus, we investigated the effects of single or double treatment with JWH-015 or MPSS on the expression of GR α and GR β in activated human PBMCs (Fig. 4). As could be expected, GR α levels were reduced, although not significantly, following MPSS or JWH-015 treatment, indicating

an activation of this protein, which is in keeping with an antiinflammatory effect. Instead, MPSS + JWH-015 treatment did not affect GR α expression levels in activated cells (Fig. 4a). Interestingly, neither MPSS nor JWH-015 alone induced significant changes of GR β protein levels in activated PBMCs compared to control cells (Fig. 4b); however, GR β expression was significantly increased when activated PBMCs were treated with a combination of the two drugs, suggesting that GR β mediates the GR α /CB $_2$ interaction.

GR α , besides being regulated by GR β , is also controlled by hetero-oligomeric complexes containing heat-shock protein 90 (hsp90) and heat-shock protein 70 (hsp70), which prevent its translocation to the cell nucleus and its antiinflammatory action in the absence of GCs [14, 15]. Thus, we investigated the formation of hsp90/hsp70/GR α complexes upon single or double activation of GR α and CB $_2$ by immunoprecipitation (Fig. 5). Activated PBMCs treated with MPSS or JWH-015 alone displayed low expression of hsp90 (Fig. 5a). Conversely, a ~3-fold increase in hsp90 expression was observed following double treatment with MPSS and JWH-015, and was completely abolished by SR144528 (Fig. 5a), but not by RU-486 (data not shown). Furthermore, while activated PBMCs treated with MPSS showed low expression of hsp70, JWH-015-treated cells showed a significant ~2-fold increase in its expression (Fig. 5b). Such an increase in hsp70 was also observed in double-treated cells and was completely abolished by SR144528 (Fig. 5b). These data suggest a key role for CB $_2$ in the GC/eCB interactions. To provide further mechanistic insights, we performed a screening of protein kinases that might be engaged by GR α and CB $_2$ using a phospho-kinase array kit. Interestingly, out of the 44 different phospho-kinases analyzed, only p-JNK and p-STAT6 showed significant differences in double-treated PBMCs compared to single-treated cells (Fig. 6). The proinflammatory p-JNK showed a ~1.5-fold increase when activated PBMCs were treated with MPSS and JWH-015 together, whereas the antiinflammatory Th2-driving p-STAT6 was significantly reduced (approximately by half) by the combination of the two drugs.

Discussion

This study demonstrates for the first time the existence of a negative interaction between CB $_2$ and GR α on neuroprotective and antiinflammatory effects, both in rats and humans. Furthermore, our study indicates that such an interaction is mediated by CB $_2$ and might engage JNK and STAT6 pathways. Different neurodegenerative diseases, including multiple sclerosis, stroke, traumatic brain and spinal cord injury, as well as Alzheimer's disease, have been recently considered suitable targets for both eCB- and glucocorticoid-based

