Stress-induced neuroinflammation of the spinal cord is restrained by Cort113176 (Dazucorilant) a specific glucocorticoid receptor modulator

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Stress-induced Neuroinflammation of the Spinal Cord is Restrained by Cort113176 (Dazucorilant), A Specific Glucocorticoid Receptor Modulator

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Abstract

Glucocorticoids exert antiinflammatory, antiproliferative and immunosupressive effects. Paradoxically they may also enhance inflammation particularly in the nervous system, as shown in Cushing´ syndrome and neurodegenerative disorders of humans and models of human diseases. ."The Wobbler mouse model of amyotrophic lateral sclerosis shows hypercorticoidism and neuroinflammation which subsided by treatment with the glucocorticoid receptor (GR) modulator Dazucorilant (CORT113176). This effect suggests that GR mediates the chronic glucocorticoid unwanted effects. We now tested this hypothesis using a chronic stress model resembling the condition of the Wobbler mouse Male NFR/NFR mice remained as controls or were subjected to a restraining / rotation stress protocol for 3 weeks, with a group of stressed mice receiving CORT113176 also for 3 weeks. We determined the mRNAS or reactive protein for the proinflamatory factors HMGB1, TLR4, NFkB, TNFα, markers of astrogliosis (GFAP, SOX9 and acquaporin 4), of microgliosis (Iba, CD11b, P2RY12 purinergic receptor) as well as serum IL1β and corticosterone. We showed that chronic stress produced high levels of serum corticosterone and IL1β, decreased body and spleen weight, produced microgliosis and astrogliosis and increased proinflammatory mediators. In stressed mice, modulation of the GR with CORT113176 reduced Iba + microgliosis, CD11b and P2RY12 mRNAs, immunoreactive HMGB1 + cells, GFAP + astrogliosis, SOX9 and acquaporin expression and TLR4 and NFkB mRNAs vs. stress-only mice. The effects of CORT113176 indicate that glucocorticoids are probably involved in neuroinflammation. Thus, modulation of the GR would become useful to dampen the inflammatory component of neurodegenerative disorders.

Keywords Neuroinflammation · Glucocorticoids · Glucocorticoid receptor modulators · Cort113176 · Stress

Introduction

Glucocorticoids are systemic hormones that regulate the functions of diverse cell types in both physiological and pathologically conditions. Both fast, nongenomic effects as well as slow receptor-mediated effects account for glucocorticoid actions. In the latter case, the main glucocorticoids (cortisol in humans, corticosterone in rodents) bind to two types of receptors, the high affinity mineralocorticoid receptor (MR) (NR3C2, nuclear receptor subfamily 3, group C, member 2) and the low affinity glucocorticoid receptor (GR) (NR3C1, nuclear receptor subfamily 3, group C, member 1) [1]. In molecular terms, ligand-activated GR and MR bind as homo or heterodimers to glucocorticoid-response elements on the DNA, regulating in a positive or

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negative way the transcription of target genes [1, 2]. Additionally, GR may directly bind to DNA and interact with other transcription factors causing gene repression. Thirdly, gene transrepression can take place by direct protein-protein interaction (tethering) between the GR and the transcription factors activator protein-1 (AP-1) and NFkB [3–5]. Therefore, mechanisms involving the GR offer the opportunity for pharmacological modulation of this receptor when dysregulation of glucocorticoid action is involved in the initiation or aggravation of pathology.

Glucocorticoids are well known for their anti-inflammatory, anti-proliferative and immunosuppressive effects. These properties have been used for the treatment of diseases with a dysregulated immune system affecting the nervous system, bronchial system, bowel, skin, hematological, bone, or joints [6, 7]. Although beneficial effects are due to suppression of inflammatory genes, paradoxically, glucocorticoids may enhance inflammation in peripheral organs and particularly in the nervous system [7–12]. Examples of the latter effect include Cushing syndrome, major depressive disorder, post-traumatic stress disorder, brain aging and animal models presenting prolonged glucocorticoid excess [13–16]. Interestingly, neurodegenerative diseases such as Alzheimer`s, Parkinson´s, Huntington´s and amyotrophic lateral sclerosis (ALS) show abnormal glucocorticoid dynamics, consisting of HPA axis hyperactivation, elevation of blood cortisol levels and abnormal circadian rhythms of cortisol [17–21]. Remarkably, these disorders also present increased production and/or levels of peripheral and central inflammatory factors [21].

In previous studies we used the Wobbler mouse model of ALS to elucidate if hypercorticosteronemia associates with neuroinflammation. Wobblers show a point mutation of the vacuolar protein sorting-associated protein 54 (Vps54) leading to motoneuron degeneration and aberrant glial cell function [22]. Wobblers show dysregulated HPA axis, increased levels of corticosterone in brain, plasma and adrenal glands, focal adrenal hypertrophy and enhanced response to stress [14, 23]. Concomitantly, the mutant mice show faulty hippocampal and spinal cord parameters. To disclose if changes of hippocampus are reversible by inhibition of GR, Wobbler mice received for 4 days the specific GR antagonist CORT108297. Treatment with CORT108297 increases doublecortin (DCX) + neuroblasts in the subgranular zone of the hippocampus, diminishes astrogliosis and changes the phenotype of Iba1 + microglia from a reactive to a quiescent form without changing the hypercorticosteronemia [24]. Besides hippocampus, pathological changes are also prominent in the spinal cord of Wobbler mice, which show microgliosis, astrogliosis and high expression of proinflammatory factors [14, 23–28]. Treatment of Wobblers for 3 weeks with the GR modulator CORT113176 (Dazucorilant) reduces proinflammatory markers and density of astrocytes and microglial cells [29, 30]. We hypothesized that CORT113176 interferes with GR function or could favor restoration of the balance between pro- and anti-inflammatory signaling pathways in coordination with the MR [29]. That brain and spinal cord neuroinflammation of the Wobbler mouse decreases by treatment with GR modulators [24, 30, 31], supports the paradoxical proinflammatory effect of glucocorticoids.

Classically, mifepristone (RU496) has been used to inhibit the GR in hypercorticoidisms and to prevent secondary effects of glucocorticoids. However, mifepristone is a non-selective compound with potent antagonism for progesterone receptors [32]. Therefore, specific inhibitors/modulators of higher affinity have been developed. In this regard, the GR modulators CORT113176 and CORT108297 have been used to reverse β-amyloid toxicity, loss of memory processes, hippocampal pathology and neuroendocrine overshooting, supporting a pathological role of GR activation in the context of CNS diseases [32–35]. In control NFR/NFR mice, we have shown that treatment with corticosterone for 5 days reduces DCX + neuroblasts and induces astrocyte hypertrophy in hippocampus dentate gyrus. In these circumstances, treatment with CORT108297 antagonizes the corticosterone effects [24]. Furthermore, efficacy of CORT113176 as antagonist is higher than CORT108297, a property that can be due to differences in the affinity of both compounds for GR [36] or different capacity to recruit coregulators that would condition their function [33, 37]. Within this family of compounds, CORT118335 shows mainly brain antagonism, but for CORT108297 there is also agonism. For these reasons they are considered GR modulators [32].

