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The selective glucocorticoid receptor modulator CORT108297 restores faulty hippocampal parameters in Wobbler and corticosterone-treated mice

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ABSTRACT

Mutant Wobbler mice are models for human amyotrophic lateral sclerosis (ALS). In addition to spinal cord degeneration, Wobbler mice show high levels of blood corticosterone, hyperactivity of the hypothalamic–pituitary–adrenal axis and abnormalities of the hippocampus. Hypersecretion of glucocorticoids increase hippocampus vulnerability, a process linked to an enriched content of glucocorticoid receptors (GR). Hence, we studied if a selective GR antagonist (CORT108297) with null affinity for other steroid receptors restored faulty hippocampus parameters of Wobbler mice. Three months old genotyped Wobbler mice received s.c. vehicle or CORT108297 during 4 days. We compared the response of doublecortin (DCX)+ neuroblasts in the subgranular layer of the dentate gyrus (DG), NeuN+ cells in the hilus of the DG, glial fibrillary acidic protein (GFAP)+ astrocytes and the phenotype of Iba1+ microglia in CORT108297-treated and vehicle-treated Wobblers. The number of DCX+ cells in Wobblers was lower than in control mice, whereas CORT108297 restored this parameter. After CORT108297 treatment, Wobblers showed diminished astrogliosis, and changed the phenotype of Iba1+ microglia from an activated to a quiescent form. These changes occurred without alterations in the hypercorticosteronemia or the number of NeuN+ cells of the Wobblers. In a separate experiment employing control NFR/NFR mice, treatment with corticosterone for 5 days reduced DCX+ neuroblasts and induced astrocyte hypertrophy, whereas treatment with CORT108297 antagonized these effects. Normalization of neuronal progenitors, astrogliosis and microglial phenotype by CORT108297 indicates the usefulness of this antagonist to normalize hippocampus parameters of Wobbler mice. Thus, CORT108297 opens new therapeutic options for the brain abnormalities of ALS patients and hyperadrenocorticisms.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motoneuron degenerative disease in humans [1]. Recent findings have also disclosed that in addition to spinal cord and brain stem pathology, ALS patients show abnormalities of hippocampus neurochemistry and function [2–4].

Animal models of motoneuron degeneration have been used to unravel pathogenetic mechanisms of ALS. The Wobbler mouse suffers a spontaneous mutation of the Vsp54 gene resulting in degeneration of lower and brain stem motoneurons [5], whereas in similarity with ALS, Wobblers also present a dysfunctional hippocampus [6]. Several of these abnormalities, with the exception of hippocampus neurogenesis, are normalized by treatment with neuroprotective doses of progesterone [6].

Several reports have demonstrated in ALS patients and Wobbler mice alterations of the endocrine system, which may aggravate neurodegeneration. For example, the hypothalamic–pituitary–adrenal (HPA) axis is highly dysfunctional in ALS patients [7]. ALS subjects show a loss of the cortisol circadian rhythm due to a significant morning increase in circulating cortisol,

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coupled to a decreased response to stress [7]. Our group has shown increased morning levels of circulating cortisol in patients with the sporadic form of ALS [8], and a blunted cortisol awakening response correlating with disease severity has been observed by Roozendaal et al. [9]. In common with ALS patients, dysregulation of the HPA axis, high levels of circulating corticosterone and aberrant response to stress characterized animal models with neurodegeneration, including Alzheimer's transgenic mice (3XTg-AD), streptozotocin-induced type I diabetic mice, type 2 diabetic rats, Wobbler mice and transgenic SOD1G93A mice [10–14].

Previous studies in Wobbler mice have shown increased basal plasma corticosterone, hyperresponse to stress [11] and up-regulation of microglial reactivity and microglia-related pro-inflammatory factors [15–19]. Chronic stress, excess levels of glucocorticoids and microglial activation are negative regulators of hippocampus neurogenesis [20–25]. Instead, adrenalectomy, treatment with the GR antagonist Mifepristone or inflammation blockade avoids the impairment of neurogenesis [24,26,27]. If hyperadrenocorticism plays a role in the hippocampal disturbances of Wobbler mice, antagonizing the glucocorticoid receptor (GR) should relieve the reported abnormalities of neurogenesis and glial cell reactivity [6]. To answer this question, we selected the GR antagonist CORT108297 (*R*)-4*a*-ethoxy-1-(4-fluorophenyl)-6-(4-trifluoromethylbenzenesulfonyl)-4,4*a*,5,6,7,8-hexahydro-1*H*-1,2,6-triazacyclopenta[*b*]naphthalene), a non-steroidal competitive compound with high and selective affinity towards GR (Ki 0.9 nM) and an almost 1000-fold lower affinity for the PR (progesterone), ER (estrogen), AR (androgen) or MR (mineralocorticoid) receptors [28]. We studied the effects of CORT108297 on doublecortin (DCX), a marker of neuronal progenitors [29], astrocytes immunoreactive for the glial fibrillary acidic protein (GFAP), microglia immunopositive for the marker ionized calcium binding adapter molecule 1 (Iba1) >and the specific nuclear protein (NeuN) marker of mature neurons in the hippocampus from symptomatic Wobbler mice. Second, we analyzed if CORT108297 also normalized some of these parameter in control mice receiving a corticosterone load. The results obtained with Wobbler mice and corticosterone-treated mice indicated that the GR modulator normalized hippocampus abnormalities. Thus, we suggest that CORT108297 may be therapeutically useful for endogenous hyperadrenocorticisms and to prevent secondary effects of glucocorticoid treatment on the brain.