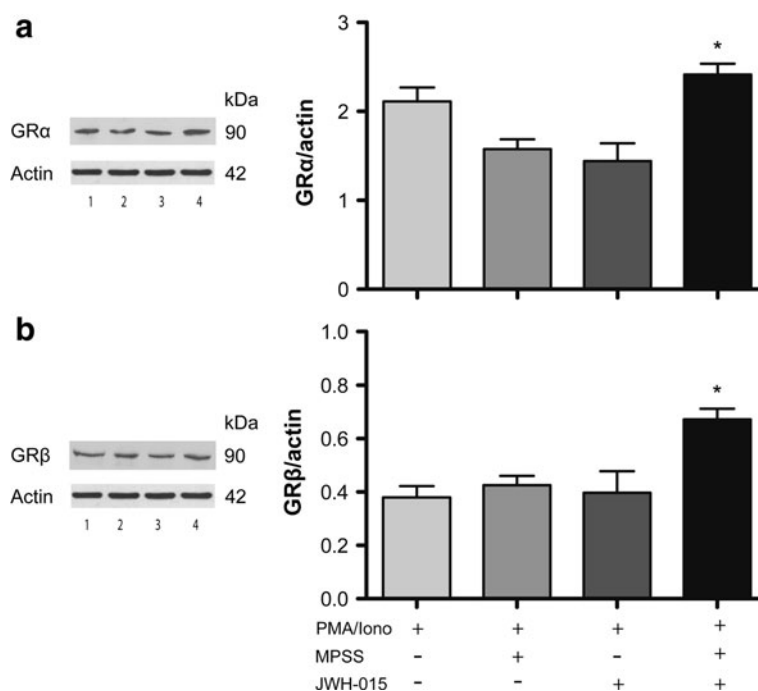


Fig. 4 Combined stimulation of GR α and CB $_2$ affects the expression of GR α and GR β in peripheral lymphocytes. **a** Representative immunoblotting and densitometry of GR α expression normalized to the loading control (Actin) in PMA/Iono-activated peripheral blood lymphocytes untreated (lane 1) or treated with MPSS (lane 2), JWH-015 (lane 3) or a combination of them (lane 4). One-way ANOVA ($F = 19.10$; $p < 0.01$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD

($n = 4$). * $p < 0.05$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015. **b** Representative immunoblotting and densitometry of GR β expression normalized to the loading control (Actin) in PMA/Iono-activated peripheral blood lymphocytes untreated (lane 1) or treated with MPSS (lane 2), JWH-015 (lane 3) or a combination of them (lane 4). One-way ANOVA ($F = 39.25$; $p < 0.0001$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 4$). * $p < 0.05$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015

drugs [26–33]. In this context, the present findings of that a negative interaction between eCBs and GCs receptors seem particularly relevant. HCB-based remote cell death is a well-established model to address neurodegeneration [34] as it has been associated with necrosis and apoptosis of neurons [35], glial activation and inflammation [23], as well as mitochondrial damage and autophagy [24, 36, 37]. In this model, neuroprotective effects of both GR α and CB $_2$ have been recently reported in terms of cell survival, glial activation and functional recovery [23, 34, 38], as well as oxidative and nitrative stress [24, 36]. Against this background, we wondered whether GR α and CB $_2$ ligands and their subsequent signaling could interact with each other. Double treatment resulted in striking differences compared to each treatment alone. In particular cell survival, which was highly enhanced after single treatment with either CB $_2$ or GR α agonists, was almost abolished after double treatment. Analysis of mitochondrial damage and glial activation yielded similar results, and also functional recovery scores corroborated the morphological and biochemical data. Therefore, our findings, though unexpected, clearly indicate a negative functional interaction exists between CB $_2$ and GR α in the central nervous system (CNS).

It is now well recognized that most neuroinflammatory diseases of the CNS are characterized by massive infiltrations of discrete populations of peripheral blood immune cells that are recruited within the CNS at the level of the blood brain barrier [39]. This recruitment is often driven by the endogenous immune cells of the CNS, i.e., microglia and astrocytes, which, following activation, locally release chemokines and cytokines that diffuse into the bloodstream, thereby attracting leukocytes at sites of inflammation [40, 41]. Among the recruited leukocytes, CD4 and CD8 T lymphocytes are the main players. Thus, our data on human activated T cells, by extending the evidence of a negative interaction between CB $_2$ and GR α receptors to humans, widen the clinical implications of this cross-talk. Indeed, cytokine release from these T cell subsets is highly indicative of neurodegenerative-associated inflammatory processes [42, 43]. In the present study, we mainly focused on TNF- α and IFN- γ production by both CD4 and CD8 T lymphocytes since they represent the main cytokines that characterize the Th-1 proinflammatory response and have been reported to be inhibited by either CB $_2$ or GR α activation [5, 44–46]. In keeping with our observations on the animal model, the concomitant activation of CB $_2$ and GR α prevented their