We now tested the hypothesis that neuroinflammation associates to adrenal dysfunction using a high inflammation, high corticosterone model that resemble the situation of the Wobbler mouse. Chronic stress produces prolonged activation of the HPA axis and results in maladaptive responses (increasing allostatic load) with detrimental effects in the nervous system [38]. Thus, prolonged high stress levels of adrenal glucocorticoids increase reactivity and proliferation of microglía and astrocytes in prefrontal cortex and hippocampus, with enhanced expresión of proinflammatory mediators [39–42]. Spinal cord astrocytosis or astrocyte hypertrophy have also been described in the spinal cord from animals subjected to stress or corticosterone treatment [13, 24, 43]. We thus analyzed the involvement of GR on spinal cord neuroinflammation, and compared the expresión of proinflammatory factors within the HMGB1-TLR4-NFkB pathway, microgliosis, astrogliosis and related markers of glial dysfunction between stressed mice and stressed mice receiving CORT113176. Our data provided

support that treatment with a GR modulator rescued the spinal cord from stress-related neuroinflammation, which could be advantageous for the treatment of ALS, multiple sclerosis, and spinal cord injury.

Materials and Methods

Experimental Animals

Mice of the NFR/NFR strain originally obtained from NIH (Bethesda, MD, USA) were bred and maintained in the animal room facility of the Instituto de Biologia y Medicina Experimental. The NFR/NFR strain is the background strain of Wobbler mice, in which CORT113176 prevented unwanted effects of endogenous glucocorticoids. To better reproduce these effects in stressed mice, the same strain was used. Mice were kept in ventilated cage racks under controlled temperature (22 ° C), a 12/12 h light dark cycle with lights on at 7 am. Mice were fed Purina mouse chow and water ad libitum. Five-month-old animals were divided into three groups (A) control non-stressed mice; (B) mice subjected to restrain and rotation stress; (C) mice subjected to restrain and rotation stress and receiving CORT113176 (Dazucorilant, ([4a(R)-1-(4-fluorophenyl)-6-(4-trifluoromethylphenyl)sulfonyl)-4,4a,5,6,7,8-hexahydro-4aH-pyrazolo[3,4 g]isoquinolin-4a-yl][pyridine 2yl] methanone1H-pyrazolo, Corcept Therapeutics, Menlo Park, CA, USA) .CORT113176 shows a Ki value for GR of < 1 nM and does not interact with androgen receptors, estrogen receptors or MR [36]. The protocol used for stress and CORT113176 administration is graphically presented in Fig. 1 with full details of the procedure given in the legend.

The weight of the mice was recorded once a week for a period of 3 weeks. For immunohistochemistry and immunofluorescence procedures, mice were first anesthetized with a mixture of ketamine (75 mg/kg, i.p, cat.#326F, Holliday Labs, Argentina) and xylazine (6 mg/kg, i.p, cat.#050,Richmond Vet., Argentina).and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4. For PCR analysis, anesthetized mice were decapitated and spinal tissue kept frozen at −80º C until used. All animal procedures were evaluated and approved by the Institutional Animal Care and Use Committee following the Guide for the Care and Use of Laboratory Animals (Animal Welfare Assurance, NIH certificate # F16-00065 A5072-01).

Immunohistochemistry and Immunofluorescence Procedures

For microglia labeling, dissected cervical spinal cords were embedded in Tissue-Tek (OCT compound, cat.# KMA.0100.00 A, Cell Path, Newtown, U.K.) 16 µm sections were cut in a cryostat set at −10ºC, and stained for microglia using a rabbit anti-Iba1 antibody (1:2000, Cat. #019-19741, RRID: AB_839504, Wako, Japan). The secondary antibody was a goat anti-rabbit IgG conjugated to Alexa Red 555 (cat # 21,428, RRID: AB_141784, Invitrogen, Life Technologies, Eugene, OR, USA) Sections were cover-slipped with Fluoromont (cat. # 0100-01, Southern Biotech, Birmingham, AL, USA) and Iba1 + immunofluorescent microglial

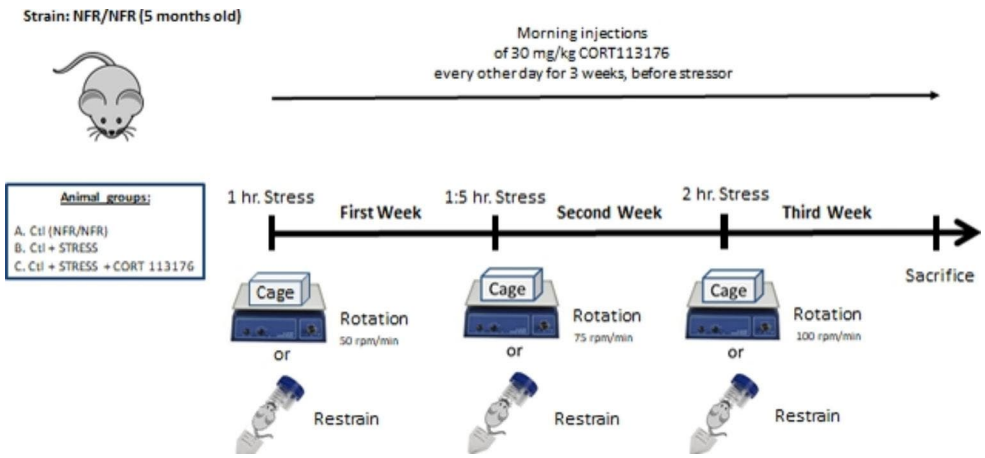

Fig. 1 Five months old male mice of the NFR/NFR strain were left as control (**A**), subjected to stress (**B**) or subjected to stress and given COR113176. at the beginning of the 1st week (**C**). For restraining stress, mice were introduced into Falcon tubes with open ends to allow normal breathing. Rotation stress consisted of maintaining mice in Plexiglas cages on top of a platform rotating at speeds from 0 to 100 rpm/min. Stress lasted for 1 h the 1st week, 1.5 h the 2nd week and 2 h the 3rd week. Mice were stressed once a day and the regimen of the stressor was alternated to avoid habituation. Mice in group C were given morning (8 a.m.) injections of 30 mg/kg CORT113176 every other day for 3 weeks, whereas groups A and B received oil vehicle only. CORT113176 was dissolved in castor vegetable oil and injected s.c. on the back of the neck. During the experiment, injections of CORT113176 or vehicle were given 30 min before the employment of stressors. On day 21, mice were injected at 8 a.m. with CORT113176 or vehicle, stressed and sacrificed 2 h afterwards [31]

cells were counted in gray matter of the ventral horn [30]. Images taken with the confocal microscope were analyzed using Image J (Image Processing and Analysis in Java, NIH, MD, USA) at 200X. The number of Iba1 + microglia was quantified by this program and expressed per unit area (mm^2). Cells were counted in 5–6 sections per mice. The number of mice in this and subsequent experiments are indicated in the Figure legends.

The response of astrocytes was determined by immunohistochemistry using a GFAP antibody that stains both quiescent and reactive astrocytes. After anesthesia and perfusion, the cervical region of the spinal cord was used to compare actual data with those obtained in CORT113176-treated Wobbler mice [30]. Paraffin sections were exposed to a primary rabbit polyclonal GFAP antibody (1/500 dilution, cat.# G9269,RRID:AB_477035, Sigma-Aldrich, USA)) followed by a biotinylated goat antirabbit secondary antibody (1:200 dilution, cat# 7014, RRID:AB_477035, Sigma-Aldrich). Thereafter, sections were treated following an ABC kit instructions (cat# PK2200, RRID: AB_2336835, Vector Labs, CA, USA), with peroxidase activity revealed by diaminobenzidine tetrachloride (DAB, Sigma). GFAP + cells were quantitated by a computerized image analysis system (Bioscan Optimas VI, Edmonton, WY, USA) equipped with a Panasonic GPKR222 camera connected to an Olympus BH2 microscope. GFAP + cells were counted in the ventral horn gray matter from at least 6 sections per mice, and results were averaged per animal. Data were expressed as the mean number of labeled cells ± SEM per unit area (mm^2).