2. Materials and methods

2.1. Mice

Wobbler mice (phenotype WR, genotype wr/wr) and homozygous healthy control littermates (NFR/NFR) were obtained from the Instituto de Biología y Medicina Experimental mouse colony. Mice were kept under conditions of controlled humidity and temperature (22 °C), with lights on from 07:00 am to 07.00 pm and fed standard mice chow with vitamin supplementation (Ensure, Abbott, Zwolle, Holland). The Wobbler phenotype was identified according to Rathke-Hartlieb et al. [18], employing an Alu I restriction polymorphism of a Cct4 amplification product for testing the allelic status at the wr locus. Cct4 diagnostic primers and restriction enzymes for genotyping were purchased from Promega (Madison, WI, USA). Animals were housed in group cages containing 2–3 Wobblers and 1 control mouse. This social interaction prolonged the life span and improved the health status of the Wobbler mice (Junier, personal communication). In the present experiments, we employed 3 months old Wobbler mice with motor deficit represented by tremor, ambulatory difficulty, slight flexion of proximal limbs, diminished muscle strength and weight loss. Animal of both sexes were used in comparable numbers in all groups since neither the onset nor the progression of the disease correlated with gender [30]. A group of Wobbler mice received s.c. vehicle or 3.5 mg/mouse/day during 4 days of the GR antagonist CORT108297 dissolved in a mixture of 3 w/v carboxymethylcellulose, 0.1% Tween 20 in 0.9% NaCl. Wobblers and their controls were killed 2 h after the last sc administration of the GR antagonist.

In a second experiment, control NFR/NFR mice (the background strain of the Wobblers) were divided into three groups. One group received vehicle, a second group received 45 mg/kg corticosterone in sesame oil daily for 5 days, and a third group received corticosterone plus 3.5 mg/mouse/day of CORT 108297 in the mixture described above. All mice in this experiment were killed on the 5th day, 2 h after receiving the last dose of the chemicals.

Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Assurance Certificate no. A5072-01) and were approved by the Institute's Animal Care and Use Committee. Efforts were taken to keep the number of animals at a minimum to obtain statistical significance.

2.2. Doublecortin (DCX) staining of neuroblasts

Wobbler mice show deficient hippocampal neurogenesis [6]. To unravel if hyperadrenocirticism played a role on this event, we studied the response of DCX+ cells to CORT108297 in Wobblers and corticosterone-treated control mice. Mice anesthetized with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg) were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. After an overnight incubation in 4% PFA, brains were transferred to Tris-buffered saline (TBS), pH: 7.4, sectioned frontally at 50 μm using a vibrating microtome and processed for free-floating immunocytochemistry. To label neuroblasts we employed a DCX antibody [29]. To this end, coronal brain sections were first exposed to methanol: H_2O_2 (100:1) during 10 min at room temperature washed and blocked for 30 min in PBS containing 10% rabbit serum at 37 °C. Sections were incubated overnight with a goat polyclonal anti-DCX antibody (1:250, sc-8066, Santa Cruz Biotechnology, CA, USA) followed by a biotinylated anti-goat IgG made in rabbit (1:200, Sigma Chemical Company, St. Louis, MO, USA) and processed following the ABC instruction kit (Vector laboratories, Burlingame, CA, USA). For development, we used diaminobenzidine chloride 0.25 mg/ml (Sigma Chemical Company, St. Louis, MO, USA), 0.05% H_2O_2 at room temperature (25 °C). Sections were finally dried, dehydrated and mounted with Permount (Fischer Scientific, Pittsburgh, PA, USA). Non-specific staining was assessed in the absence of primary antibody. Cell counts were restricted to the inner granular cell layer (GCL) of the dentate gyrus [30]. Thus, immunoreactive bodies filled with DAB product that came into focus and focusing down through the thickness of the section were considered for the study. DCX+ cells were counted every eighth coronal section throughout the entire rostrocaudal extension of the dentate gyrus, corresponding to Plates 26–40 from the stereotaxic atlas of the mouse brain [31]. The optical dissector method was used for cell counting, described in full in Beauquis et al. [32]. Quantitation was performed in 5 control mice, 5 untreated Wobblers and 8 Wobblers receiving CORT 108297 using a 40× objective in an Olympus BH-2 microscope and the image analysis software Bioscan Optimas II.

2.3. Neuron-specific nuclear protein (NeuN) immunostaining in the hilus of the dentate gyrus

To study if the Wobbler mutation and CORT108297 affected mature neurons as well as progenitors, we measured the number of NeuN+ neurons in the hilus of the dentate gyrus. The morphometric analysis was restricted to the hilus, because the low neuronal

Table 1
Serum corticosterone levels of control and Wobbler mice with and without CORT108297 and control mice receiving a corticosterone load with or without CORT108297.

Control	Wobbler	Wobbler + CORT108297	Control	Control + corticosterone	Control + corticosterone + CORT108297
91 ± 26	453.3 ± 105[a]	372.3 ± 60[a]	78 ± 92	670 ± 188[b]	491 ± 63 [b]

Results expressed as ng/ml serum (mean ± S.E.M.).
[a] $p < 0.01$ vs. control ($n = 6$–8 animals per group).
[b] $p < 0.01$ vs. control ($n = 5$ animals per group), statistical comparison by ANOVA and post hoc test.

density in this area allows a more accurate quantification [33]. Free-floating sections (50 μm) were incubated with a monoclonal NeuN antibody (anti-Neuronal Nuclei MAB 2377, Chemicon-Millipore, California, USA) at a 1/500 dilution overnight. Slices were rinsed three times in PBS 0.1% Triton X-100 for 15 min before application of a 1/200 dilution of the anti-mouse second antibody:(Vector) prepared in 1% horse serum and 1% Triton-X100 in PBS. Thereafter, a procedure similar to that used for DCX immunostaining was followed for development and quantification of NeuN+ cells. Results were expressed as the number of NeuN+ cells per hilus, and numbers of animals were 6 per group.

