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THE PROGESTERONE RECEPTOR AGONIST NESTORONE HOLDS BACK PROINFLAMMATORY MEDIATORS AND NEUROPATHOLOGY IN THE WOBBLER MOUSE MODEL OF MOTONEURON DEGENERATION

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Abstract—Wobbler mutant mice suffer from progressive motoneuron degeneration and glial cell reactivity in the spinal cord. To prevent development of these abnormalities, we employed Nestorone, a high-affinity progesterone receptor agonist endowed with neuroprotective, promyelinating and anti-inflammatory activities in experimental brain ischemia, preventing neuroinflammation and chemical degeneration. Five-month-old Wobbler mice (wr−/wr−) received s.c. injections of 200 μg/day/mouse of Nestorone in vegetable oil or vehicle for 10 days. Control NFR/NFR mice (background strain for Wobbler) received vehicle only. Vehicle-treated Wobblers showed typical spinal cord abnormalities, such as vacuolated motoneurons, decreased immunoreactive choline-acetyltransferase, decreased expression of glutamine synthase (GS), increased glial fibrillary acidic protein-positive (GFAP) astrogliosis and curved digits in forelimbs. These cell-specific abnormalities were normalized in Nestorone-treated Wobblers. In addition, vehicle-treated Wobblers showed Iba1+ microgliosis, high expression of the microglial marker CD11b mRNA and up-regulation of the proinflammatory markers TNFα and iNOS mRNAs. In Nestorone-treated Wobblers, Iba1+ microgliosis subsided, whereas CD11b, TNFα and iNOS mRNAs were down-regulated. NFκB mRNA was increased in Wobbler spinal cord and decreased by Nestorone, whereas expression of its inhibitor IκBα was increased in Nestorone-treated Wobblers compared to control mice and vehicle-treated Wobblers. In conclusion, our results showed that Nestorone restraining effects on proinflammatory mediators, microgliosis and astrogliosis may support neurons in their resistance against degenerative processes.

Key words: microglia, proinflammatory factors, nestorone, neuroprotection, neuroinflammation, Wobbler mouse.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal, progressive degenerative disorder that affects the spinal cord, motor cortex and brain stem. It is characterized by motoneuron degeneration and activation of astrocytes and microglial cells (Mitchell and Borasio, 2007; Valori et al., 2014). Several pharmacological treatments have failed to stop the progress of ALS, with the exception of the anti-glutamatergic drug Riluzole, which modestly prolongs survival (Mitsumoto et al., 2014). Considering the limited treatment options for ALS, preclinical studies may open therapeutic venues for neurodegenerative diseases.

In this regard, several laboratories have supported the protective, promyelinating and anti-inflammatory effects of progesterone and derivatives for experimental neuropathologies. These include spinal cord and brain trauma, ischemic stroke, diabetes mellitus, glutamate and β-amyloid toxicity, neuropathic pain, Alzheimer-like degeneration and neuro-inflammation (Brinton and Wang, 2006; Kaur et al., 2007; Leonelli et al., 2007; Labombarda et al., 2011; Coronel et al., 2011; Liu et al., 2012; De Nicola et al., 2013; Stein, 2013; Garay et al., 2014; Guennoun et al., 2015). In the nervous system, progesterone signaling is multifactorial, ranging from genomic effects after binding to nuclear receptors (PR), interaction of progesterone or its ring A-reduced metabolites with membrane progesterone receptors (mPR), modulation of neurotransmitter and opioid sigma receptors and binding to the progesterone receptor membrane component 1 (PRMC1) (Brinton et al., 2008; Petersen et al., 2013; Schumacher et al., 2014).

Synthetic progestins with high potency and safety for contraception are now available (Kumar et al., 2000; Sitruk-Ware et al., 2003). Nestorone (16-methylene-17alpha-acetoxy-19-norpregn-4-ene-3,20-dione) is a 19-nor

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Abbreviations: ALS, Amyotrophic lateral sclerosis; ChAT, choline acetyltransferase; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; GS, glutamine synthase; Nestorone, (16-methylene-17alpha-acetoxy-19-norpregn-4-ene-3,20-dione); PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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progesterone derivative showing higher affinity for PR than the natural progesterone, but lacking estrogenic, androgenic or glucocorticoid properties (Kumar et al., 2000; Sitruk-Ware et al., 2003). In addition to its use in reproduction, Nestorone has shown strong effects in the nervous system. Thus, Nestorone provides neuroprotection against cerebral ischemia, increases adult rat neural progenitor cell proliferation, enhances myelin repair in a chemical demyelination model, increases the brain regenerative capacity, and constitutes an effective treatment for experimental autoimmune encephalomyelitis (EAE) and for stimulating myelin regeneration after cuprizone-induced demyelination (Liu et al., 2010, 2012; Hussain et al., 2011; Garay et al., 2014; El Etr et al., 2015). Regarding its mechanism of action in the nervous system, it is likely that central effects of Nestorone require the PR. First, Nestorone exerts a higher transactivation effect of the PR than progesterone itself (Liu et al., 2012). Second, Nestorone shows very low conversion into membrane active 3α, 5α-reduced derivatives endowed with GABAA receptor affinity (Liu et al., 2012). Third, Hussain et al. (2011) have shown in a cerebellar demyelination model, that Nestorone-induced remyelination does not take place if mice bear a non-functional PR (PRKO mice).

However, the pharmacological effectiveness of Nestorone for motoneuron degeneration diseases is unknown. The Wobbler mouse suffers a mutation in the gene coding for VPS54 (vacuolar vesicular protein sorting) and has been used as a pre-clinical trial for ALS (Pioro and Mitsumoto, 1995; Staunton et al., 2011; Nieto-Gonzalez et al., 2011; Canzi et al., 2012; Moser et al., 2013; Thielsen et al., 2013). In our hands, treatment of symptomatic Wobbler mice with progesterone decreases motoneuron degeneration and astrogliosis, prolongs life span, and increases muscle strength, the expression of neurotrophic factors, the cholinergic enzyme choline acetyltransferase (ChAT) and the glutamate detoxifying enzyme glutamine synthase (GS) (González Deniselle et al., 1999, 2002b, 2004, 2007, 2012; Meyer et al., 2010, 2012). The present study was based on the hypothesis that synthetic progestins such as Nestorone, currently developed for contraception or in HRT, may also bring beneficial effects in neurodegenerative disorders (Schumacher et al., 2008). More specifically, we addressed whether Nestorone curtails pathology and inflammation of the degenerating spinal cord of Wobbler mice.