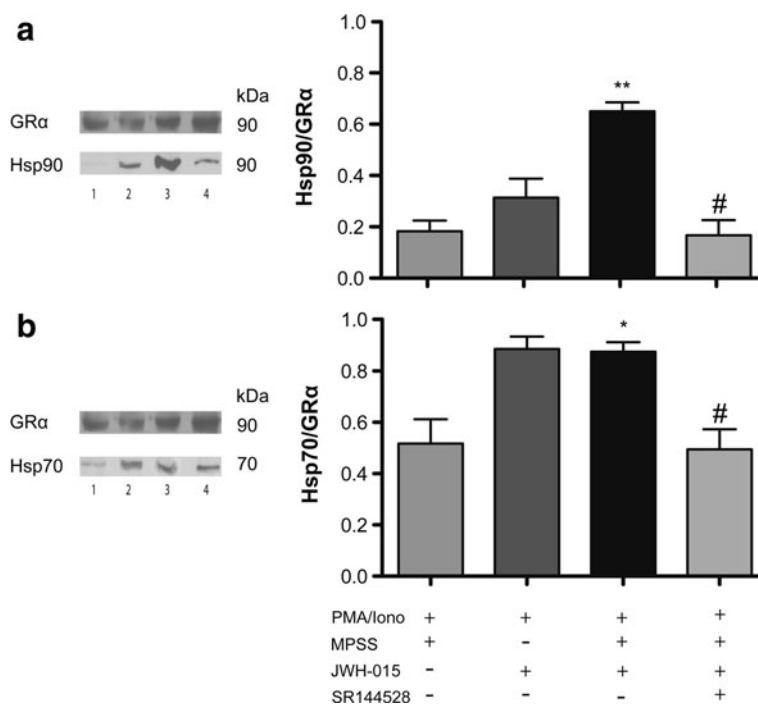


Fig. 5 Combined stimulation of GR α and CB $_2$ enhances hsp70 and hsp90 expression in peripheral lymphocytes. **a** Representative immunoblotting and densitometry of GR α -precipitated hsp90 expression in PMA/Iono-activated peripheral blood lymphocytes treated with MPSS (lane 1), JWH-015 (lane 2), a combination of them (lane 3) or CB $_2$ antagonist SR144528 (lane 4). One-way ANOVA ($F = 33.82$; $p < 0.01$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 4$). ** $p < 0.01$ versus PMA/Iono-MPSS; # $p < 0.05$ versus PMA/Iono-MPSS + JWH-015.

b Representative immunoblotting and densitometry of GR α -precipitated hsp70 expression in PMA/Iono-activated peripheral blood lymphocytes treated with MPSS (lane 1), JWH-015 (lane 2), a combination of them (lane 3) or CB $_2$ antagonist SR144528 (lane 4). One-way ANOVA ($F = 19.75$; $p < 0.01$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 4$ per group). * $p < 0.05$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015; # $p < 0.05$ versus PMA/Iono-MPSS + JWH-015

antiinflammatory effects by blocking the production of both TNF- α and IFN- γ . Furthermore, the isolation of peripheral blood lymphocytes allowed us to unravel the role of the two receptors in mediating such a negative interaction. By using selective antagonists, we found that the negative interaction between CB $_2$ and GR α was erased only by SR144528, suggesting that the receptor cross-talk is driven by CB $_2$. The selectivity of drug manipulation of receptor is often a matter of debate. Indeed, JWH-015 could also activate CB $_1$, but only when used at high nanomolar concentrations (>432 nM) [47], concentrations quite far from those used here. The high affinities toward CB $_2$ and the very low affinities toward CB $_1$ of the selective agonists used (JWH-015 and GP 1A) clearly rule out any doubts on the specificity of CB $_2$ in sustaining the observed effects.

GC resistance or insensitivity has been recently reported to be the main cause of the lack of response to the manifold therapeutic benefits of GR agonists in various disorders of proliferation and chronic inflammatory diseases [20, 25, 48–50]. This can occur through multiple mechanisms, including differences in cellular localization, transcriptional activity and sensitivity of GR isoforms, as well as GR-independent

mechanisms such as differences in those chaperones and co-chaperones that could alter GR heterocomplex signaling [51, 52]. Hence, our immunoblotting and immunoprecipitation data of the two main GR subunits (α and β) seem of major relevance, because they demonstrate a significant increase in GR β after concomitant CB $_2$ and GR α activation. Recent evidence suggests that the ratio of GR α :GR β expression is indeed critical to the GCs responsiveness of various cells, where higher ratios correlate with GC sensitivity, while lower ratios correlate with GC resistance [53]. In particular, the increased expression of GR β in the development of GC-resistant forms of immune-related diseases is increasingly well documented [54–57]. In this context, our findings not only contribute to validate the theory of GR β as a dominant negative inhibitor of GR α -induced transcriptional activity, but also offer a putative mechanism for the observed negative interaction between CB $_2$ and GR α .