2.4. Glial fibrillary acidic protein (GFAP) immunofluorescene staining of astrocytes in hippocampus

Increased number of GFAP+ astrocytes showing a reactive phenotype are present in Wobbler hippocampus [6]. To study if the GR antagonist CORT10897 modulated astrogliosis, we employed immunofluorescence staining for GFAP. Sixteen micrometer cryostat sections from the dorsal hippocampus were post-fixed in 4% PFA and kept frozen at −80 °C until used. Sections were defrosted, washed in PBS and washed again in PBS containing 0.5% Triton X100. Slices were preincubated with 3 v/v goat serum in 0.5% PBS-Triton at 37 °C during 10 min and then incubated with a 1/250 dilution of the primary GFAP antibody (rabbit polyclonal anti-GFAP, cat. G-9269, Sigma, St. Louis, MO, USA) made in Triton-PBS buffer, 2% goat serum to block non-specific binding. After incubation during 18–20 h at 4 °C, a goat anti-rabbit IgG conjugated to Alexa Green 488 (1/1000 dilution) (Invitrogen, Molecular Probes, Eugene, OR, USA) was added in Triton-PBS, 2% goat serum solution. Following 1 h incubation in the dark, sections were washed, dried, mounted with Fluoromount-G (0100-01, SouthernBiotech, Alabama, USA) and examined in a confocal Nikon Eclipse E 800 microscope equipped with Nikon 11691 photographic equipment. Images taken with the confocal microscope were analyzed using Image J (Image Processing and Analysis in Java, NIH, MD, USA) at 600×. The number of GFAP+ astrocytes per unit area was quantified in the hilus of the dentate gyrus. Astrocytes were counted in 5 sections comprising right and left hemispheres from 5 animals per experimental group.

2.5. Iba1 immunofluorescence staining of microglia

Several studies have shown that microgliosis characterizes the Wobbler disease [15–19]. Therefore, we investigated if CORT10829 modified the microglial reactive profile. To this purpose, free-floating brain sections were obtained as described for DCX immunostaining. Sections were washed 3 times in PBS and blocked with 5% bovine serum albumin (BSA) in PBS for 30 min. For staining of microglia we used as primary antibody a 1/2000 dilution of rabbit anti-Iba1 antibody (Cat. no. 019-19741, Wako, Japan) prepared in 5% BSA, 0.1% Triton X100 in PBS. After incubation for 2 days at 4 °C, sections were washed with PBS and incubated for 1 h at room temperature with a goat anti-rabbit IgG conjugated to Alexa Green 488 prepared in the same solution used for the first antibody. After washing in PBS, sections were dried at room temperature and coverslipped with Fluoromont-G. Photographic observations and quantification of Iba+ immunofluorescent microglial cells were performed as described above for the immunfluorescent staining of GFAP+ astrocytes. To determine microglial reaction, Iba1+ cells were counted in the outer molecular layer of the dentate gyrus, region in which microglial cells and their processes were easily identified without the interference of neuronal bodies. Two different phenotypes of Iba1+ cells were considered for counting, following the classification of Kreutzberg [34]. (a) The ramified quiescent type bearing several stained processes and (b) the activated type showing fewer stained processes and ameboid morphology. Cell counting comprised at least 5 sections per mice. Number of mice was $n = 5$ per group. In both cases, data were expressed as % of "ameboid" Iba1+ cells respect of total Iba1+ cells present in the section.

2.6. Plasma corticosterone assays

For corticosterone determination, the steroid was first extracted with dichloromethame from mouse serum and then determined by RIA as previously described [35]. Corticosterone antiserum was provided by Dr. A. Bélanger, Laval University, Quebec, Canada.

2.7. Statistical analysis

All results were expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA followed by the post-hoc Newman–Keuls test. Statistical analysis was performed with Prism 4 GraphPad software (San Diego, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Effects of CORT108297 on serum corticosterone levels

The ANOVA test demonstrated a significant increased variation of serum corticosterone in Wobblers compared to control mice ($F_{2,18} = 6.05$; $p < 0.001$). Table 1 shows the corticosterone values and post-hoc statistical analysis of the three groups. Plasma >corticosterone was increased 5-fold in Wobbler mice compared to control NFR/NFR mice ($p < 0.01$). Following CORT108297, plasma corticosterone levels were slightly lower, but not significantly different from the untreated Wobbler group. ANOVA also showed significant variations in the experiment with control mice receiving corticosterone ($F_{2,10} = 6.125$; $p < 0.01$). Table 1 shows that corticosterone-loading of control NFR/NFR mice increased serum corticosterone 8-fold compared to control mice ($p < 0.01$). CORT108297 treatment of corticosterone-treated mice did not changed serum corticosterone levels compared to control mice receiving the steroid only ($p > 0.5$). Thus, high corticosterone values from endogenous or exogenous sources persisted in Wobbler mice and corticosterone-treated rats in the presence of the GR antagonist.