In this regard, it has been proposed that cells involved in the inflammatory response such as astrocytes and microglia secrete toxic factors for motoneurons, both in ALS patients and the SOD1 transgenic mice model of ALS (Frakes et al., 2014; Re et al., 2014). Early activation of microglia and astrocytes with increased signs of neuroinflammation has been described in the spinal cord of Wobbler mice (Rathke-Hartlieb et al., 1999; Boillee et al., 2001; De Paola et al., 2012). Taking into account the possible pathogenic role of glial cells in this disorder (Frakes et al., 2014; Re et al., 2014), and the anti-inflammatory effects of Nestorone in models of multiple sclerosis and experimental demyelination (Garay et al., 2014; El Etr et al., 2015), we decided to investigate if the progestin holds back spinal cord inflammation and neuropathology of Wobbler mice. To accomplish this task, we determined in vehicle- and Nestorone-treated Wobbler mice the expression of several proinflammatory mediators and enzymes, as well as the number of Iba1+ microglia and glial fibrillary acidic protein-positive (GFAP+) astrocytes, which are accepted sources of proinflammatory factors. Simultaneously, we investigated Nestorone effects on motoneuron vacuolation and immunostaining for the cholinergic marker choline acetyltransferase (ChAT). The present results support that curtailing neuroinflammation and glial reactivity with Nestorone decreased the spinal cord pathology found in Wobbler's motoneuron degeneration.

EXPERIMENTAL PROCEDURES

Experimental animals

Control (NFR/NFR) and homozygous Wobbler mice (wr/wr) obtained from the Instituto de Biologia y Medicina Experimental colony were housed under conditions of controlled humidity, temperature (22 °C) with lights on from 0700 h to 1900 h. The Wobbler phenotype was identified according to (Rathke-Hartlieb et al., 1999), 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 (U.S.A.). Five-month-old male mice where used in all experiments. There are no gender differences in the onset or progression of the Wobbler disease. Regarding the steroid responses, the content of spinal cord PR in males is similar to that of females (Labombarda et al., 2000), suggesting that in this region males are fully responsive to genomic effects of progestins. For our experiments, Wobbler mice were divided into two groups: one group received daily s.c. injections of 200 μg/day/mouse of Nestorone (The Population Council, New York, USA) dissolved in vegetable oil during 10 days, and the other group received vehicle only. The chosen dose was based on Nestorone's 100-fold higher potency than progesterone, which at the dose of 20 mg offered neuroprotection to Wobbler mice (Meyer et al., 2010, 2012). However, considering the peculiarities of Nestorone metabolism (Liu et al., 2012), it is possible that lower doses may still be effective. For the different experiments, mice were previously anesthetized with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg). Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Assurance Certificate # A5072-01) and were approved by the Institute's Animal Care and Use Committee and the CICUAL of the Faculty of Medicine, University of Buenos Aires.

Real-time PCR of proinflammatory mediators

Following deep anesthesia, the spinal cords were carefully removed. Cervical spinal cords were frozen in dry ice and immediately stored at −80 °C until used for

qPCR studies. Tissues were homogenized with a Polytron homogenizer and total RNA was extracted using Trizol reagent (Life Technologies-Invitrogen, CA, USA). The concentration and purity of total RNA was determined by measuring the optical density at 260 and 280 nm. All samples were precipitated with ethanol and then dissolved in distilled water at a concentration of 1 μg/μL. Total RNA was subjected to Dnase 1 (Life Technologies-Invitrogen) treatment (2 U for 10 min at room temperature (RT) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 μg of total RNA using a SuperScript III Rnase H Reverse transcriptase kit (Life Technologies-Invitrogen) for 60 min at 50 °C in the presence of random hexamer primers. The sequence of primers for amplification of mouse TNFα, iNOS, TLR4, NFκB, IκBα and CD11b is shown in Table 1. PCR protocols have been published before (Garay et al., 2011, 2012). Cyclophilin b was chosen as a housekeeping gene based on the similarity of mRNA expression across all samples templates. Relative gene expression data were analyzed using the ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA, USA), calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001), and it was expressed as fold induction with respect to its respective control. Specificity of PCR amplification and the absence of dimers were confirmed by melting curve analysis. For amplification, 10 ng of cDNA was used and qPCR was performed under optimized conditions: 95 °C at 10 min followed by 40 cycles at 95 °C for 0.15 s and 60 °C for 1 min. Primer concentrations varied between 0.2 and 0.4 μM. Groups were composed of seven animals each (i.e., control, Wobbler and Wobbler + Nestorone).

Immunofluorescence staining for Iba1

Mice were deeply anesthetized and perfused intracardially with phosphate-buffered saline (PBS, pH 7.4) followed by freshly prepared 4% paraformaldehyde in PBS. Cervical cords were dissected out from the spine, stored in the same fixative for 2 h (4 °C), cryoprotected in 20% sucrose with the same phosphate buffer, and stored overnight (4 °C). This step was followed by embedding and freezing in Tissue-Tek (OCT compound, Miles Inc., USA). Transverse sections (30 μm) were cut in a cryostat maintained at −20 °C and placed on gelatin-coated slides (Meyer et al., 2012). Sections were washed three-times in PBS and heated for 10 min at 120 °C to retrieve antigens in 10 mM sodium citrate buffer pH 6.0. Afterward, sections were blocked with 5% bovine serum albumin (BSA) in PBS for 10 min at 37 °C. For staining of microglia we used a 1/1500 dilution of rabbit anti-Iba1 antibody (Cat. #019-19741, Wako, Japan) prepared in 5% BSA, 1% Triton X100 in PBS. After incubation for 3 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 Red 555 prepared in the same solution used for the 1st antibody. After washing in PBS, sections were dried at room temperature and cover slipped with Fluoromont-G. Photographic observation and quantitative analysis of Iba+ immunofluorescent microglial cells was performed in the ventrolateral funiculus of the spinal cord (Meyer et al., 2014). Images taken with the confocal microscope were analyzed using Image J (Image Processing and Analysis in Java, NIH, MD, USA) at 600X. The number of Iba1+ microglia was quantified and expressed per unit area (mm^2). Cells were counted in four sections from seven animals per experimental group.

Immunocytochemistry for ChAT, GFAP and GS

The effect of the Wobbler mutation and of Nestorone treatment on neuronal and glial immunostaining was studied in the ventral horn of the spinal cord, using previously published procedures (Meyer et al., 2010, 2012). For ChAT 16-μm cryostat sections were post-fixed in 4% paraformaldehyde and kept frozen at −80 °C until used. In the case of GFAP, cervical spinal cords were removed from anesthetized mice and post fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2 for 2.5 h, dehydrated in graded ethanols and embedded in paraffin and 5-μm-thick sections were used. In both cases, sections were treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase and then incubated overnight at 4 °C with primary antibodies, i.e., a 1/500 dilution of the ChAT goat polyclonal antibody (AB 144P, Chemicon International Inc., CA) or a 1/250 dilution of the GFAP rabbit polyclonal antibody (G-9269, Sigma). After washing, sections were exposed to a biotinylated rabbit antigoat for ChAT or goat antirabbit secondary antibody for GFAP (1/200 dilution, 60 min). For GS, 5-μm-thick spinal cord sections were treated with mouse IgG block reagent (Vector M.O.M. Immunodetection Kit, Vector Labs), washed and incubated overnight with a 1/200 dilution of a purified monoclonal mouse anti-GS (#610517, BD Biosciences). As second antibody, we used a 1/200 dilution of a biotin-conjugated goat antimouse IgG (Vector M.O.M. Immunodetection Kit), and sections were further processed according to the ABC kit instructions (Vector Labs, CA, USA). In all