Glutamine synthase (GS) staining was performed in sections pretreated with mouse IgG blocking reagent (cat.#PK2200,Vector M.O.M. Immunodetection Kit, Vector Labs) and then incubated with monoclonal mouse anti- GS (1:200 dilution, cat. #610,517, BD Biosciences, RRBD_AB 387,879, CA, USA). This step was followed by incubation with a secondary monoclonal antimouse (M.O.M. Immunodetection Kit cat# PK2200, Vector Labs. Cells were counted in 5–6 Sects. (5–6 images were taken from the right side of the ventral horn of the spinal cord and 5–6 images from the left side, leaving a total of 10–12 quantified images) from each animal. GS + cells, were quantitated by a computerized image analysis system (Bioscan Optimas VI, Edmonton, WY, USA) equipped with a Panasonic GPKR222 camera connected to an Olympus BH2 microscope.

qPCR of Glial Cell Markers and Inflammatory Mediators

The cellular response to stress and CORT113176 was also analyzed at the transcriptional level by measuring mRNA of the astrocyte markers aquaporin 4 (AQP4) and SOX9 [44, 45] and two microglia markers: CD11b and the purinergic receptor P2RY12 [46, 47]. Regarding the inflammatory mediators we selected those pertaining to the HMGB1 → TLR4 → NFkB pathway [48, 49]. As shown in previous work, CORT113176 down-regulates the increased levels of these inflammatory factors in the spinal cord of Wobbler mice [31]. All mRNA levels were measured by real time PCR using previously published procedures [50]. The cervical spinal cord was used for qPCR analysis, to better compare results with those in the Wobbler mouse in which pathology is restricted to the cervical región [30, 31]. Sequences of primers are shown in Table 1.

Briefly, total RNA was extracted from spinal cord with Trizol (cat.#15,596,026,Life Technologies-Invitrogen, CA, USA), and remaining DNA removed by treatment with DNase1 (cat.# EC 3.1.21.1, Promega, Madison, WI, USA). Then, we used a M1705 MMLV reverse transcriptase (cat# EC 2.2.2.49; Promega) for PCR amplification of DNA templates in the presence of random hexamer primers. Cyclophilin was used as the house keeping gene. A real time Step-one Plus sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to establish gene expression profiles and mRNA expression was analyzed by the $2^{-\Delta ct}$ method [51]. Results were expressed as fold induction over group (A), composed of non-stressed mice.

ELISA for IL1β and TNFα

Levels of serum IL-1β and spinal cord TNFα were quantified by ELISA. A volume of 100 µl of serum or tissue

Table 1 Sequence of primers for PCR analysis

Gene	Gene Bank Accession No.	Forward Primer (5′- 3′)	Reverse Primer (5′- 3′)
CD11b	***NM_008401***	AAACCACAGTCCCGCAGAGA	CGTGTTCACCAGCTGGCTTA
TLR4	***NM_021297***	GGCTCCTGGCTAGGACTCTGA	TCTGATCCATGCATTGGTAGGT
NFκB p50	***NM_ 008689***	TCCACTGTCTGCCTCTCTCGTC	GCCTTCAATAGGTCCTTCCTGC
P2RY12	***NM_027571.4***	TTTCAGATCCGCAGTAAATC-CAA	GGCTCCCAGTTTAGCATCACTA
AQP4	***NM_009700.3***	CTGGAGCCAGCATGAATCCAG	TTCTTCTCTTCTCCACGGTCA
SOX9	***NM_011448.4***	GGACAACACATGCCTCTGCAA	TCTCCAGCCACAGCAGTGAG-TAA
Cyclophilin b	***NM_022536***	GTGGCAAGATCGAAGTG-GAGAAAC	TAAAAATCAGGCCTGTG-GAATGTG

homogenate was used to quantify these cytokines. Homogenates from the spinal cord were prepared in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na3VO4, and 1% Triton 100, pH 7. 4) containing a protease inhibitor cocktail (Roche Diagnostics). We employed the BioLegend ELISA Max Deluxe set kit (San Diego, CA, USA) for IL-1 β (Cat# 432604,5 plates) and for TNFα (Cat# 430904,5 plates), in accordance with the manufacturer's instructions Each sample was quantified in triplicate.

Determination of Serum Corticosterone

Trunk blood was taken in the morning after decapitation of anesthetized mice. Serum was collected and steroids were extracted with dichloromethame. Corticosterone was determined by RIA using an antiserum provided by Dr. A. Bélanger, Laval University, Quebec, Canada. For this assay, inter and intra assays coefficients of variation were 5.9% and 4.9% [14], respectively, with sensitivity set at 0.3 ng/ml. Results were expressed as ng/ml of serum corticosterone.

Statistical Analysis

Data were analyzed by one-way ANOVA followed by the post-hoc Newman-Keuls test with the exception of the effect of treatments on the weight of the animals which was analyzed by repeated measures two-way ANOVA. Statistical analysis was performed with Prism 9 GraphPad software (San Diego, CA, USA). The level of statistical significance was set at *and $^{\#}p<0.05$, ** and $^{\#\#}p<0.01$, and *** and $^{\#\#\#}p<0.001$.

Results

Changes of Body Weight, Spleen Weight and Serum Corticosterone in Mice Subjected to Stress Plus or Minus CoRT113176

As shown in Fig. 2A body weight of control mice was maintained or slightly increased throughout the 3 week period. Instead, stressed mice with or without CORT113176 treatment showed a significantly decreased body weight at the end point of the 3 week period. However, stressed mice receiving CORT11376 were moderately but significantly heavier than the stress-only group. Statistical analysis using two-way ANOVA with repeated measures showed significant effects of time ($F(_{3,39)=}$ 12.91, $p<0.001$), effects of treatment ($F(_{2,13})=5.174, p=0.022$) and effects of time x treatment interaction ($F_{(6,39)=}$ 6.536, $p<0.001$). Regarding spleen weight, one-way ANOVA test showed significant group differences according to treatment ($F_{(2,23)}=13.11$, $p<0.0002$). The multiple comparison test showed a significant decrease in spleen weight in stressed mice ($p<0.001$ vs. control Fig. 2B) whereas spleen weight was recovered in stressed mice receiving CORT113176 ($p<0.01$ vs. stress only group). As expected, stressed mice showed high levels of serum corticosterone (Fig. 2C). Quantitative analysis by ANOVA showed significant group differences