3.2. Effects of CORT108297 on DCX+ neuroblasts of Wobbler mice and of corticosterone-treated control mice

Previous results have shown a deleterious effect of the Wobbler mutation on neuronal progenitors in the hippocampus [6]. In

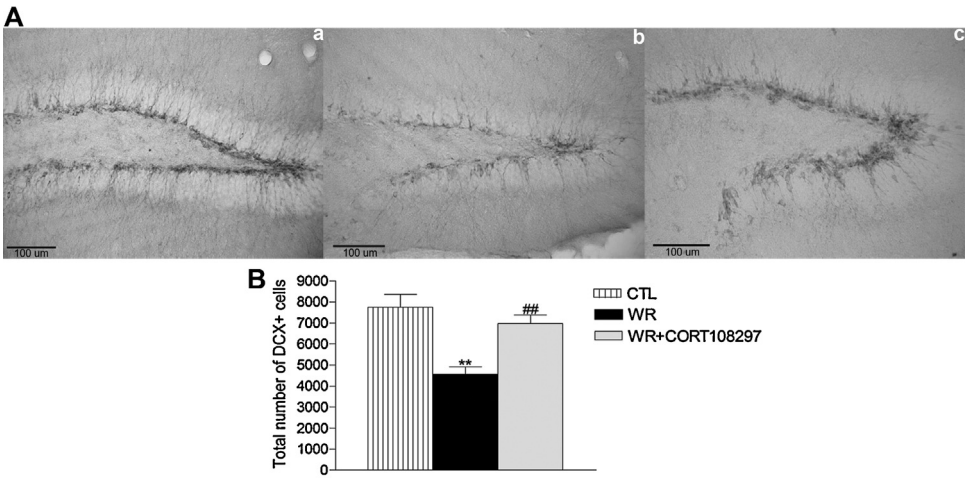

Fig. 1. Effects of a 4 day treatment with CORT 108297 on doublecortin (DCX) immunopositive neuroblasts in the dentate gyrus of hypercorticosteronemic Wobbler mice. (A) Representative microscope images of DCX+ cells in NFR/NFR control mice, Wobbler mice and Wobblers receiving CORT108297. (a) Control mice showed abundant DCX+ cells in the inner zone with staining of cell processes extending into the granule cell layer. (b) Untreated Wobbler mice showed sparse number of DCX+ cells in the inner zone with weak staining of bodies and few stained processes. (c) Wobbler mice treated with CORT108297 showed enhanced DCX expression, suggesting a more mature phenotype compared to untreated Wobblers. Inside bar: 100 μm. (B) Quantitative analysis of DCX+ cell number in the dentate gyrus. ANOVA and post-hoc test showed a significant decrease of DCX+ cells in Wobbler mice (Wr) compared to controls (Ctl) (** $p < 0.01$), and a significant rise of DCX+ cell number in Wobblers receiving CORT108297 compared to untreated Wobblers ($^{\#\#}$ $p < 0.01$). Results represent the mean ± S.E.M. of $n = 5$–8 mice per group.

the present work, we also used DCX immunostaining to investigate the effects of CORT 108297 on this cell population. Images obtained by light microscopy (Fig. 1A, a–c) demonstrated that soma of DCX+ cells were mostly located in the inner GCL. However, differences in morphology, in addition to number, were apparent in the three groups. Whereas abundant DCX+ cells with ramified cell processes were present in control NFR/NFR mice (a), Wobblers showed paucity of DCX+ cells of low staining intensity and fewer processes (b). Treatment of Wobbler mice with the GR antagonist enhanced DCX staining, and highly branched cells were observed, mainly in the upper blade of the dentate gyrus (c).

Statistical analysis using ANOVA showed significant differences between control, Wobbler and Wobbler + CORT108297 ($F_{2,15} = 10.96$; $p < 0.001$). The post-hoc test revealed that DCX+ cells were reduced by half in Wobbler mice ($p < 0.01$) compared to control NFR/NFR mice (Fig. 1B). Furthermore, a significant stimulatory effect on DCX+ cell number followed CORT108297 (Wobbler vs. Wobbler + CORT108297: $p < 0.01$) (Fig. 1B).

We also studied if changes of DCX+ neuroblasts observed in Wobblers without and with CORT108297 also applied to mature neurons. To elucidate this issue, we measured the number of NeuN+ neurons in the hilus of the dentate gyrus, since their low density makes cell counting feasible compared to other hippocampus regions. Results obtained in control mice (1456 ± 124.2 per whole hilus, $n = 6$), Wobbler mice (1927 ± 221.1, $n = 6$) and Wobbler mice receiving CORT108297 (1820 ± 141.2, $n = 6$) did not show significant differences according to ANOVA and post-hoc tests ($F_{2,15} = 2.165$; $p = 0.14$).

In the second experiment designed to test CORT108297 effects on DCX+ neuronal progenitors in corticosterone-treated control NFR/NFR mice, results mimicked those obtained for untreated Wobbler mice. Staining profiles of DCX+ cells in the GCL of the dentate gyrus showed that glucocorticoid loading induced a depletion of both the number of DCX+ cell bodies and cell processes (Fig. 2A, a vs. b). After receiving CORT108297 plus corticosterone, the number and staining profile of DCX+ cells was increased (Fig. 2A, c). ANOVA demonstrated significant differences between control, control + corticosterone and control + corticosterone plus CORT108297 ($F_{2,10} = 4.22$; $p < 0.05$). The post-hoc test demonstrated that raising corticosterone levels by exogenous steroid application imposed a significant 40% reduction in the number of DCX+ cells ($p < 0.05$ vs. untreated group) (Fig. 2B). The reduction of neuronal progenitors was prevented when CORT108297 was additioned to corticosterone treatment (CORT108297 + corticosterone vs. corticosterone: $p < 0.01$) (Fig. 2B). These data indicated that antagonism of GR up-regulated the number of DCX+ neuronal progenitors in hypercorticosteronemia from endogenous or exogenous sources.