Table 1. Mouse forward and reverse prime sequences

Gene	Gene Bank Accession No.	Forward primer (5′–3′)	Reverse primer (5′–3′)
CD11b	***NM_008401***	AAACCACAGTCCCGCAGAGA	CGTGTTCACCAGCTGGCTTA
TNFα	***NM_013693***	GAAAAGCAAGCAGCCAACCA	CGGATCATGCTTTCTGTGCTC
TLR4	***NM_021297***	GGCTCCTGGCTAGGACTCTGA	TCTGATCCATGCATTGGTAGGT
iNOS	***NM_010927***	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCGNF
NFκB	***NM_009045***	GCCTACCCGAAACTCAACTTC	CTCTTTGGAACAGGTGCAGAC
IκBα	***NM_010907***	TTGGTCAGGTGAAGGGAGAC	GTCTCGGAGCTCAGGATCAC

immunoreactions, the peroxidase activity was revealed using diaminobenzidine tetrachloride (DAB, 0.25 mg/ml, SIGMA, St. Louis, MO) as substrate in the presence of 0.01% H_2O_2 for 7 min in the dark. The sections were given a final rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

Determination of motoneuron vacuolation

Motoneuron vacuolation density was measured according to González Deniselle et al. (2004). Following deep anesthesia with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg), mice were perfused transcardially with a solution containing 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4. Following laminectomy, cervical spinal cords were removed and small blocks of tissue were obtained by cutting transverse sections of 2–3 mm maximum length. Blocks were immersed for 2.5 h in 4% PFA, postfixed in graded ethanols and embedded in paraffin. Paraffin sections (5 μm) were stained with cresyl violet for light microscopy observations and quantitation of cells showing cytoplasmic vacuolation.

Quantitative analysis of immunoreactive cells

A computerized image analyzer (Bioscan Optimas VI, Edmonton, WY, USA) equipped with a Panasonic GPKR222 camera connected to an Olympus BH2 microscope was used for quantitative analysis. Cross-sections were examined and digitized photographs were taken under identical lighting conditions at a 400× magnification (Ferrini et al., 1995; González Deniselle et al., 2007). GFAP+, GS+ and ChAT+ cells were counted in the gray matter ventral horn, whereas Iba1+ cells were counted in the ventrolateral funiculus of anatomically matched sections of the spinal cord. Cell counting comprised at least four sections per mice. Number of mice was seven for each group (control, Wobbler and Wobbler + Nestorone). In all cases, cell density was calculated and data were expressed as the mean number of labeled cells ± SEM per unit area (mm^2).

Statistical analysis

Results were expressed as mean ± SEM. Data corresponding to proinflammatory mediators and immunostaining were analyzed by a one-way ANOVA followed by the post-hoc Newman–Keuls test. Data corresponding to forepaw anatomy were analyzed by an unpaired "*t*" test. Statistical analyses were performed with Prism 4 GraphPad software (San Diego, CA, USA). uSignificance was set at $p < 0.05$.

RESULTS

Effects of Nestorone on Iba1+ microglia and CD11b mRNA

The effects of the Wobbler mutation and Nestorone treatment on microglial activation was analyzed by Iba1 immunostaining and CD11b mRNA analysis. Counting of Iba1+ cells in the spinal cord revealed a significant group effect in the ANOVA ($F_{2,18} = 63.95$; $p < 0.001$). A post-hoc test demonstrated a 1.7-fold higher density of Iba1+ cells in Wobblers compared to controls (Fig. 1A; $p < 0.001$). The Wobbler microgliosis was significantly reduced after mice received Nestorone for 10 days (Fig. 1A; $p < 0.001$ vs. untreated Wobblers). Nestorone also modified the phenotype of Iba1+ cells (Fig. 1C). Thus, Iba1+ cells displayed a highly ramified morphology in untreated Wobblers (Fig. 1C WR); whereas cells with less ramified processes prevailed in Nestorone-treated Wobblers (Fig. 1C WR NESTORONE).

The level of CD11b mRNA also provided an index of microglial cell activation. The ANOVA revealed groups differences ($F_{2,16} = 6.54$; $p < 0.01$). Pairwise comparisons showed a moderate but significant increment of this marker in untreated Wobblers compared to control mice (Fig. 1B, $p < 0.05$). However, Nestorone treatment significantly down-regulated CD11b mRNA compared to steroid-naïve Wobbler mice (Fig. 1B; $p < 0.01$). Therefore, results of Iba1 immunocytochemistry and CD11b mRNA analysis supported a decreasing effect of Nestorone on both markers of microglial activation.

Effects of Nestorone on proinflammatory mediators

We next studied if activation of Wobbler microglia, as shown above by the up-regulation of Iba1 immunostaining and CD11b mRNA, was associated with changes in the proinflammatory mediators TNFα, iNOS, NFκB, IκB and TLR4 (Fig. 2A–E). Levels of TNFα mRNA showed significant group differences in the ANOVA ($F_{2,12} = 11.94$; $p < 0.001$). Pairwise comparisons demonstrated a 1.7-fold higher TNFα mRNA expression in Wobblers compared to control mice ($p < 0.001$), whereas a significant reduction of this factor followed Nestorone treatment ($p < 0.01$ vs. untreated Wobblers) (Fig. 2A). Significant group differences were also obtained for iNOS mRNA ($F_{2,18} = 8.35$, $p < 0.01$). The post-hoc test revealed that Wobbler mice expressed 1.5-fold higher iNOS mRNA vs. control mice ($p < 0.01$). However, Nestorone treatment blunted the high iNOS expression of Wobbler mice ($p < 0.01$ vs. untreated Wobblers) (Fig. 2B).