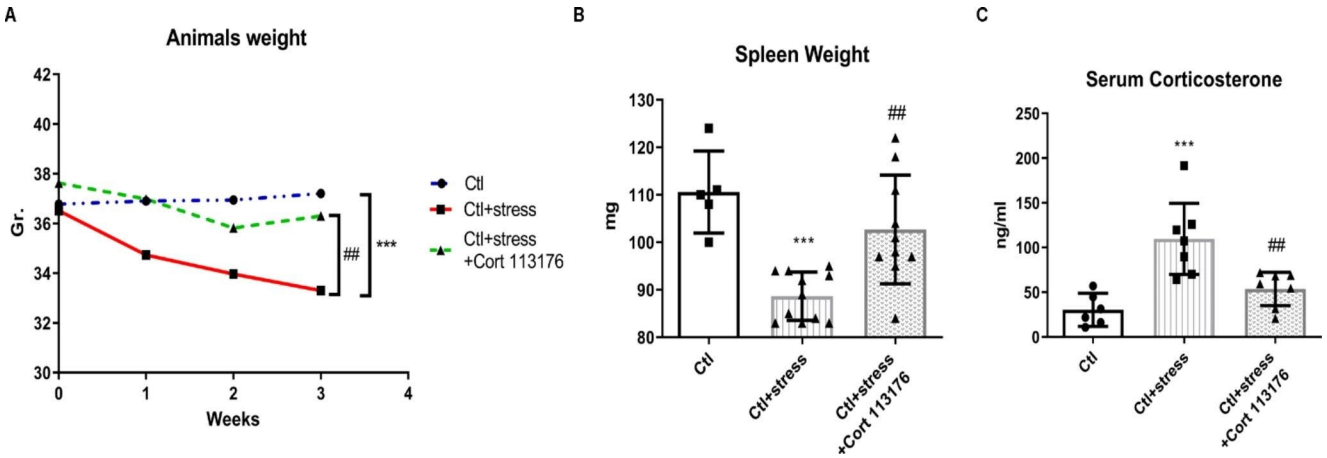

Fig. 2 **A**: Changes of body and spleen weight and plasma corticosterone in control, stressed mice and stressed mice receiving CORT113176 (Dazucorilant) for 3 weeks. Whereas control mice maintained their body weight during the 3 weeks experimental period, stressed mice were lighter vs. controls. Body weight decline was partially corrected by CORT113176. *Two-way ANOVA análisis of this parameter showed significant effects of time ($p<0.0001$), treatment ($p=0.022$) and time x treatment interaction ($p<0.0001$). Multiple comparison test at the 3rd week showed significant differences between control and stress groups (*** $p<0.001$) and stress vs. stress + CORT113176 groups (## $p<0.01$)***B***: Weight of spleen was reduced in stressed mice vs. controls (***$p<0.001$). CORT113176 treatment of stressed mice increased spleen weight vs. the stress only group (## $p<0.01$). (n for controls = 5; stress and stress + CORT113176 groups 10 mice per group). **C**: Plasma corticosterone was increased by stress (***$p<0.001$) vs. control mice, and decreased after CORT113176 treatment of stressed mice ($^{\#\#}$ $p<0.01$). Results represent 7 mice per group

($F_{(2,17)} = 12.45$, $p = 0.0005$). Thus, serum corticosterone was 4-fold higher in stressed mice vs. control animals ($p < 0.001$) whereas CORT113176 treatment of the stressed group significantly decreased serum corticosterone ($p < 0.01$ vs. stress only mice).

Effects of Stress and CORT113176 on Markers of Microglia Activation

Microglia becomes highly reactive in response to stress, with glucocorticoids playing a mediating role on microglia priming [40, 41, 52, 53]. In the present work, we employed control mice, stressed mice and stressed mice receiving the GR modulator CORT113176 to measure the response of microglia markers to the mentioned experimental conditions. Significant group differences for the microglia marker CD11b (cluster of differentiation molecule 11b) were found in the ANOVA test ($F_{(2,16)} = 7.602$,$p < 0.0048$). Post-hoc analysis demonstrated that stress lasting for 3 weeks significantly increased CD11b mRNA expression compared to control mice (Fig. 3A, $p < 0.01$). The increased CD11b mRNA of stressed mice was prevented by modulation of the GR with CORT113176 ($p < 0.01$ vs. the stress-only group) (Fig. 3A). ANOVA analysis also showed strong group differences for Iba1 immunofluorescent microglia ($F_{(2,12)=}100.8$, $p < 0.0001$) (Fig. 3C). Post-hoc analysis showed higher number of Iba1 + cells in the spinal cord of the stress group vs. the control group ($p < 0.001$) that was reduced in the stress + CORT113176 treated mice ($p < 0.001$ vs. stress-only mice) (Fig. 3C). Microscopy images of the mentioned changes of Iba1 + cells produced by stress and stressed mice receiving the GR modulator are shown in Fig. 3D.

Comparable results to those obtained for CD11b and Iba1 were found for P2RY12 mRNA, a purinergic receptor associated to activated microglia in neurodegenerative and neuroinflammatory disorders [54, 55]. We found group differences for P2RY12 mRNA in the ANOVA analysis ($F_{(2,18)} = 5.507$, $p = 0.0136$). A multiple comparison test revealed a moderate, although significant increase of P2RY12 in the stressed group ($p < 0.05$ vs. control) that decreased to control levels after treatment with CORT113176 ($p < 0.05$ vs. stressed-only group; NS vs. control mice). (Fig. 3B).

Comparable results to those obtained for CD11b and Iba1 were found for P2RY12 mRNA, a purinergic receptor associated to activated microglia in neurodegenerative and neuroinflammatory disorders [54, 55]. We found group differences for P2RY12 mRNA in the ANOVA analysis ($F_{(2,18)} = 5.507$, $p = 0.0136$). A multiple comparison test revealed a moderate, although significant increase of P2RY12 in the stressed group ($p < 0.05$ vs. control) that decreased to control levels after treatment with CORT113176 ($p < 0.05$ vs. stressed-only group; NS vs. control mice). (Fig. 3B).

Effects of Stress and CORT113176 on Astrocytes in the Spinal cord

Normal astrocytes are associated with neuroprotection and metabolic function of neurons, although under pathological circumstances they become a source of proinflammatory factors, with astrogliosis developing after chronic stress and glucocorticoid exposure [24, 43, 56]. In the present study, group differences were found in stressed mice without or with CORT113176 treatment. ANOVA analysis showed significant changes in GFAP + astrocyte number ($F_{(2,12)=}37.85$, $p < 0.0001$) and in the mRNA of the astrocyte-specific nuclear marker SOX9 ($F_{(2,19)} = 4.534$, $p < 0.0246$). Furthermore, multiple comparison tests showed a significant stress-induced GFAP + astrogliosis (Fig. 4A, $p < 0.001$) and higher mRNA of the astrocyte nuclear marker SOX9 (Fig. 4C, $p < 0.05$) vs. control mice. Changes produced by stress were restored after GR inhibition with CORT113176. The reducing effect was shown for GFAP immunolabeling and SOX9 mRNA (Fig. 4A, C and $p < 0.001$ and $p < 0.05$ vs. stress-only group, respectively).

The astrocyte response to experimental conditions included the water channel acquaporin4 (AQP4) immunostaining and mRNA (Fig. 4D and F). Significant group differences were found for AQP4 immunoreactive area ($F_{(2,12)} = 13.54$,$p = 0.008$).The multiple comparison test showed higher levels for the stress group ($p < 0.001$ vs. control mice), which subsided when stressed mice received CORT113176 (Fig. 4D, $p < 0.01$ vs. stress only group). Immunofluorescence images of AQP4 protein (Fig. 4E) showed higher labeling of astrocyte-like cells and microvasculature in the stressed group compared to the control and stress + CORT113176 groups. Changes were also obtained for AQP4 mRNA ($F_{(2,15)} = 6.385$, $p = 0.0099$). A multiple comparison test showed that the stress-induced rise in AQP4 ($p < 0.05$ vs. control) was reduced by CORT113176 treatment ($p < 0.01$ vs. stress-only group) (Fig. 4F). To further localize AQP4 we performed a double label colocalization of AQ4 with GFAP using different Alexa fluor dyes. We found that AQ4 (green label) colocalized with astrocyte (red label) around microvessels (Suplementary Fig. 1).