3.3. *Effects of CORT108297 on hippocampus astrocytes of Wobbler mice and corticosterone-treated mice*

Hippocampus astrogliosis is a common feature of Wobbler mice [6,36]. In line with these reports, we found changes of GFAP+ astrocytes in the dentate gyrus of the mutant mice. As shown in the microscope images of Fig. 3A, GFAP+ cells showed a quiescent phenotype in control mice (a), in contrast to GFAP+ cells bearing a stellate, reactive morphology in the Wobbler dentate gyrus (b). This profile was modified in Wobblers receiving CORT108927, in which a quiescent cellular shape was reinstated (c). ANOVA showed significant group differences between control, Wobbler, and Wobbler + CORT108297 ($F_{2,15} = 8.91$, $p < 0.01$). The post-hoc test demonstrated increased astrocyte density in Wobblers compared to control mice (Fig. 3B, $p < 0.01$) with a return to control levels after blockage of GR with CORT108927 in the Wobbler group (Fig. 3B, $p < 0.01$). Thus, the GR antagonist produced a down-regulation of hippocampus astrogliosis.

The response of GFAP+ astrocytes was also studied in NFR/NFR control mice receiving a corticosterone load alone or in combination with CORT108297. Immunostaining of hippocampus astrocytes revealed that corticosterone treatment induced astrocyte hypertrophy compared to control untreated mice (Fig. 3A, d vs. e), whereas addition of the GR inhibitor restored the quiescent phenotype (f). Quantitative assessment of astrocyte immunoreactive size showed significant group differences ($F_{2,33} = 24.2$; $p < 0.001$). The post-hoc test demonstrated increased GFAP-immunoreactive astrocyte size in corticosterone-treated mice as opposed to control mice (106.6 ± 5.2 vs. 50.5 ± 4.3 μm^2, $p < 0.001$). This value was significantly decreased in the combined corticosterone plus CORT108297-treated group (72.5 ± 5.3, $p < 0.001$ vs. corticosterone only group) However, in contrast to results in the Wobbler

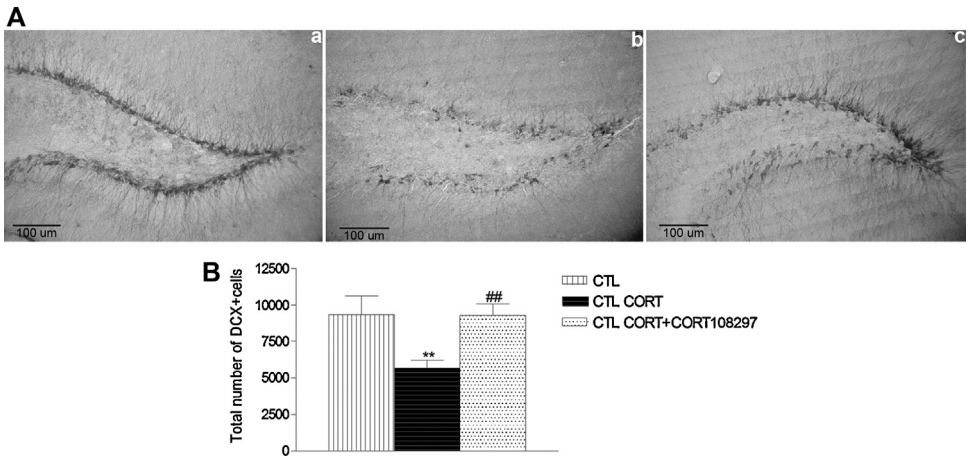

Fig. 2. Effects of a 4 day treatment with CORT 108297 on DCX immunopositive neuroblasts in the dentate gyrus of NFR/NFR control mice receiving vehicle or a corticosterone load. (A) (a) Representative image in a control mouse showed abundant DCX+ cells in the inner zone with staining of processes extending into the granule cell layer. (b) A corticosterone-receiving mouse showed reduced staining of DCX+ cells with sparce processes in the inner granular cell layer. (c) In contrast, a mouse receiving both corticosterone plus CORT108297 showed enhanced DCX expression pattern, suggesting a more mature cell phenotype. Inside bar: 100 μm. (B) Quantitative analysis of DCX+ cell numbers in the dentate gyrus. ANOVA and post-hoc test showed a significant decrease of DCX+ cells in corticosterone-loaded mice (Ctl CORT) compared to untreated mice (Ctl) (** $p < 0.01$), and a significant rise of DCX+ cell number in corticosterone-treated mice receiving CORT108297 (Ctl CORT + CORT108297) compared to the corticosterone only group ($^{\#\#}$ $p < 0.01$). Results represent the mean ± S.E.M. of $n = 5$ mice per group.

experiment, astrocyte hyperplasia was not found following corticosterone treatment, because astrocyte number remained at the control level after addition of CORT108297 (Fig. 3C).