Furthermore, we also analyzed other mechanisms underlying GC resistance through which CB $_2$ signaling might affect GR α -induced beneficial effects. Since the integrity of the mature GR heterocomplex is required for optimal binding of GR α agonists and subsequent activation of the

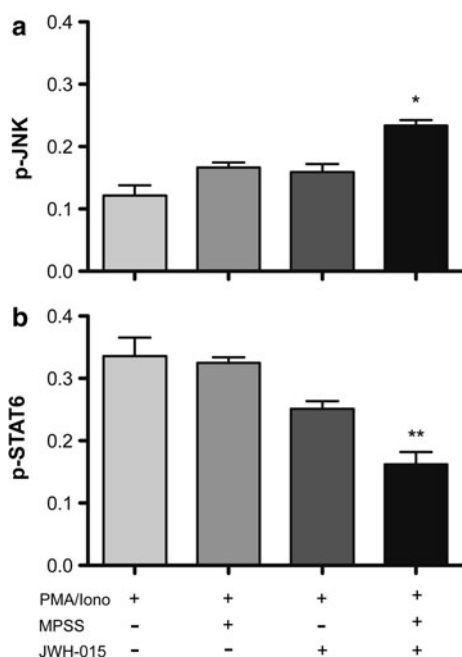


Fig. 6 Combined stimulation of GR α and CB $_2$ modulates JNK and STAT6 expression in peripheral lymphocytes. **a** Human phospho-kinase array kit and densitometry graph of p-JNK in PMA/Iono-activated peripheral blood lymphocytes treated or not with MPSS, JWH-015 or a combination of them. One-way ANOVA ($F = 30.70$; $p < 0.01$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 4$ per group). # $p < 0.05$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015. **b** Human phospho-kinase array kit and densitometry graph of p-STAT6 in PMA/Iono-activated peripheral blood lymphocytes treated or not with MPSS, JWH-015 or a combination of them. One-way ANOVA ($F = 34.58$; $p < 0.01$) followed by Bonferroni multiple comparison test was performed. Data are reported as mean \pm SD ($n = 4$ per group). ## $p < 0.01$ versus PMA/Iono-MPSS and PMA/Iono-JWH-015

transcriptional response, abnormalities in the chaperones and co-chaperones that make up the heterocomplex may contribute to such GC resistance. Indeed, several pieces of evidence showed that alterations in hsp90 and hsp70 were associated with decreased cellular sensitivity to GCs in several disorders, including malignancies, asthma, and multiple sclerosis [48, 58, 59]. In this scenario, our observed increase in both hsp90 and hsp70 following concomitant activation of CB $_2$ and GR α is of particular relevance in elucidating the molecular mechanism underlying their negative interaction. However, the increase in hsp70 was also observed following single stimulation of CB $_2$. This finding is in keeping with the previous observation that CB $_2$ agonism significantly increased the expression of hsp70 in neurons of the same rat model of remote cell death [24]. Therefore, it can be suggested that CB $_2$ activation per se induces an early increase in hsp70 expression, followed by a marked increase in hsp90 that was indeed observed when GR α was concomitantly activated. The role of CB $_2$ in mediating such hsp70/90-dependent CB $_2$ /GR α interaction was further

confirmed by the complete reversion of receptor cross-talk upon SR144528 pre-treatment.

Taken together, our findings suggest that the lack of neuroprotection and antiinflammation upon simultaneous activation of CB $_2$ and GR α can occur through at least two mechanisms: one mediated by GR β and the subsequent alteration of the GR α :GR β ratio, and the other one involving hsp90- and hsp70-mediated alteration of the mature GR heterocomplex.

In order to further investigate the molecular basis of the CB $_2$ /GR α interaction, we performed a preliminary proteomic multiarray screening of the various phospho-proteins known to be downstream of CB $_2$ and/or GR α . Interestingly, only JNK and STAT6 were significantly modulated upon concomitant activation of the two receptors compared to their individual stimulation. However, this preliminary screening is far from comprehensive, and further studies are required to clarify the real mechanism at the basis of the observed CB $_2$ /GR α negative cross-talk. The unprecedented evidence of a negative interaction between these two receptors also extends the concept of eCBs as part of a wider regulatory system in which different signaling pathways interact to control manifold biological functions of the body, from mood control to inflammation and neurodegeneration [60–64]. On a final note, it should be mentioned that nowadays there is quite a demand to extend the clinical use of (endo) cannabinoid-oriented drugs to treat neurological diseases where steroid treatment is already a routine. In this light, our present findings call for caution concerning eCB/GC combinations as novel therapeutic approaches; however, they point to a possible exploitation of CB $_2$ -targeted therapeutics to cope with the ever-increasing problem of GC resistance.