Glutamine synthase (GS) is an enzyme of the glutamatergic pathway that metabolizes glutamate + NH4 into glutamine, preventing glutamate excitotoxicity [57]. ANOVA analysis showed significant group differences for GS ($F_{(2,10)} = 39.05$, $p < 0.0001$). Stress powerfully down-regulated GS vs. control mice ($p < 0.001$), whereas GS + cells were modestly although significantly increased by CORT113176 treatment ($p < 0.05$) (Fig. 4G). Images in Fig. 4H show scarce GS + cells in the stressed mice (middle graph) vs. the control or stress + CORT113176 groups (left and right graphs, respectively). Although GS is considered an

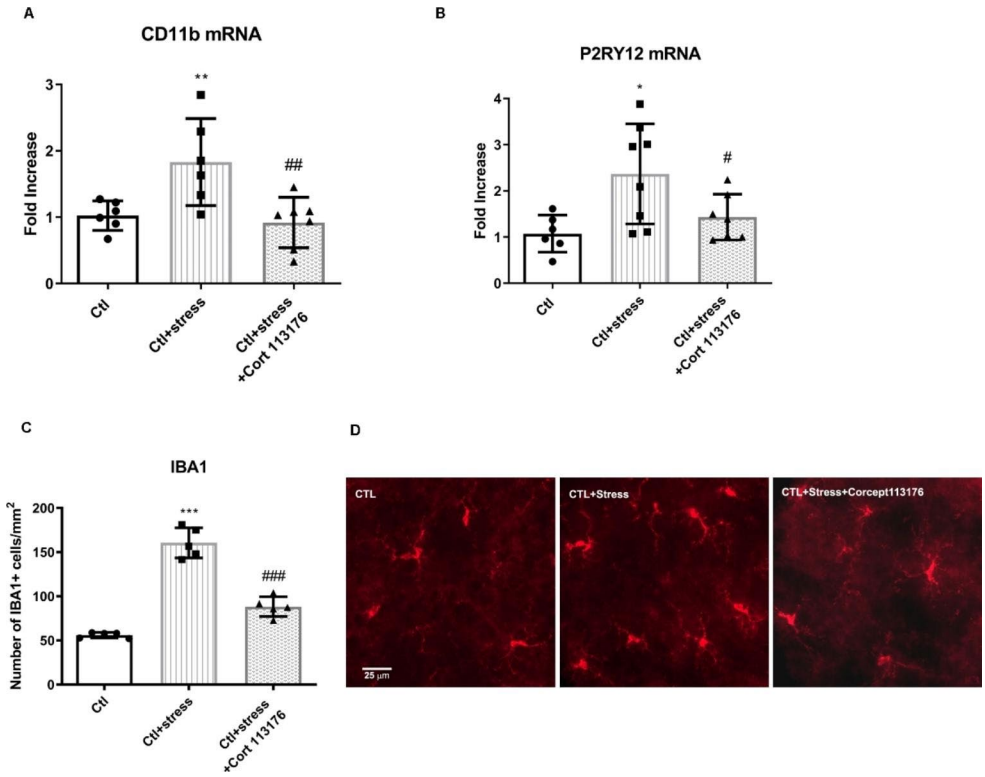

Fig. 3 Changes of microglia-related parameters produced by stress and CORT113176 (Dazucorilant). **A**:Stress significantly increased CD11b mRNA (** $p<0.01$, $n=6$) vs. control mice ($n=6$). CORT113176 + stress decreased this microglía marker vs. the stress-only group ($^{\#\#}$ $p<0.01$, $n=7$). **B**: The purinergic receptor P2RY12 mRNA levels were higher in stress mice (*$p<0.05$,$n=8$) vs. control mice ($n=6$). CORT113176 treatment decreased P2RY12 levels ($^{\#}$ $p<0.05$, $n=7$) vs. the stress only group. **C**: Number of Iba1 + cells was higher in the stress group (*** $p<0.001$) vs. control mice, whereas CORT113176 returned Iba1 + cell number to normal ($^{\#\#\#}p<0.001$ vs. stress only group). Cells were counted in 5–6 sections per mice ($n=5$ mice per group).**D**: Immunofluorescent staining of Iba1 + cells shows lower cell density in control and stressed + CORT113176-treated mice (left and right images) vs. the stress group (middle image). Inside bar: 25 µm

astrocyte-produced protein, its cellular location has been disputed [58]. However, studies using antibody combinations producing green and red fluorescent labeling of each marker and confocal microscopy, show scarce double-labelled GS-GFAP + cells (Fig. 4B, right image, arrowhead), whereas most GFAP + cells were GS negative (Fig. 4B left and middle images) supporting previous contentions that part of GS + cells in the spinal cord may be oligodendrocytes [59, 60].

Effects of Stress and CORT113176 on Proinflammatory Mediators

Stress-induced glucocorticoid secretion activates the synthesis and release of proinflammatory mediators in the brain [16, 21, 40, 41]. Our data demonstrated that similar effects take place in the spinal cord. As shown in Fig. 5, stress up-regulated the expression of inflammation-related molecules TLR4 mRNA, NFkBp50 mRNA, HMGB1 + cells and TNF α protein in the spinal cord and IL1β in serum. ANOVA showed significant group differences in the spinal cord for NFkBp50 mRNA ($F_{(2,16)}=7.374$, $p=0.0054$), TLR4 mRNA ($F_{(2,17)}=6.876$, $p=0.0065$),TNFα protein ($F_{(2,11)}=6.255$, $p=0.0153$), and serum IL1β ($F_{(2,18)}=19.53$,$p<0.0001$). Multiple comparison tests showed that stress increased levels of TLR4 mRNA (Fig. 5A, $p<0.05$), NFkBp50 mRNA (Fig. 5B, $p<0.01$), TNFα protein (Fig. 5C, $p<0.05$), and serum IL1β (Fig. 5D, $p<0.001$) vs. control mice.

Administration of CORT113176 for 3 weeks to stressed mice prevented or attenuated proinflammatory mediators, as demonstrated by decreased levels of TLR4 mRNA ($p<0.01$), NFkBp50 mRNA ($p<0.01$), TNFα protein ($p<0.05$) and serum IL1β ($p<0.001$) (Fig. 5A-D). Additionally, ANOVA showed significant group differences in the alarmin HMGB1 + cells/mm^2 ($F_{(2,11)}=16,54$,$p=0.0005$). Multiple comparison test showed increased labelling for HMGB1 in stressed mice (Fig. 5E, $p<0.001$ vs. controls) and decreased staining in the CORT113176-treated stressed group ($p<0.01$). Images of Fig. 4F show enhanced number of HMGB1 + cells in the stressed group and their decrease by antagonizing the GR with CORT113176. Therefore, modulation of the GR with CORT113176 prevented the