3.4. Effect of the Wobbler mutation and CORT108297 treatment on hippocampus microglia

As stated earlier, microglia activation has been described in Wobbler mice central nervous system [15–17,19]. Taking into consideration these reports, we analyzed if antagonism of the GR changed the reactive morphology of microglia in the dentate gyrus. The images of Fig. 4A show that Iba1+ microglia of control mice mostly presented a ramified appearance (a), whereas the phenotype changed to a round, ameboid form with few stained processes in vehicle-receiving Wobbler mice (b). As shown in (c) CORT108297 treatment reinstated the ramified phenotype in Wobbler mice. Quantitation by ANOVA (Fig. 4B) of the % ameboid-like microglia respect of total microglia in the three experimental groups demonstrated significant group differences ($F_{2,11} = 10.36$, $p < 0.01$). Thus, % reactive microglia was significantly higher in Wobblers compared to control or Wobbler mice receiving the GR antagonist. Thus, CORT108297 returned the phenotype of Iba1+ microglia from a reactive, ameboid-like cell, to a ramified or quiescent form.

4. Discussion

The present work investigated if targeting the GR with CORT108297 influenced hippocampal neurogenesis, astrogliosis, neuronal number and microgliosis of Wobbler mice. Additional studies were performed to unveil if antagonism of GR also modulated neurogenesis and astrocytes of corticosterone-loaded control mice. Both paradigms (i.e. Wobblers and corticosterone-treated mice) shared in common a pronounced hypercorticosteronemia, derived from endogenous sources or from exogenous steroid administration. Our results showed that GR blockage increased neuronal progenitors, decreased astrogliosis and modified microglial phenotype in the dentate gyrus of Wobbler mice. In our second study, the GR modulator given to control mice subjected to high corticosterone treatment, also normalized the defective neurogenesis and decreased astrocyte hypertrophy. Therefore, it is likely that excess GR activation triggers hippocampal abnormalities in both experimental situations. This finding agrees with the hypothesis that elevated glucocorticoid levels adversely modified hippocampus morphology and function, according to current views on central glucocorticoid action [37,38].

CORT108297 is of great advantage due to its high GR selectivity. According to Clark et al. [28] none of the 4-fluorophenylpyrazole compounds – from which CORT108297 derive – displaced 50% binding at ER, AR, MR or PR at 10 μM. The most commonly used GR antagonist Mifepristone (RU-486) also increases neurogenesis in rats subjected to inescapable stress, in mice after chronic corticosterone exposure [27,28] and also prevents stress-induced apoptosis of newborn cells in the dentate gyrus [26]. Baglietto-Vargas et al. [39] have recently disclosed that Mifepristone regulates Alzheimer-like pathology in 3×-Tg-AD mice. However, Mifepristone also blocks progesterone-induced survival of newborn cells in control male mice [40]. A bias with Mifepristone derives from its high affinity for PR (1.3 nM) [28], which makes unclear whether its effects result from mixed inhibition of both steroid receptors. Therefore, a more selective, high affinity GR antagonist seems desirable to discriminate the receptor type involved. Along this line, Zalachoras et al. [41] has recently described a beneficial effect of CORT108297 on cell proliferation in the dentate gyrus of male rats receiving corticosterone for 3 weeks. In the same study, the authors observed lower number of DCX+ cells in corticosterone-treated rats; however, CORT108297 given during the last 4 days of corticosterone treatment was unable to recover DCX+ cell number. The apparent differences in the response of corticosterone-treated animals between our work and Zalachoras et al. [41] can be interpreted on the basis of differences in species (mice vs. rats), length of treatment (4 days vs. 21 days) and doses of CORT108297 (116 mg/kg daily by s.c. injection vs. 50 mg/kg daily by gavage).

The neurogenic potential of CORT108297 also emerged when the doses and method of application described for corticosterone-treated control mice were used in Wobbler mice. In untreated Wobblers, a low number of DCX+ cells in the dentate gyrus correlated with a 5-fold increase in serum levels of corticosterone. In these mice, CORT108297 treatment significantly increased by 50% the population of DCX+ cells, consistent with an effect on cell differentiation. This change was not due to decreased corticosterone