Significant group differences were also obtained in the ANOVA for NFκB mRNA ($F_{2,14} = 4.77$; $p < 0.02$). After the post-hoc test, higher NFκB mRNA expression was found in untreated Wobblers vs. control mice (Fig. 2C; $p < 0.05$). Nestorone treatment of Wobbler mice induced a significantly lower NFκB mRNA expression compared to untreated Wobbler mice (Fig. 2C; $p < 0.05$). The ANOVA also found significant group differences regarding the expression of IκBα inhibitor of NFκB ($F_{2,19} = 4.49$; $p < 0.05$). Although pairwise comparison showed similar IκBα mRNA levels in control and untreated Wobblers (Fig. 2D), this molecule was up-regulated after Nestorone treatment of Wobbler mice ($p < 0.05$ vs. untreated Wobblers). Lastly, we studied TLR4, a molecule positioned along the signaling pathway of NFκB. TLR4 mRNA showed significant group differences in the ANOVA ($F_{2,17} = 6.717$; $p < 0.001$). Post-hoc comparisons showed that TLR4

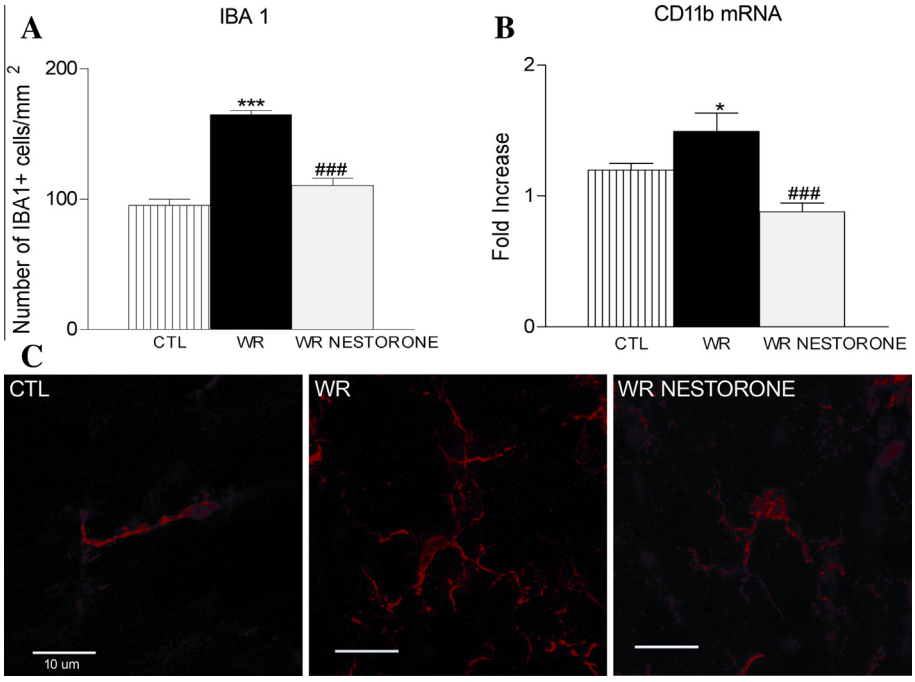

Fig. 1. Microglial markers in the spinal cord ventral horn of control mice, steroid-naïve Wobblers and Nestorone-treated Wobbler mice. (A) The number of Iba1+ cells was higher in Wobbler (Wr) than in control mice (CTL, $^{***}p < 0.001$). Nestorone (NEST) treatment decreased Iba+ 1 cell number compared to untreated Wr mice ($^{###}p < 0.001$). (B) Wr mice showed higher expression of CD11b mRNA compared to CTL ($^{*}p < 0.05$). Treatment with NEST decreased CD11b mRNA expression in Wr ($^{###}p < 0.001$ vs. untreated Wr). Statistical analysis by one-way ANOVA and the post-hoc Newman–Keuls test. (C) Immunofluorescence staining for Iba1 in the three experimental groups. Photomicrographs show Iba1+ cell in a CTL mouse (left hand image, CTL), a highly ramified Iba1+ cell in Wr (middle image, WR) and a less ramified phenotype of an Iba1+ cell in Wr+ NEST mouse (right hand image, Wr NESTORONE). Inside bar = 100 μm.

mRNA was increased in Wobbler spinal cord ventral horn (Fig. 2E, $p < 0.01$ vs. control) but in contrast to other proinflammatory factors studied herein, it did not decline after Nestorone treatment. In summary, Wobbler microgliosis was associated with a clear up-regulation of TNFα, iNOS and TLR4 mRNA, whereas attenuation of microgliosis by Nestorone was accompanied by reductions of TNFα, iNOS and NFκB and by increased IκBα expression.

Effects of Nestorone on Wobbler mouse spinal cord neuropathology

We also analyzed if down-regulation of microglia and proinflammatory mediators associated with improved Wobbler neuropathology. Studied parameters in motoneurons included the aberrant vacuolated phenotype and immunostaining for choline-acetyltransferase (ChAT). In glial cells we analyzed labeling for GFAP and GS (González Deniselle et al., 1999, 2002b, 2004, 2007, 2012; Meyer et al., 2010, 2012). The ANOVA revealed significant group differences in the number of vacuolated motoneurons ($F_{2,18} = 23.99$; $p < 0.001$). As shown in Fig. 3A, vacuolated motoneurons were absent from control NFR/NFR mice, in contrast with the abundant vacuolated phenotype in the spinal cord from untreated Wobblers ($p < 0.001$ vs. controls). Vacuolated cell number was drastically reduced by 70% following Nestorone treatment (Fig. 3A, $p < 0.001$ vs. untreated Wobblers). Fig. 4A shows a group of vacuolated motoneurons in an untreated Wobbler (arrowheads), and more normally appearing motoneurons in a Nestorone-treated Wobbler (Fig. 4B).

The pattern of ChAT immunostaining was inversely related to motoneuron vacuolation. The ANOVA showed significant group differences for ChAT ($F_{2,16} = 4.75$; $p < 0.05$). Pairwise comparisons demonstrated a significant lower number of ChAT+ neurons in untreated Wobblers compared to controls (Fig. 3B; $p < 0.001$), and a significant replenishment in Nestorone-treated Wobblers (Fig. 3B, $p < 0.01$ vs. untreated Wobblers). The improved ChAT immunostaining observed in a Wobbler receiving Nestorone is shown in Fig. 4D, as opposed to the low staining for this maker in an untreated Wobbler (Fig. 4C).

In agreement with early studies, the spinal cord of Wobbler mice was heavily populated with astrocytes strongly stained with the GFAP antibody. The ANOVA showed significant group differences for GFAP+ astrocyte number ($F_{2,17} = 119.0$; $p < 0.001$) The post-hoc test demonstrated that astrogliosis of untreated Wobblers was up-regulated 2000-fold compared to the number of astrocytes in the ventral horn of control mice (Fig. 3C; $p < 0.001$). Most astrocytes of the mutant mice displayed a spider-like shape with strong staining of cell processes (Fig. 5A) (Meyer et al., 2010). Although GFAP+ astrocyte number was markedly reduced by Nestorone treatment (Fig. 3C, $p < 0.001$ vs. untreated Wobblers), it was not totally suppressed, because the density of GFAP+ cells in the progestin-treated group