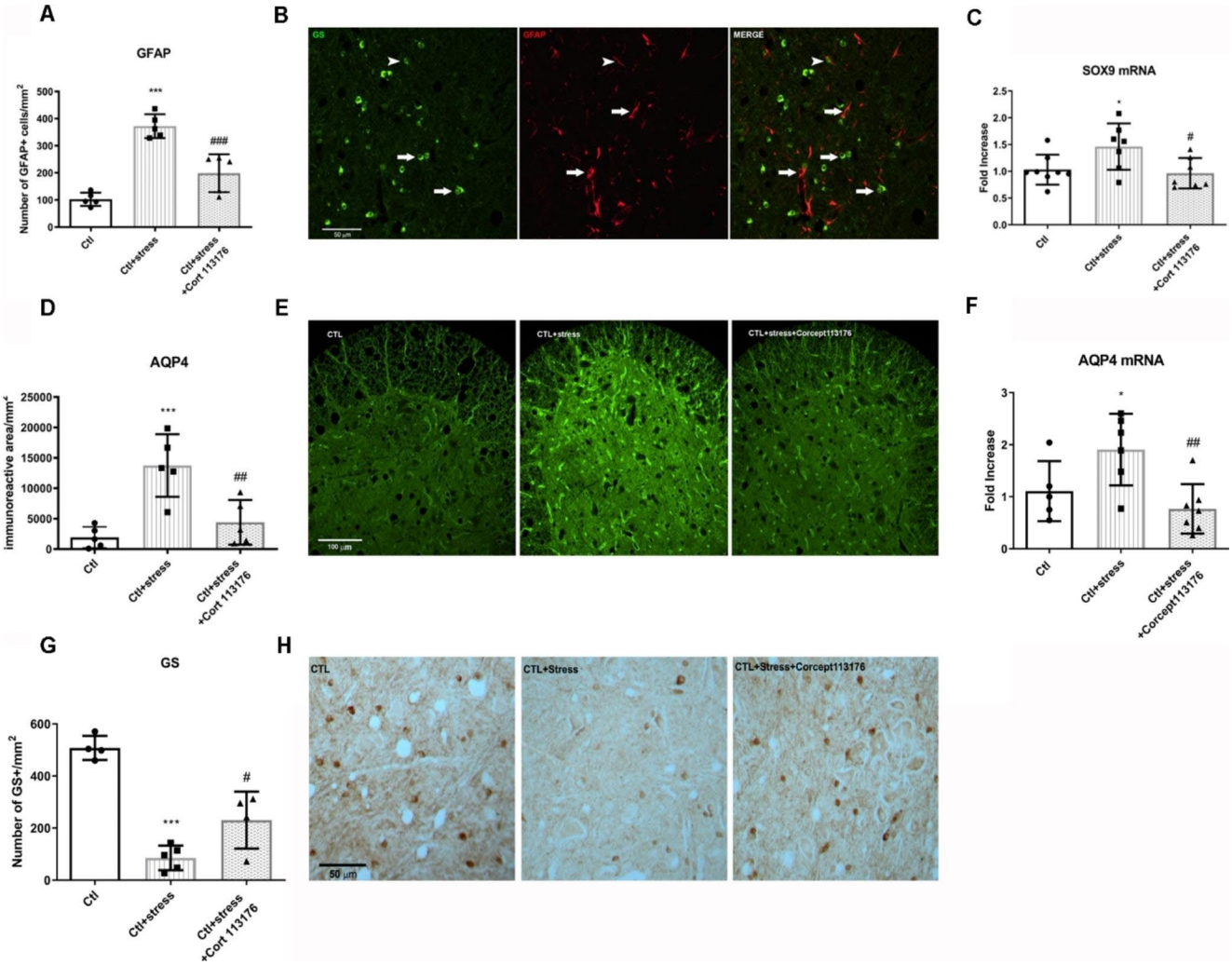

Fig. 4 Changes of astrocyte-related parameters produced by stress and CORT113176 (Dazucorilant). **A**: The astrocyte specific protein GFAP was upregulated by stress vs. control mice (*** $p < 0.001$) but GFAP + cell density declined when CORT113176 was given to stressed mice (### $p < 0.001$) ($n = 5$ animals per group). **C**: mRNA levels of the astrocyte nuclear marker SOX9 was increased in stressed mice (*$p < 0.05$) and was reduced after CORT113176 treatment (# $p < 0.05$) ($n = 7–8$ animals per group). **D**: immunolabeling for AQP4 revealed higher number of antigen + cells in stressed mice (*** $p < 0.001$ vs. control mice) that was significant decreased after CORT113176 treatment of stressed mice (## $p < 0.01$) ($n = 5$ mice per group). **E**: immunofluorecent images of AQP4 showed higher staining of astrocytes and vessels in the stressed group (middle panel) vs. the control or CORT113176-treated mice (left and right images, respectively). **F**: AQP4 mRNA expression was increased in stressed mice (*$p < 0.05$ vs. controls), whereas CORT113176 reduced AQP4 mRNA levels (## $p < 0.01$ vs. stress only group). **G**: Immunocytochemistry for glutamine synthase (GS) revealed fewer GS + cells in the stressed group vs. control (*** $p < 0.001$) and vs. CORT113176-treated mice (# $p<0.05$ vs. stress-only mice). **H**: light microscopy images support the statistical analysis of **G** regarding GS, with the stressed group presenting less + cells than the other 2 groups. Inside bar: 50 µm. The cell type expressing GS is debatable. Figure 4**B** shows double-colocalization analysis. Left panel: green fluorescent GS + cells (arrows); middle panel red staining GFAP cells (arrows); right panel: merge image with few double.labeled cells (orange, arrowhead)

stress-induced increase of several proinflammatory markers in the spinal cord and serum.

Discussion

The main findings of the present experiments led us to conclude (1) the spinal cord is vulnerable to the effect of chronic stress; (2) chronic stress associates with high levels of serum corticosterone and induces neuroinflammation; (3) the GR is a mediator of the proinflammatory effects of corticosterone, and (4) modulation of the GR with CORT113176 counteracted unwanted effects of glucocorticoids in the spinal cord.

Thus, we demonstrated that a 3 weeks experience of variable restraining / rotating stress paradigm produced signs of spinal cord pathology in mice. The observed changes resembled those reported for the Wobbler mice