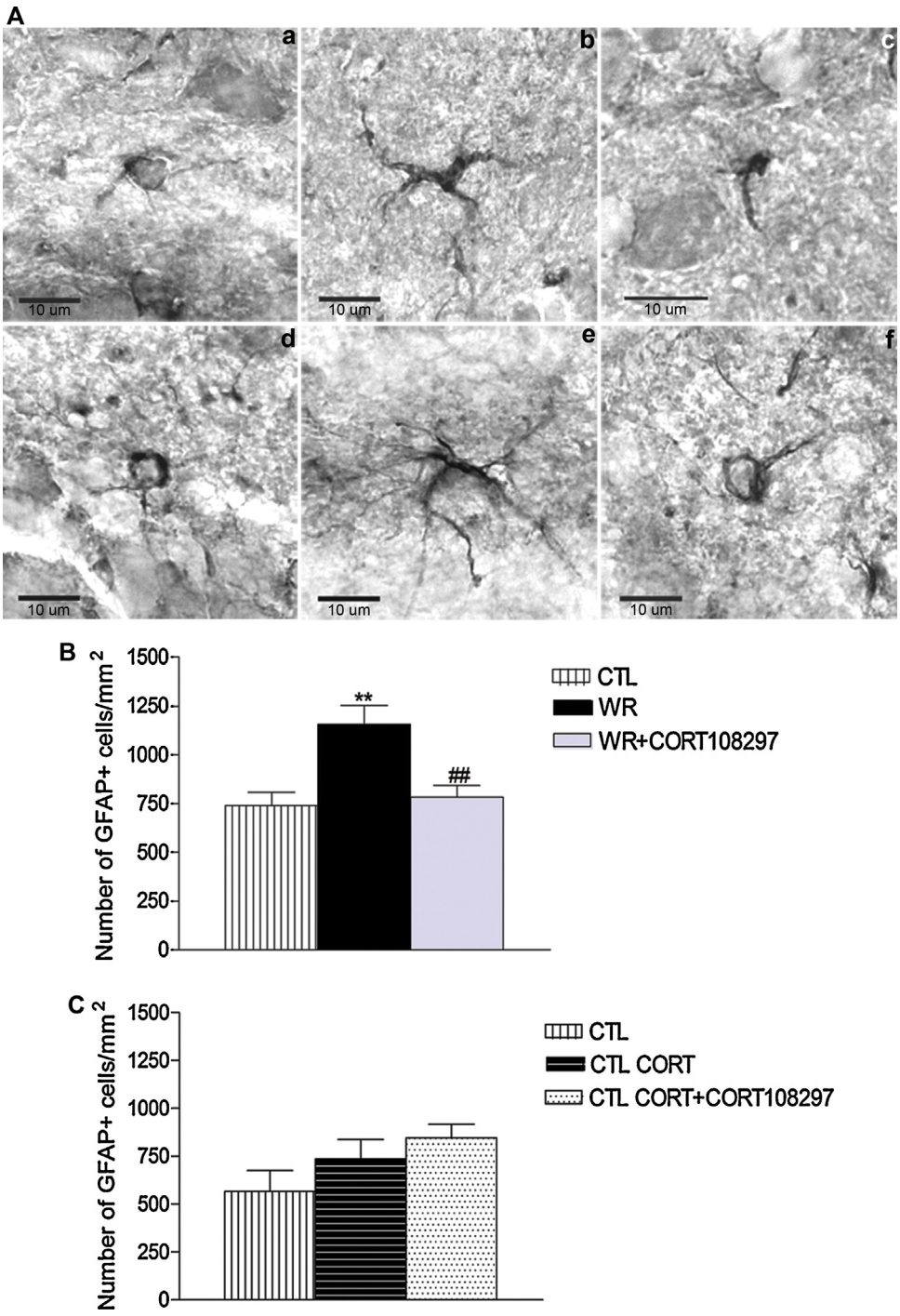

Fig. 3. Effects of CORT108297 on the morphology and number of GFAP+ astrocytes in the hippocampus of Wobbler and corticosterone-treated control mice. (A) (a) representative microscope image of a control NFR/NFR dentate gyrus showing an astrocyte with a quiescent phenotype. (b) astrocyte showing a stellate, reactive profile in an untreated Wobbler mouse (c) down regulation of astrocyte reactive profile in a Wobbler receiving CORT108297. (d) quiescent astrocyte in a control NFR/NFR mouse; (e) astrocyte with a reactive profile in a corticosterone-loaded control mice; (f) astrocyte with a normal staining profile in a mouse receiving corticosterone + CORT108297. Inside bar: 10 μm. (B) Quantitative analysis of astrocyte density in the hilus of the dentate gyrus using ANOVA and post-hoc test revealed significantly increased number of GFAP+ astrocytes per area in Wobblers (Wr) compared to control mice (Ctl) (** $p < 0.01$), and a significant decrease of astrogliosis in Wobblers receiving CORT108297 vs. untreated Wobblers (Wr + CORT108297) ($^{\#\#}$ $p < 0.01$). Results represent the mean ± S.E.M. of 5–8 mice per group. (C) Astrocyte number per area was not modified in control mice (Ctl) receiving corticosterone (Ctl CORT) or corticosterone + CORT108297 (Ctl CORT + CORT108297). However, as shown in (A), (e) corticosterone treatment increased astrocyte hypertrophy whereas blockage of GR decreased astrocyte hypertrophy.

levels, which remained in the range of naïve Wobbler mice after treatment with CORT108297. The high levels of corticosterone circulating in Wobblers are probably caused by a dysfunctional HPA axis, changes of GR and enhanced response to stress [11]. In this respect, findings in Wobbler mice resembled the HPA dysregulation, abnormal stress responses and hypercortisolemia of ALS patients [7–9]. Besides Wobblers and ALS patients, abnormalities of the control of adrenal function with elevated levels of blood cortisol or corticosterone are common to animal models of neurodegeneration, diabetes, aging and Alzheimer patients [10,13,14,42–44]. Interestinlgy, these entities have in common a dysfunctional hippocampus.