Acknowledgments This study was supported by Wings for Life Spinal Cord Research Foundation to M.T. Viscomi, by the Ministero della Salute to M. Molinari, and partly by Fondazione TERCAS (grant 2009–2012) to M. Maccarrone.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Maccarrone M (2009) The endocannabinoid system and its manifold central actions. In: Tettamanti G, Goracci G (eds) Handbook of neurochemistry and molecular neurobiology—neural lipids. Springer, Germany, pp 385–405
- Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, Friedman H (2003) The cannabinoid system and immune modulation. *J Leukoc Biol* 74(4):486–496
- Howlett AC, Blume LC, Dalton GD (2010) CB(1) cannabinoid receptors and their associated proteins. *Curr Med Chem* 17(14):1382–1393
- Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M (2010) Detailed characterisation of CB2 receptor protein expression in peripheral blood immune cells from healthy human

- volunteers using flow cytometry. *Int J Immunopathol Pharmacol* 23(1):25–34
5. Cencioni MT, Chiurchiù V, Catanzaro G, Borsellino G, Bernardi G, Battistini L, Maccarrone M (2010) Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. *PLoS One* 5(1):e8688
 6. Basu S, Dittel BN (2011) Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. *Immunol Res* 51(1):26–38
 7. Cabral GA, Griffin-Thomas L (2009) Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. *Expert Rev Mol Med* 11:e3
 8. Centonze D, Battistini L, Maccarrone M (2008) The endocannabinoid system in peripheral lymphocytes as a mirror of neuroinflammatory diseases. *Curr Pharm Des* 14(23):2370–2372
 9. Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 310(6746):329–332
 10. Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasensfitz L, Uhl GR (2006) Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann N Y Acad Sci* 1074:514–536
 11. Viscomi MT, Oddi S, Latini L, Pasquariello N, Florenzano F, Bernardi G, Molinari M, Maccarrone M (2009) Selective CB2 receptor agonism protects central neurons from remote axotomy-induced apoptosis through the PI3 K/Akt pathway. *J Neurosci* 29(14):4564–4570
 12. Chrousos GP (1995) The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med* 332(20):1351–1362
 13. Rhen T, Cidlowski JA (2005) Anti-inflammatory action of glucocorticoids—new mechanisms for old drugs. *N Engl J Med* 353(16):1711–1723
 14. Chrousos GP, Kino T (2005) Intracellular glucocorticoid signaling: a formerly simple system turns stochastic. *Sci STKE* 2005(304):pe48
 15. Kino T, Chrousos GP (2004) Glucocorticoid and mineralocorticoid receptors and associated diseases. *Essays Biochem* 40:137–155
 16. Kino T, Su YA, Chrousos GP (2009) Human glucocorticoid receptor isoform beta: recent understanding of its potential implications in physiology and pathophysiology. *Cell Mol Life Sci* 66(21):3435–3448
 17. Barnes PJ (2011) Glucocorticosteroids: current and future directions. *Br J Pharmacol* 163(1):29–43
 18. Chiurchiù V, Maccarrone M (2011) Chronic inflammatory disorders and their redox control: from molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal* 15(9):2605–2641
 19. Schacke H, Docke WD, Asadullah K (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96(1):23–43
 20. Barnes PJ (2010) Mechanisms and resistance in glucocorticoid control of inflammation. *J Steroid Biochem Mol Biol* 120(2–3):76–85
 21. Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM, Hillard CJ, Gorzalka BB, Viau V (2010) Endogenous cannabinoid signaling is essential for stress adaptation. *Proc Natl Acad Sci USA* 107(20):9406–9411
 22. Crosby KM, Bains JS (2012) The intricate link between glucocorticoids and endocannabinoids at stress-relevant synapses in the hypothalamus. *Neuroscience* 204:31–37