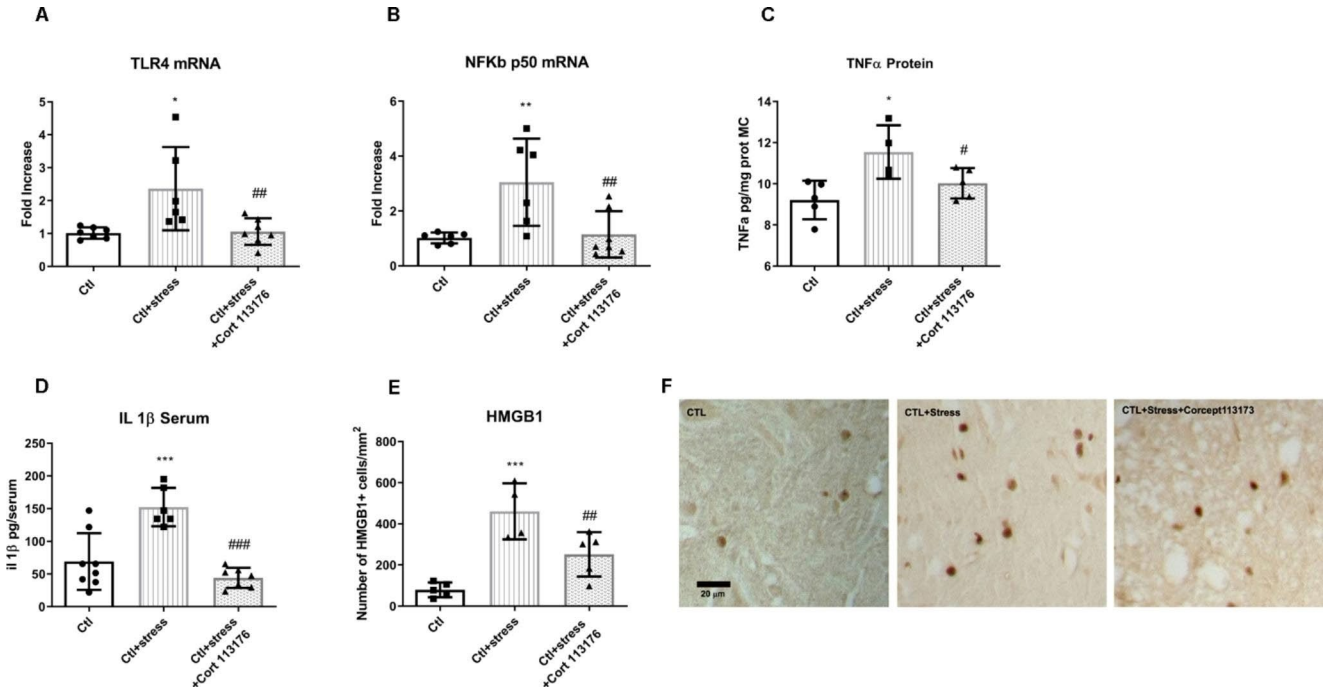

Fig. 5 Changes of proinflammatory factors in control stressed mice and stressed mice receiving CORT113176 (Dazucorilant). **A**: mRNA levels of TLR4 mRNA was increased in stressed mice (* $p < 0.05$ vs. controls) and normalized to control levels after CORT113176 treatment (## $p < 0.01$ vs. stress-only group) (n = 6 to 7 animals per group). **B**: The mRNA for the NFκBp50 subnit was higher in the stress group (** $p < 0.01$ vs. controls) and decreased after CORT113176 treatment (## $p < 0.01$) (n = 6–7 animals per group). **C**: TNFα protein measured by ELISA show higher levels in the stress grioup (* $p < 0.05$ vs. controls) amd lower levels tan stress only when mice received CORT113176 (# $p < 0.05$). (n = 5 mice per group). **D**: Serum IL1β levels were stimulated by stress (*** $p < 0.001$ vs. control mice) but highly decreased in the CORT113176 + stress mice (### $p < 0.001$) (n = 7–8 mice pre group). **E**: Density of the high mobility box group 1 protein (HMGB1) + cells were substantially increaded by stress (*** $p < 0.001$ vs. controls) and were reduced in number following CORT113176 treatment of stressed mice (## $p < 0.01$ vs. stress only mice). (n = 5 mice per goup). **F**: Light microscopy images oF HMGB1 + cells showing more immunoreactive cells in the stressed mice (middle image) compared to control or stressed mice receiving CORT113176 (left and rigth images, respectively). Inside bar: 20 µm

model of ALS studied under non-stressed conditions [29, 30]. Both models showed increased circulating corticosterone levels, decreased spleen and body weight, enhanced parameters related to astrogliosis and microgliosis and increased expression of proinflammatory factors within the HMGB1, TLR4, NFkBp50 immune-related pathway. Furthermore, these abnormalities were a likely response to hypercorticosteronemia, because they subsided when the GR modulator CORT113176 was co-administered with stress. In this regard, the response of stressed-control mice to CORT113176 resembled the response of Wobbler mice treated with this GR modulator [30].

Changes of body weight of stressed mice were expected, since high corticosterone levels produced by chronic stress activation of the HPA axis stimulates protein catabolism in muscle and lipid degradation in adipose tissue [61]. Hypercorticosteronemia also targets the spleen, a glucocorticoid-target tissue expressing GR. In this context, restrain stress increases GR activation, corticosterone-induced apoptosis and shrinkage of the spleen [62, 63]. Both parameters were significantly modified by treatment with CORT113176, which produced a partial recovery of body weight and also restored spleen weight. Along this line, there is a brain-spleen axis dysfunction during stress in which activated microglia recruits peripheral immune cells to the brain, implying that the spleen contributes to neuroinflammation [64]. The posssibility that CORT113176 restores the regulation of the brain-spleen axis opens new venues to understand the relationhip between stress-related and immune-related pathologies.

Our data are also in agreement with several publications reporting agonistic activity (i.e., inhibition of the HPA axis) of GR modulators, with normalization of circulating corticosterone. In this regard, a 7-day treatment with CORT113116 or CORT108297 prevents the rise of corticosterone induced by icv injection of amyloid β in rats [33], whereas CORT108297 given to rats for 10 days decreases hypercorticosteronemia caused by status epilepticus [65]. Moreover, treatment with CORT113176 or CORT108297 for 5 days or with CORT113176 for 18 days decreases plasma corticosterone of Wobbler mice [24, 30]. Shorter treatments are also effective. Thus, increases of plasma

corticosterone measured 15, 30 or 60 min after restrain or forced swim stress are prevented by treatment with CORT108297 [66, 67]. In the study of Gehrand et al. [66] CORT113176 in very high doses increases plasma corticosterone following hypoxic stress of neonatal rats, suggesting an antagonist role of this compound on the HPA axis. In the present experiments, 21 days of restraining/rotation stress increased serum corticosterone levels by 3.6-fold vs. control mice. This increase was significantly reduced by daily treatment with CORT113176, suggesting an agonistic-like effect at the hypothalamic or anterior pituitary level. Thus, using our experimental design, levels of serum corticosterone in stressed + CORT113176-treated mice were not significantly different from the non-stressed group. Therefore, the agonist or antagonist activity of GR modulators on the HPA axis may depend on the experimental situation, tissue in question, dosage or age of the animals. On the other hand, all published reports conclude that GR modulators show negative regulation of GR in glucocorticoid targets situated in some peripheral organs and the central nervous system.

Previous work in Wobbler mouse motoneuron degeneration demonstrate increased levels of corticosterone in plasma, spinal cord and brain, suggesting a pathogenic role for glucocorticoids in this disorder [14, 23]. These studies show that treatment of Wobblers with 30 mg/Kg CORT113176 for 21 days prevents spinal cord neuropathology, decreases reactive gliosis, motoneuron vacuolation, plasma corticosterone and expression of proinflammatory factors. Therefore, a mixed role of CORT113176 also takes place in this model, because it shows agonist activity on the HPA axis and antagonistic activity in the spinal cord, resulting in the inhibition of reactive gliosis and neuroinflammation.