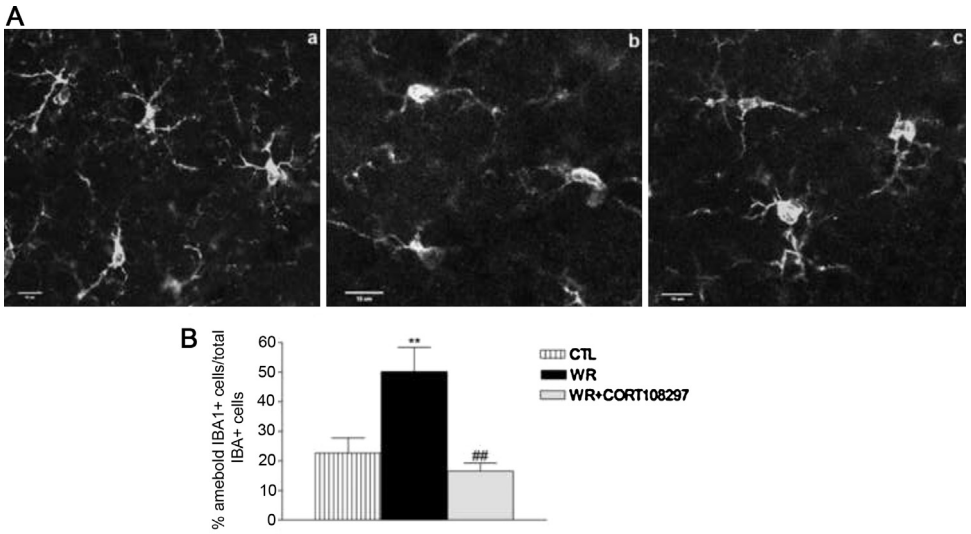

Fig. 4. Light microscope >images of Iba+ microglia in the hippocampus of control, and Wobbler mice with and without CORT108297 treatment. (A) (a) Mostly ramified Iba+ microglia characterized the dentate gyrus of control mice; (b) Iba1+ microglia showed retracted processes in Wobbler mice; (c) After CORT108297 treatment of Wobbler mice, microglia regained the ramified phenotype. B: Quantitative assessment of % "ameboid" Iba1 microglia phenotype in control (Ctl), Wobbler (Wr) and Wobbler + CORT108297-treated mice (Wr + CORT108297) showed increased % ameboid cells in Wobblers vs. control (** $p < 0.01$) and decreased % in Wobblers receiving the GR antagonist ($^{\#\#}$ $p < 0.01$). Results represent the mean ± S.E.M. of 5 sections per animal, and $n = 5$ animals per group.

A second player of the hypercorticosteronemic animals was the astrocyte, which in its reactive form (astrogliosis) is present in Wobbler hippocampus [6,36]. We have previously shown that treatment for 4 days of Wobbler mice with a corticosterone pellet implant producing a pharmacological increment of serum corticosterone, attenuated the enhanced GFAP immunoreactivity in the spinal cord [11]. Therefore, glucocorticoid treatment produced identical responses in the spinal cord and hippocampus astrocytes of Wobblers mice. Astrogliosis may constitute a secondary response to neuronal degeneration, although some authors have considered astrogliosis as a cell- autonomous event of the Wobbler mouse [45,46], the SOD1 transgenic mouse model [47] and ALS patients [48]. Wobbler astrocytes show abnormal differentiation and morphology, differential responses to several mitogens in tissue culture and reduced expression of glutamine synthetase [11,49,50]. We have hypothesized that the high density of astrocytes in the Wobbler mouse may cooperate with or even trigger factors causing neuronal death. In ALS patients, astrogliosis is considered toxic for motoneurons, because reactive astrocytes release proinflammatory mediators and prostaglandins and secrete proapoptotic factors [47,51]. In addition, astrogliosis negatively regulate neuronal progenitor cells [52]. Thus, one way to hold back the progression of the Wobbler disease and enhance neurogenesis would be to block hippocampal astrogliosis. This goal was achieved by treatment of Wobbler mice with the GR antagonist CORT108297.

The response of GFAP-expressing astrocytes to glucocorticoids has been studied by Bridges et al. [53], who found increased hippocampal levels in male and female rats receiving chronic corticosterone treatment. Instead, other reports found decreased expression of GFAP protein in hippocampus after acute or chronic corticosterone treatment of adult rats [54] or dexamethasone treatment of rat pups [55]. The last two reports are supported by the glucocorticoid inhibition of astrocyte proliferation and metabolism in culture [56–58]. The response of hippocampus astrocytes in control NFR/NFR mice was clearly different from findings in the Wobblers. Clearly, astrocytes from corticosterone-treated control mice did not show hyperplasia but a hypertrophic profile, which was prevented by CORT108297. The significant of this finding is uncertain, although it may be possible that astrocyte hyperplasia o hypertrophy depended on the time exposure to corticosterone in the context of neurodegeneration (Wobbler) vs. a short treatment with corticosterone of normal mice. Nevertheless, attenuation of astrocyte hypertrophy or hyperplasia by CORT 108297 may be related in both models to enhanced neurogénesis.

A third player of Wobbler neuropathology was the microglia, which showed a round, reactive phenotype in untreated Wobblers. As already mentioned, microglial reactivity persists in Wobbler mice during the course of the disease [15–17,19] in spite of the hypercorticosteronemia. Prima facie, this finding suggests that high corticosterone levels were not able to suppress the microglial reaction. However, more recent concepts of glucocorticoid effects sustains that these steroids are endowed with both proinflammatory and anti-inflammatory actions on the innate and adaptive immune systems [59]. Thus, glucocorticoid actions on inflammatory processes may be context dependent. For example, prolonged activation of the HPA axis aggravates inflammation, with sustained production of proinflammatory factors [60]. Under some conditions, glucocorticoids may event exert proinflammatory effects [61–65]. If these mechanisms prevail in Wobbler mice, they may explain why blockage of corticosterone action at the GR with CORT108297 returned microglia to a resting, ramified phenotype. We suggest that attenuation of microglia reactivity by CORT108297 could help to restoring neurogenesis. In other situations, microglia activation shows restraining effects on neurogenesis [22,24,25]. Further experiments are planned to investigate the expression of proinflammatory and anti-inflammatory molecules in Wobbler mice receiving CORT108297 treatment and their relationship with neurogenesis.

5. Conclusions

Our results allowed us to conclude that normalization of neuronal progenitors, astrocyte reactivity and microglial reactivity by CORT108297 indicates a role of GR activation in the faulty hippocampus parameters of Wobbler mice and corticosterone-treated control mice. The results bring new therapeutic expectations for the brain abnormalities of ALS patients and hyperadrenocorticisms.

Disclosure statement

Dr. Hazel Hunt is employed by CORCEPT Therapeutics, which developed and provided CORT108297 for the experiments. All other authors report no conflict of interest.

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