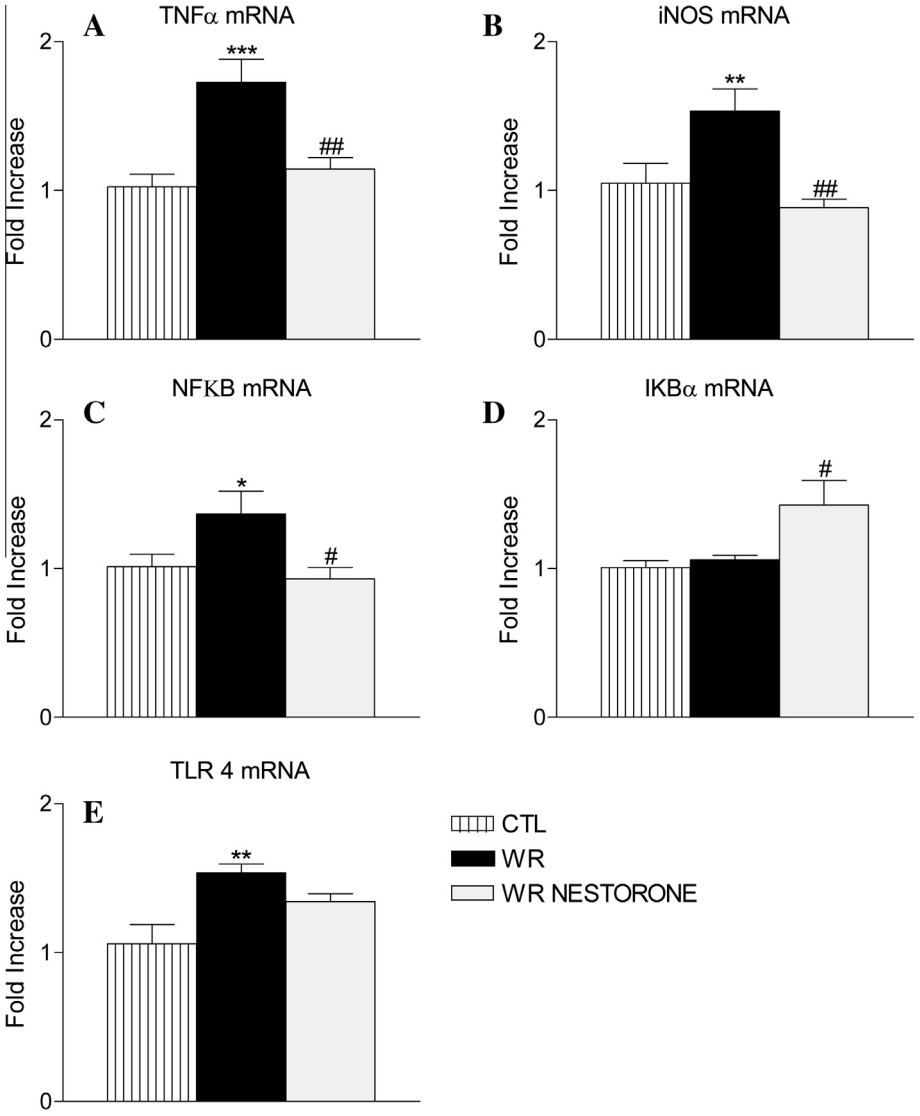

Fig. 2. Effect of Nestorone treatment on proinflammatory mediators in the spinal cord of Wobbler mice. (A and B) Wr mice showed significantly increased levels of TNFα mRNA (A, $^{***}p < 0.001$) and iNOS mRNA (B; $^{**}p < 0.01$) compared to CTL. Both inflammatory markers were decreased following NEST treatment (A and B: $^{\#\#}p < 0.01$ vs. untreated Wr). (C) NFκB mRNA was higher in untreated Wobblers vs. control mice ($^{*}p < 0.05$) but reduced in Wobblers receiving Nestorone ($^{\#}p < 0.05$). (D) Significantly higher expression of IκBα mRNA was found in Wr + NEST vs. CTL or steroid-untreated Wr groups ($^{\#}p < 0.05$). (E) Higher levels of TLR4 mRNA were measured in steroid-untreated Wr vs. CTL mice ($^{**}p < 0.01$). NEST lacked effect on this marker. Statistical analysis by one-way ANOVA and the post-hoc Newman–Keuls test. Abbreviations as in the legend to Fig. 1.

remained above control levels (Fig. 3C, $p < 0.001$). However, astrocytes of the Nestorone-treated Wobblers showed a more protracted phenotype, with smaller soma and fewer stained processes (Fig. 5B). Differences of astrocyte neuroanatomy were demonstrated quantitatively by measurement of the GFAP + immunoreactive area. The latter showed significant differences by ANOVA ($F_{2,54} = 36.41$, $p < 0.001$). Group comparison showed smaller astrocytes in control mice ($38.1 \pm 3.4\ \mu m^2$, $n = 13$) compared to untreated Wobbler mice (98.5 ± 7.0, $n = 22$, $p < 0.001$). Nestorone treatment of Wobbler mice decreased GFAP + astrocyte area compared to untreated Wobblers (49.3 ± 3.2, $n = 22$, $p < 0.001$). There were no significant differences in astrocyte area between control and Nestorone-treated Wobbler mice.

Finally, GS immunostaining showed an opposite response compared to GFAP. The ANOVA indicated significant group differences ($F_{2,18} = 18.16$; $p < 0.001$). Pairwise comparisons further showed that the number of GS immunostained cells/mm^2 was reduced in Wobblers compared to control mice (Fig. 3D; $p < 0.001$; Fig. 5C). Nestorone treatment markedly increased the number of GS + cells vs. untreated Wobblers (Fig. 3D; $p < 0.001$; Fig. 5D). Double immunofluorescence staining for GFAP/GS revealed some colocalization of these markers (results not shown), in confirmation of