The present report provides support that stress-induced hypercorticosteronemia associates with a neuroinflammatory condition with up-regulation of inflammatory factors. At first sight, this hypothesis seems controversial with the long accepted view that glucocorticoids suppress immune reactions and inflammatory factors [61, 68]. Because of these properties, glucocorticoids are widely used for the treatment of asthma, rheumatoid arthritis, respiratory distress syndrome, skin diseases, hematological cancers, inflammatory diseases, CNS trauma and transplant rejection [69]. However, there may be a switch from an anti- to a proinflammatory effect of glucocorticoids, depending on dosage, tissue environment, time of exposure and molecular mechanisms of GR signaling at target genes [7, 9, 10, 12, 29, 70]. As already mentioned, glucocorticoid proinflammatory effects have been demonstrated in the nervous system. Thus, in the hippocampus and frontal cortex stress-induced glucocorticoid secretion increases LPS-induced NFκB activation and induces the inflammatory factor TNFα and the nitric oxide synthesizing enzyme iNOS [10, 71, 72], whereas direct treatment with glucocorticoids causes neuronal damage and neuroinflammation [73]. Frank et al. have shown that stress-induced neuroinflammation is glucocorticoid-dependent, because these hormones mediate microglia reactivity, induction of the alarmin HMGB1 and potentiate NRLP3 inflammasome activation [40, 41]. These authors have shown that severe stress and corticosterone treatment induce, whereas adrenalectomy or mifepristone treatment decreases HMGB1 expression, suggesting that effects on HMGB1 are due to glucocorticoids direct effects on microglia. Thus, likely events taking place in the spinal cord of stressed mice would involve HMGB1, its receptor TLR4, and activation and nuclear translocation of NFkB with transcription effects on inflammatory cytokine genes. This cascade takes place in several inflammatory conditions [48, 49]. Although the cell types showing HMGB1 immunoreactivity were not discriminated, we have shown before colocalization of HMGB1 with the astrocyte marker GFAP and HMGB1 with the microglía marker Iba1 by means of double-immunofluorescence techniques and confocal microscopy [31]. Therefore, its is likely that changes of this proinflammatory marker in the present experiments involved both microglía and astrocytes.

Although the high content of GR makes the hippocampus highly susceptible to glucocorticoid oversecretion [8, 12, 69, 73], the spinal cord is not spared from vulnerabilty [42, 74]. Since both microglía and astrocytes (in addition to ventral and dorsal horn neurons) express immunoreactive GR [30], it is likely that CORT113176 directly affects this receptor in glial cells. The current stressed experiments support this view. Thus, GR hyperactivation becomes a danger signal increasing CNS pathology, while inhibition of this receptor with the GR antagonist mifepristone or with GR modulators dampens CNS vulnerability [24, 32–35, 65–67]. Considering the existing background, we analyzed if chronic stress in mice leads to spinal cord inflammation, an event that could be pharmacologically antagonized with a GR modulator. We found that after stress, mice developed microgliosis, increased Iba1 + labeling, high expression of the mRNAs of the microglia marker CD11b and the purinergic receptor P2RY12. This last receptor is expressed in microglia ramifications, is activated by ATP, is involved in motility and migration towards sites of injury or degeneration, activates the NLRP3 inflammasome and enhances release of IL-6 secretion by endothelial cells [46, 47, 54, 55]. Thus, important evidence supports a role pf P2RY12 in neuroinflammation. These possibilities suggest that stress activation of P2RY12 mRNA is related to the inflammatory response, which was inhibited by the GR modulator CORT113176.

Therefore, inflammatory mediators may be the common pathway for stress-related disorders involving glucocorticoids. Major components of this inflammatory pathway include HMGB1, TLR4, purinergic receptors, TNFα and NFkB for activated microglia and HMGB1, NFkB, TNFα and AQP4 for

reactive astrocytes, besides others not reported in this communication. Regulation of proinflammatory genes by glucocorticoid binding to GR expressed by astrocytes and microglia may explain proinflammatory actions of adrenal-derived steroids during chronic stress [7, 29]. We also adhere to the hypothesis that stress-mediated glucocorticoid elevation could prime the inflammatory phenotype of microglia, one likely source of inflammatory mediators [40, 41]. This possibility is reinforced by experiments showing that stress and corticosterone induced, whereas adrenalectomy or mifepristone treatment mitigates HMGB1 expression and microglia priming [71]. Therefore, glucocorticoids are probably involved in neuroinflammation because treatment of stressed mice with CORT113176 reduced Iba+microgliosis, CD11b and P2RY12 mRNAs, immunoreactive HMGB1+cells and TLR4 and NFkB mRNAs vs. stress-only mice. Thus, microglia are not the only source of inflammatory factors, because astrocytes under pathological conditions change their role from protective to damaging [75, 76]. Along this line, we showed increased number of GFAP+astrocytes in the spinal cord, together with high expression of the astrocyte water channel AQP4 mRNA and protein and of the astrocyte specific nuclear marker SOX9 [77]. A dual function of AQP4 has been observed, because when pathological conditions prevail, AQP4 is involved in astrogliosis, inflammation and release of cytokines [78, 79]. Furthermore, in our stressed mice model, we showed a reduction of immunoreactive glutamine synthase (GS), an enzyme considered a marker of astrocytes. Since stress lowers GS, less glutamate will be deaminated to glutamine, with subsequent increase of glutamate and generation of excitoxicity [58]. In addition to astrocytes, GS+cells may be located in oligodendrocytes [59, 60]. Whether stress influences colocalization of this enzyme in astrocytes and oligodendrocytes warrants further investigation. Furthermore, expression of TLR4 and response to HMGB1 also occurs in astrocytes resembling expression of these factors in microglia [75]. Therefore, it was most rewarding that CORT11376 treatment down-regulated astrogliosis and other parameters of inflammation including neurotoxicity. These data suggest that blockage of GR with CORT113176 disabled pathological functional phenotypes of astrocytes and microglia.

The cellular pathways employed by glucocorticoids as mediators of stress-induced inflammation of the spinal cord needs to be analyzed, in addition to the molecular mechanism employed by GR modulators leading to neuroprotection. The fact that CORT113176 counteracts the effects of stress on inflammatory mediators (i.e., HMGB1, TLR4, NFkB, etc.) seems a reasonable argument favouring antagonism. Instead, GR actions via its classical antiinflammatory role or the supression of stress-induced high corticosterone levels suggest agonistic effects. Therefore, the molecular mechanisms of this class of GR modulators needs further appraisal, because they show combined agonist/antagonist properties [37]. Having this caveat in mind, deactivation of the GR may be a useful pharmaceutical strategy for attenuating the damaging effect of high glucocorticoid levels detected in neurodegenerative diseases including ALS [29]. In this regard, a current phase II clinical trial (Beta.clinical trial.gov, NCT05407324) is recruiting patients to test CORT113176 (Dazucorilant) effects in ALS. The possibility exists, therefore, that modulation of the GR would become useful for the treatment of inflammatory pathologies [80] in addition to neurodegenerative disorders.

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Authors' contributions Maria Meyer and Maria Claudia Gonzalez Deniselle analyzed data and performed the experiments; Analia Lima perform cell immunolabelling; Onno Meijer and E.Ronald de Kloet corrected the manuscript, emphasized the agonist/antagonist nature of glucocorticoid receptor modulators and advised regarding the selection of cell markers; Hazel.Hunt. and Joseph Belanoff made valuable comments and corrected the manuscript; Maria Claudia Gonzalez Deniselle and Alejandro F. De Nicola wrote the final version. All authors read and approved the final manuscript.

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Data Availability All data presented in this study are included in the Materials and Methods section, in the corresponding References section and are available from the corresponding author on request.

Declarations

Ethics approval All animal procedures were evaluated and approved by the Institutional Animal Care and Use Committee following the Guide for the Care and Use of Laboratory Animals (Animal Welfare Assurance, NIH certificate granted to our Institute is # F16-00065 A5072-01).

Consent to participate All listed authors agreed to participate in this study. All listed authors gave explicit consent to submit. The work had the consent from the responsible authorities at the institutions where the work has been carried out.

Consent for publication All listed authors have approved the final manuscript before submission, including the names and order of authors.

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

Compliance with Ethical Standards The present report did not include human subjects.

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