 23. Viscomi MT, Florenzano F, Latini L, Amantea D, Bernardi G, Molinari M (2008) Methylprednisolone treatment delays remote cell death after focal brain lesion. *Neuroscience* 154(4):1267–1282
 24. Oddi S, Latini L, Viscomi MT, Bisicchia E, Molinari M, Maccarrone M (2012) Distinct regulation of nNOS and iNOS by CB2 receptor in remote delayed neurodegeneration. *J Mol Med* 90(4):371–387
 25. Goleva E, Li LB, Eves PT, Strand MJ, Martin RJ, Leung DY (2006) Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 173(6):607–616
 26. Rossi S, Bernardi G, Centonze D (2010) The endocannabinoid system in the inflammatory and neurodegenerative processes of multiple sclerosis and of amyotrophic lateral sclerosis. *Exp Neurol* 224(1):92–102
 27. Rog DJ (2010) Cannabis-based medicines in multiple sclerosis—a review of clinical studies. *Immunobiology* 215(8):658–672
 28. Sandercock PA, Soane T (2011) Corticosteroids for acute ischaemic stroke. *Cochrane Database Syst Rev* 9:CD000064
 29. Hillard CJ (2008) Role of cannabinoids and endocannabinoids in cerebral ischemia. *Curr Pharm Des* 14(23):2347–2361
 30. Bracken MB (2012) Steroids for acute spinal cord injury. *Cochrane Database Syst Rev* 1:001046
 31. Teasell RW, Mehta S, Aubut JA, Foulon B, Wolfe DL, Hsieh JT, Townson AF, Short C, Spinal Cord Injury Rehabilitation Evidence Research Team (2010) Spinal cord injury rehabilitation evidence research team. A systematic review of pharmacological treatments of pain after spinal cord injury. *Arch Phys Med Rehabil* 91(5):816–831
 32. Baastup C, Finnerup NB (2008) Pharmacological management of neuropathic pain following spinal cord injury. *CNS Drugs* 22(6):455–475
 33. Dhawan N, Puangco J, Jandial R (2008) In search of a treatment for Alzheimer's disease and potential immunosuppressive therapeutic interventions. *Neurol Endocrinol Lett* 29(4):410–420
 34. Viscomi MT, Florenzano F, Latini L, Molinari M (2009) Remote cell death in the cerebellar system. *Cerebellum* 8(3):184–191
 35. Viscomi MT, Florenzano F, Conversi D, Bernardi G, Molinari M (2004) Axotomy dependent purinergic and nitergic co-expression. *Neuroscience* 123(2):393–404
 36. Pacher P, Mackie K (2012) Interplay of cannabinoid 2 (CB2) receptors with nitric oxide synthases, oxidative and nitrate stress, and cell death during remote neurodegeneration. *J Mol Med (Berl)* 90(4):347–351
 37. Viscomi MT, D'Amelio M, Cavallucci V, Latini L, Bisicchia E, Nazio F, Fanelli F, Maccarrone Moreno S, Cecconi F, Molinari M (2012) Stimulation of autophagy by rapamycin protects neurons from remote degeneration after acute focal brain damage. *Autophagy* 8(2):222–235
 38. Viscomi MT, Oddi S, Latini L, Bisicchia E, Maccarrone M, Molinari M (2010) The endocannabinoid system: a new entry in remote cell death mechanisms. *Exp Neurol* 224(1):56–65
 39. Rezai-Zadeh K, Gate D, Town T (2009) CNS infiltration of peripheral immune cells: D-day for neurodegenerative disease? *J Neuroimmune Pharmacol* 4(4):462–475
 40. Ransohoff RM, Kivisakk P, Kidd G (2003) Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol* 3(7):569–581
 41. Engelhardt B (2008) The blood-central nervous system barriers actively control immune cell entry into the central nervous system. *Curr Pharm Des* 14(16):1555–1565
 42. Huang X, Reynolds AD, Mosley RL, Gendelman HE (2009) CD4+ T cells in the pathobiology of neurodegenerative disorders. *J Neuroimmunol* 211(1–2):3–15
 43. Neumann H, Medana IM, Bauer J, Lassman H (2002) Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci* 25(6):313–319
 44. Franchimont D, Galon J, Gadina M, Visconti R, Zhou Y, Aringer M, Frucht DM, Chrousos GP, O'Shea JJ (2000) Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *J Immunol* 164(4):1768–1774