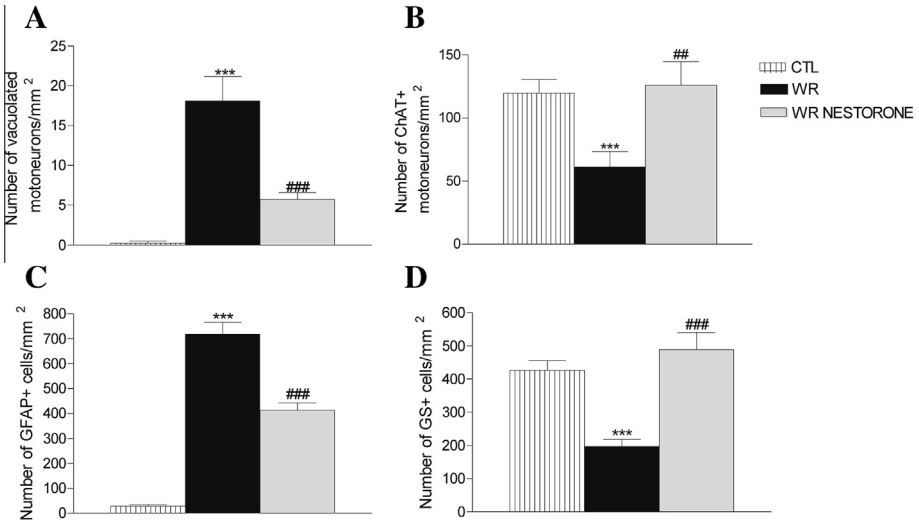

Fig. 3. Reparative effects of Nestorone treatment on Wobbler spinal cord neuropathology. (A) The abundant vacuolated motoneurons of Wr ($^{***}p < 0.001$ vs. CTL mice) were decreased in NEST-treated Wr ($^{###}p < 0.001$ vs. untreated Wr). (B) ChAT immunostaining was reduced in Wr mice ($^{***}p < 0.001$ vs. CTL), whereas the enzyme was replenished after NEST treatment ($^{##}p < 0.01$ vs. untreated Wr). (C) The strong GFAP + astrogliosis of Wr ($^{***}p < 0.001$ vs. CTL) was decreased by NEST treatment ($^{###}p < 0.001$ vs. untreated Wr). (D) Glutamine synthase (GS) immunostaining was lower in Wr than CTL mice ($^{***}p < 0.001$), whereas the enzyme recovered its normal labeling following NEST ($^{###}p < 0.001$ vs. untreated Wr).

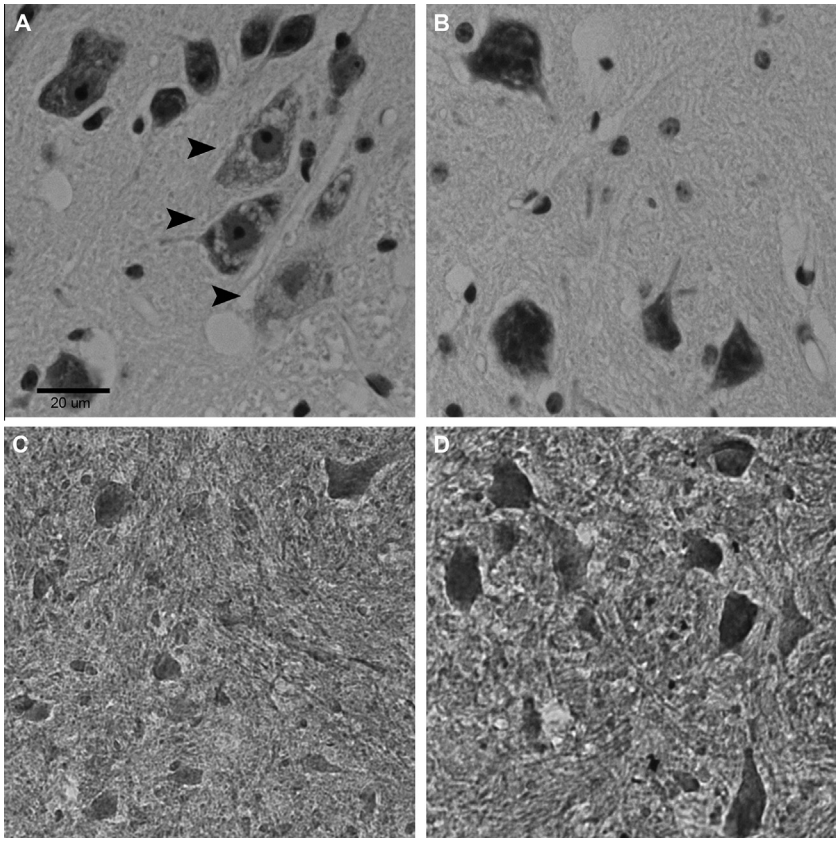

Fig. 4. Nestorone effects on Wobbler mouse motoneuron pathology. Typical photomicrographs of the cervical spinal cord ventral horn from untreated Wr and Nestorone-treated Wr mice. Top images show vacuolated motoneurons from an untreated Wr (A) and normally stained motoneurons after Nestorone treatment (B). Bottom images show fade choline-acetyltransferase (ChAT) labeling in an untreated Wr (C) and stronger staining for this marker in a Nestorone-receiving Wr (D). Bars = 50 μm.

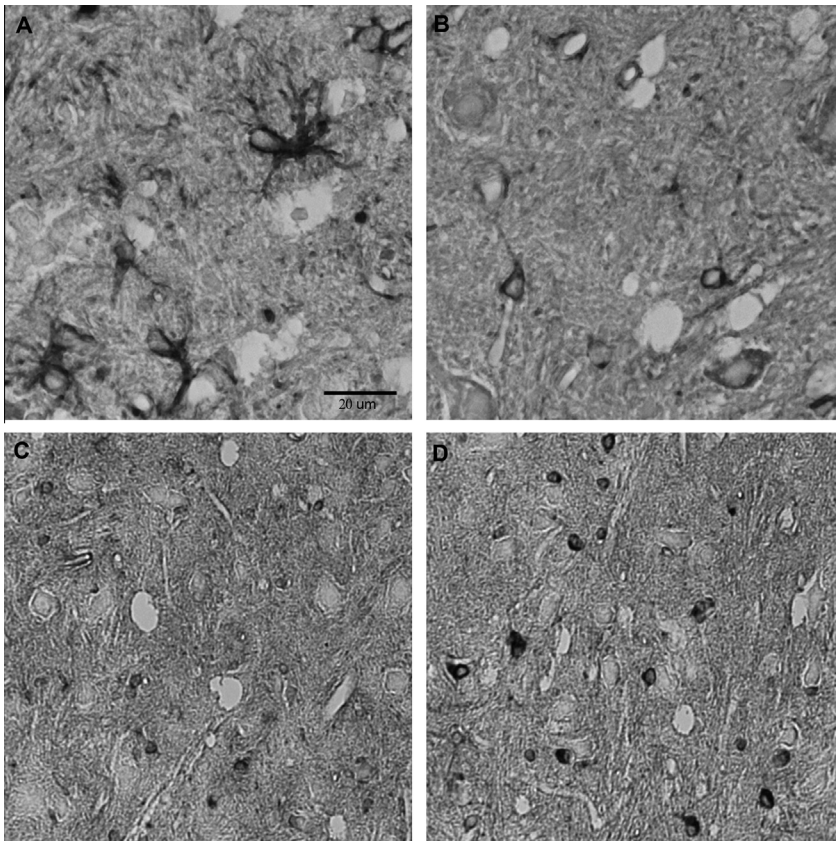

Fig. 5. Nestorone effects on Wobbler mouse glial pathology. Representative photomicrographs of reactive GFAP + astrocytes in untreated Wr (A) and a quiescent-like phenotype in Wr receiving Nestorone (B). Few glutamine synthase (GS) + cells are observed in an untreated Wr (C), whereas more strongly stained GS + cells are visualized after Nestorone treatment (D). Bar = 50 μm.

earlier findings sustaining that a population of Wobbler mouse spinal cord astrocytes are GS negative (Blondet et al., 1995; Meyer et al., 2012).

Clinical findings

Although Wobbler mice were treated by Nestorone for only 10 days, it produced a small improvement in forepaw anatomy. Because only steroid-untreated and Nestorone-treated Wobblers were compared, results were analyzed by an unpaired "*t*" test. Before steroid treatment, Wobbler mice showed in both forepaws atrophy and 90° curled digits (grade 2.1 ± 0.07). The same animals assessed 10 days after Nestorone treatment showed atrophy but 75° curled digits (grade 1.75 ± 0.1; $t = 2.200$; df = 12; $n = 7$ animals per group; $p < 0.05$). Thus, Nestorone seems to slightly ameliorate functional deficits of the disease in this model.

DISCUSSION

The present study revealed that Nestorone treatment exerted a beneficial effect by opposing the morphological and neurochemical abnormalities of Wobbler spinal cord. Thus, Nestorone treatment decreased microgliosis and astrogliosis, down-regulated proinflammatory mediators, decreased motoneuron vacuolation and enhanced the expression of a neuronal cholinergic enzyme. Clinically, Nestorone-induced changes in the cervical spinal cord were associated with a slight improvement of forelimb digits. Nestorone is a very selective and high-affinity ligand of the PR, suggesting that effects of this compound in Wobbler mice were mediated by the PR.

In our study, Nestorone decreased microgliosis, which is in line with the demonstrations that progesterone and some progestins are immunomodulators under physiological and pathological conditions including pregnancy, central nervous system injury, brain ischemia, microglia cultures and experimental multiple sclerosis (Miyaura and Iwata, 2002; He et al., 2004; El Etr et al., 2005; Gibson et al., 2005; Pettus et al., 2005; Dang et al., 2011; Garay et al., 2012; Habib et al., 2013). Activation of microglial cells has already been described in the Wobbler mouse. In a time-course study, Rathke-Hartlieb et al. (1999) have shown that damaged motoneurons of Wobbler mice are accompanied by microgliosis, pointing out that inflammation may play an important role in neurodegeneration. Boillee et al. (2001) have further reported that microglial cells are activated at the very onset of the Wobbler disease, before detection of motoneuron death. Using confocal microscopy of the reactive microglia, these authors detected microglia with long processes ensheathing vacuolated motoneurons. They have interpreted this observation as consistent with the maintenance of microglia in a state of continuous

activation by the degenerating motoneurons. In agreement with these (Rathke-Hartlieb et al., 1999; Boillee et al., 2001) and other published data (De Paola et al., 2012), the present study also demonstrated strong microgliosis in Wobbler mice, as revealed by increased immunostaining for Iba1 and increased expression of CD11b mRNA. Features of microglia activation were substantially modified by Nestorone treatment, which depleted the number of Iba1+ cells and reduced CD11b mRNA. In addendum, the remaining Iba1+ cells in the Nestorone-treated group showed scarce processes instead of the highly ramified form exhibited in untreated Wobblers, suggesting that immunomodulation by Nestorone modified the activated morphology and marker expression of microglia.

Astrocytes, which have been recognized as an important source of pro-inflammatory mediators in ALS (Phani et al., 2012), were also examined in the Wobblers for their response to Nestorone. For this purpose, we used GFAP immunocytochemistry for labeling and quantification of the quiescent and reactive astrocytes. Astrogliosis of Wobbler mice was markedly responsive to Nestorone treatment, in agreement with the astrocyte response previously observed for progesterone (González Deniselle et al., 2002b; Meyer et al., 2012). In our experiments, Nestorone decreased both the number as well as the GFAP immunoreactive area of astrocytes of Wobbler mice. Astrogliosis is a hallmark of Wobbler neurodegeneration, and the reactive astrocytes from these mice change their neuroprotective profile to become neurotoxic, proinflammatory cells. It has been reported that pathological Wobbler astrocytes produce high levels of nitric oxide (NO) and proinflammatory factors, show high expression of the proliferation marker S100 β, respond abnormally to transforming growth factors, and express low levels of GS and glutamate transporters (González Deniselle et al., 1999; Hantaz-Ambroise et al., 2001; Corvino et al., 2003; Diana et al., 2010). Astrogliosis is a premature finding in the spinal cord of newborn Wobbler mice, suggesting an initiator role (Laage et al., 1988; Sica, 2012) and unpublished results of Meyer et al.). In ALS patients, astrogliosis is considered toxic for motoneurons, because reactive astrocytes release inflammatory mediators, express high levels of iNOS and COX2 and secrete pro-apoptotic factors (Barbeito et al., 2004; Julien, 2007). Re et al. (2014) have recently proposed that motoneuron loss in ALS is programed by the reactive astrocytes. Both in SOD1-transgenic mice and ALS patients, metabolic abnormalities of astrocytes expressing a mutation of SOD1 may contribute to motoneuron injury (Cassina et al., 2008). Reactive astrocytes may also play a toxic role in Alzheimer's disease, post-stroke disorder, HIV-associated dementia, models of multiple sclerosis and Parkinson's disease (Garay et al., 2007; Doyle et al., 2010; Avila-Munoz and Arias, 2014; Sofroniew, 2014). The current data indicate that holding back astrogliosis with Nestorone could partially prevent the spinal cord from neurodegeneration.

GS is an important enzyme synthesizing glutamine from glutamate and ammonia. GS is normally localized in astrocytes; however, Blondet et al. (1995) have reported that gliosis in the Wobbler mouse involves a subpopulation of astrocytes strongly positive for GFAP but GS-negative, leading to an impaired regulation of the glutamate cycle. Furthermore, significant decreases in glutamate uptake and reduced glutamate transporters GLT1 and GLAST have been reported in Wobbler mouse astrocytes (Diana et al., 2010). Our present data confirmed previous results of Blondet et al. (1995) and Meyer et al. (2010), showing a decrease in GS+ cells in Wobbler mouse spinal cord. We have shown here that Nestorone restored GS+ cells in the Wobbler mouse, in agreement with previous data with progesterone (Meyer et al., 2010). Up-regulation of GS by Nestorone may be another mechanism of neuroprotection, because glutamate released at the synapses will be metabolized to glutamine, avoiding glutamate excitotoxicity. New studies are needed to substantiate if Nestorone regulates glutamate astrocyte transporters GLT1 and GLAST in addition to its effect on GS. How Nestorone regulates the population of glial cells is not yet clear. PR is expressed by astrocytes but not microglia (Sierra et al., 2008). Whether Nestorone uses different signaling mechanisms or receptors in glial cells, or whether its actions involve cross-talk between different neural cell populations, the final outcome was a reduction in astrogliosis and microgliosis.