45. Kunicka JE, Talle MA, Denhardt GH, Brown M, Prince LA, Goldstein G (1993) Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell Immunol* 149(1):39–49
46. Monick MM, Aksamit TR, Geist LJ, Hunninghake GW (1994) Dexamethasone inhibits IL-1 and TNF activity in human lung fibroblasts without affecting IL-1 or TNF receptors. *Am J Physiol* 267(1 Pt 1):L33–L38
47. Murataeva N, Mackie K, Straiker A (2012) The CB2-preferring agonist JWH015 also potently and efficaciously activates CB1 in autaptic hippocampal neurons. *Pharmacol Res* 66(5):437–442
48. Matysiak M, Makosa B, Walczak A, Selmaj K (2008) Patients with multiple sclerosis resisted to glucocorticoid therapy: abnormal expression of heat-shock protein 90 in glucocorticoid receptor complex. *Mult Scler* 14(7):919–926
49. Meijer OC, Karssen AM, De Kloet ER (2003) Cell- and tissue-specific effects of corticosteroids in relation to glucocorticoid resistance: example from the brain. *J Endocrinol* 178(1):13–18
50. Dai J, Buijs R, Swaab D (2004) Glucocorticoid hormone (cortisol) affects axonal transport in human cortex neurons but shows resistance in Alzheimer's disease. *Br J Pharm* 143(5):606–610
51. Tissing WJE, Meijerink JPP, den Boer ML, Pieters R (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. *Leukemia* 17(1):17–25
52. Gross KL, Lu NZ, Cidlowski JA (2009) Molecular mechanisms regulating glucocorticoid sensitivity and resistance. *Mol Cell Endocrinol* 300(1–2):7–16
53. Lewis-Tuffin LJ, Cidlowski JA (2006) The physiology of human glucocorticoid receptor beta (hGRbeta) and glucocorticoid resistance. *Ann N Y Acad Sci* 1069:1–9
54. Christodoulouopoulos P, Leung DY, Elliott MW, Hogg JC, Muro S, Toda M, Laberge S, Hamid QA (2000) Increased number of glucocorticoid receptor- β -expressing cells in the airways in fatal asthma. *J Allergy Clin Immunol* 106(3):479–484
55. Hamid QA, Wenzel SE, Hauk PJ, Tscopoulos A, Wallaert B, Lafitte JJ, Chrousos GP, Szefer SJ, Leung DYM (1999) Increased glucocorticoid receptor β in airway cells of glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 159(5 Pt 1):1600–1604
56. Honda M, Orii F, Ayabe T, Imai S, Ashida T, Obara T, Kohgo Y (2000) Expression of glucocorticoid receptor β in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. *J Gastroenterol* 118(5):859–866
57. Sousa AR, Lane SJ, Cidlowski JA, Staynov DZ, Lee TH (2000) Glucocorticoid resistance in asthma is associated with elevated in vivo expression of the glucocorticoid receptor β -isoform. *J Allergy Clin Immunol* 105(5):943–950
58. Kojika S, Sugita K, Inukai T, Saito M, Iijima K, Tezuka T, Goi K, Shiraishi K, Mori T, Ozaraki T, Kagami K, Ohyama K, Nakazawa S (1996) Mechanisms of glucocorticoid resistance in human leukemic cells: implication of abnormal 90 and 70 kDa heat shock proteins. *Leukemia* 10(6):994–999
59. Qian X, Zhu Y, Xu W, Lin Y (2001) Glucocorticoid receptor and heat shock protein 90 in peripheral blood mononuclear cells from asthmatics. *Chin Med J (England)* 114(10):1051–1054
60. Gorzalka BB, Dang SS (2012) Endocannabinoids and gonadal hormones: bidirectional interactions in physiology and behavior. *Endocrinology* 153(3):1016–1024
61. Hill MN, Tasker JG (2012) Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. *Neuroscience* 204:5–16
62. Lopez HH (2010) Cannabinoid-hormone interactions in the regulation of motivational processes. *Horm Behav* 58(1):100–110
63. Parolaro D, Rubino T, Viganò D, Massi P, Guidali C, Realini N (2010) Cellular mechanisms underlying the interaction between cannabinoid and opioid system. *Curr Drug Targ* 11(4):393–405
64. Ferré S, Lluís C, Justinova Z, Quiroz C, Orru M, Navarro G, Canela EL, Franco R, Goldeberg SR (2010) Adenosine-cannabinoid receptor interactions. Implications for striatal function. *Br J Pharm* 160(3):443–453