In order to analyze the functional outcome of changes in glial cells, we analyzed the expression of the proinflammatory factors TNFα, iNOS, NFκB, IκB and TLR4 mRNAs. De Paola et al. (2012) have previously reported increased expression of TNFα and its colocalization with CD11b in Wobbler mice spinal cord. In confirmation of this finding, we found high levels of TNFα mRNA in untreated Wobbler spinal cord, suggesting its role in neuroinflammation. TNFα becomes a damaging molecule after interaction with its receptor TNFR1, leading to cytolysis and apoptosis (Kim et al., 2011). Notably, Nestorone reduced TNFα expression in Wobbler spinal cord, supporting its immunomodulatory effect. Secondly, increased mRNA of iNOS was found in vehicle-treated Wobblers, which probably led to neurotoxic levels of NO. High activity of NOS has been already shown in Wobbler spinal cord (Clowry and McHanwell, 1996), and its neurotoxic role was postulated because treatment with the enzyme inhibitor 7-nitroindazole delays motoneuron degeneration (Ikeda et al., 1998). We have previously reported that early treatment with progesterone caused a significant reduction in NADPH diaphorase-active NOS in motoneurons and astrocytes of Wobbler mice (González Deniselle et al., 2004). NO is considered a free radical causing oxidative stress and damage to the mitochondrial respiratory chain in the Wobbler spinal cord (González Deniselle et al., 2012). Therefore, reduction of iNOS mRNA may contribute to Nestorone's neuroprotective and anti-inflammatory effects.

Wobbler mouse spinal cord also showed higher expression of NfκB mRNA compared to normal control mice, but the increase, however, was modest compared to other pro-inflammatory factors such as TNFα. This is not unexpected, since NFκB is considered a rapid action transcription factor, the activation of which may not require new protein synthesis. Instead, it has been

shown that the main mechanism of NfκB activation is nuclear translocation rather than increase in its expression (Gilmore, 2006). Several target genes are transcribed by NFκB action in the nucleus, including TNFα, IL1-β and toll-like receptors (Oeckinghaus and Ghosh, 2009). Therefore, it is likely that NFκB activation preceded the increased inflammation measured in the Wobbler spinal cord. Interestingly, the PR agonist Nestorone was able to antagonize NFκB transcription in the Wobbler mice. In this regard, Nestorone antagonism may have taken place by two different mechanisms. First, by repression of NFκB transcription; second, by increased expression of the inhibitor IκBα. The protein transcribed from IκBα is known to combine with NFκB in the cytoplasm, avoiding its nuclear translocation. In summary, the NFκB pathway seems another target contributing to the anti-inflammatory and neuroprotective effects of Nestorone.

TLR4 activation is another proinflammatory molecule that triggers inflammation and neurotoxicity. Increased expression of TLR4 has been found in reactive glia of the spinal cord of ALS patients (Casula et al., 2011), and treatment of Wobbler mice with an antagonist of TLR4 have shown a protective effect (De Paola et al., 2012). We also found increased expression of TLR4 in Wobbler spinal cord; however, Nestorone inactivity toward this molecule suggests that TLR4 expression is spared from the progestin effects.

The above-mentioned evidence suggested that increased expression of proinflammatory markers and the oxidative environment may aggravate motoneuron vacuolation, the hallmark of Wobbler disorder. Vacuolated motoneurons show morphological features resembling the type II cytoplasmic form of cell death called paraptosis (Clarke, 1990). In particular, mitochondria from degenerating motoneurons show membrane disruption, crystolysis and vacuolation (González Deniselle et al., 2002a). Neurochemical studies of these mitochondria revealed decreased respiratory chain activity, decreased oxidative phosphorylation, low activity of MnSOD and increased intra-mitochondrial NOS (Santoro et al., 2004; Dave et al., 2005; González Deniselle et al., 2012). Mechanistically, motoneuron and mitochondrial vacuolation suggest the participation of free radicals including NO, peroxynitrite and lipid peroxides. In the Wobbler spinal cord, NO is synthesized by intra-mitochondrial NOS (mNOS) and iNOS expressed by glial cells (Clowry and McHanwell, 1996; González Deniselle et al., 2012). Whereas protective effects of Nestorone on motoneurons could be mediated by a neuronal PR, antagonism of inflammatory mediators and oxidative stress may also shield motoneurons from degeneration.

Motoneuron vacuolation is only a histological observation, which does not reveal the functional consequences. To disclose this possibility, we analyzed ChAT, the immuno-histochemical marker for functional motoneurons. ChAT immunostaining confirmed the severe loss of staining in vehicle-treated Wobbler mice, suggesting that motoneurons were functionally impaired (González Deniselle et al., 2007). The reduction of ChAT immunostaining has been shown before in Wobbler mice (Yung et al., 1994; Bigini et al., 2007; González Deniselle et al., 2007) and ALS patients (Kato, 1989; Oda et al., 1995). Depletion of ChAT-IR was not irreversible, because Nestorone significantly increased ChAT+ neuronal staining. The Nestorone-enhanced ChAT immunostaining correlated with decreased curve digits of the treated Wobblers, suggesting improved forelimb innervation. We have shown before that effect of progesterone on ChAT correlates with increments of muscle weight (González Deniselle et al., 2007). These data indicated that Nestorone reduction of vacuolation resulted in improved cholinergic function of motoneurons.

Our data support a considerable role for neuroinflammation during progression of motoneuron degeneration. The amplified response of the local immune system is not confined to Wobbler mice, because in ALS patients and the SOD1 transgenic mice model of ALS, astrocytes and microglia also release proinflammatory factors which harm the surrounding neurons (Phani et al., 2012). In our hands, Nestorone behaved as an anti-inflammatory factor for arresting spinal cord neurodegeneration, without showing important side effects at the dose and time schedule employed in our investigation. Thus, drowsiness was not observed in Nestorone-treated mice, which clinically remained more active and also partially recovered forepaw anatomy. The absence of drowsiness may be due to the much lower conversion of Nestorone into 3α,5α-reduced metabolites with GABAA receptor affinity, in comparison with progesterone reduced metabolites (Liu et al., 2012).

Further work is needed to elucidate if Nestorone effects are long lived and free of side effects in Wobblers, in similarity with the prolonged effects of this progestin in EAE (Garay et al., 2014).

CONCLUSIONS

The present results showed that Nestorone, a high-affinity ligand of the progesterone receptor, exerts potent anti-inflammatory effects in the Wobbler mice model of motoneuron degeneration. The spinal cord of Wobblers expressed higher levels of proinflammatory mediators including TNFα, NFκB, TLR4 and the enzyme iNOS, concomitant with GFAP+ astrogliosis and IBA1+, CD11b microgliosis, in confirmation of the important role played by neuroinflammation in neurodegeneration. Nestorone down-regulated all proinflammatory factors – with the exception of TLR4-, increased the mRNA for the IκB inhibitor and attenuated the astrocyte and microglial reaction. These effects were accompanied by decreased spinal cord pathology involving motoneurons, as revealed by the decreased vacuolated phenotype and increased ChAT immunostaining, a marker of functional viability. Therefore, Nestorone restraining effects on inflammation may open new therapeutic strategies for the treatment of neurodegenerative diseases.

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