Neuroscience 226 (2012) 40–50

PROGESTERONE DOWN-REGULATES SPINAL CORD INFLAMMATORY MEDIATORS AND INCREASES MYELINATION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract—In mice with experimental autoimmune encephalomyelitis (EAE) pretreatment with progesterone improves clinical signs and decreases the loss of myelin basic protein (MBP) and proteolipid protein (PLP) measured by immunohistochemistry and *in situ* hybridization. Presently, we analyzed if progesterone effects in the spinal cord of EAE mice involved the decreased transcription of local inflammatory mediators and the increased transcription of myelin proteins and myelin transcription factors. C57Bl/6 female mice were divided into controls, EAE and EAE receiving progesterone (100 mg implant) 7 days before EAE induction. Tissues were collected on day 17 post-immunization. Real time PCR technology demonstrated that progesterone blocked the EAE-induced increase of the proinflammatory mediators tumor necrosis factor alpha (TNFα) and its receptor TNFR1, the microglial marker CD11b and toll-like receptor 4 (TLR4) mRNAs, and increased mRNA expression of PLP and MBP, the myelin transcription factors NKx2.2 and Olig1 and enhanced CC1 + oligodendrocyte density respect of untreated EAE mice. Immunocytochemistry demonstrated decreased Iba1 + microglial cells. Confocal microscopy demonstrated that TNFα colocalized with glial-fibrillary acidic protein + astrocytes and OX-42 + microglial cells. Therefore, progesterone treatment improved the clinical signs of EAE, decreased inflammatory glial reactivity and increased myelination. Data suggest that progesterone neuroprotection involves the modulation of transcriptional events in the spinal cord of EAE mice.

Key words: progesterone, experimental autoimmune encephalomyelitis, transcription factors, myelin proteins, proinflammatory mediators, neuroprotection.

INTRODUCTION

Multiple sclerosis (MS) is an invalidating disease that produces demyelination of the spinal cord and some regions of the brain. MS is considered of autoimmune origin, driven by myelin-specific T-cells, activated microglia/macrophages and their product tumor necrosis factor-α (TNFα), interferon-γ (IFN-γ) and metalloproteinases (McQualter and Bernard, 2007; Trapp and Nave, 2008). More recently a role for IL-17 has also been considered (Jiang et al., 2003). There is also strong evidence for neurodegeneration (Trapp and Nave, 2008). Other authors have pointed out that intrinsic defects of the oligodendrocytes, with the release of myelin proteins and debris may provoke the reaction of the peripheral and local immune systems (Matute and Perez-Cerda, 2005).

In approximately 80% of the cases MS shows a relapsing-remitting course. Importantly, a decline in the rate of relapses occurs during the third trimester of pregnancy, whereas relapses return during the 3 months post partum (Confavreux et al., 1998). The absence of MS relapses during pregnancy and their come-back postpartum are believed to be due to the increased and decreased levels of circulating sex steroids, respectively (Hughes, 2004; Druckmann and Druckmann, 2005; El-Etr et al., 2005). Therefore, the potential therapeutic benefit of sex steroid hormones for MS patients merits consideration. In this regard, it has been hypothesized that demyelination in MS may be due to a deficient content of brain neurosteroids (Leitner, 2010). In support of this hypothesis, decreased levels of progesterone's reduced metabolite allopregnanolone are found in the brain of MS patients (Noorbakhsh et al., 2011).

A common model used to study MS is experimental autoimmune encephalomyelitis (EAE) in rodents (Sun et al., 2003; Ayers et al., 2004). This model has been used to test the efficacy of a number of treatments, among them estrogenic steroids (Offner, 2004; Palaszynski et al., 2004; Elloso et al., 2005; Morales

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Abbreviations: CTRL, control; EAE, experimental autoimmune encephalomyelitis; GM, gray matter; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, phosphate-buffered saline; PLP, proteolipid protein; PNS, peripheral nervous system; RT, room temperature; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor-α; VM-VF, white matter ventral funiculus; WM-VLF, white matter ventrolateral funiculus.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.09.032

et al., 2006). Additional reports have supported that progesterone is also beneficial for EAE mice (Yates et al., 2010) in agreement with the known neuroprotective and promyelinating effects of progesterone for the healthy and pathological central and peripheral nervous systems (Leonelli et al., 2007; Schumacher et al., 2007; Brinton et al., 2008; Stein, 2008; De Nicola et al., 2009; Mensah-Nyagan et al., 2009). Our laboratory has previously reported that progesterone pretreatment of EAE mice attenuates disease progression, decreases inflammatory cell infiltration of the spinal cord, increases immunostaining for myelin basic protein (MBP) and proteolipid protein (PLP) and markers of motoneuron function and decreases axonal pathology (Garay et al., 2007, 2008, 2009). Other groups have strengthened the protective effects of progesterone in the acute phase of EAE in mice (Yates et al., 2010; Yu et al., 2010) and in a chronic model employing dark Agouti rats, an experimental model showing a protracted-relapsing EAE (Giatti et al., 2012). Further evidence using the lysolecithin model of primary demyelination suggests that in addition to stimulating myelin proteins and oligodendrocytes, progesterone also arrests the local inflammatory response (Garay et al., 2011).

The present investigation studied if progesterone pretreatment of EAE mice directly modulates transcriptional events in the spinal cord. To this end, we determined progesterone effectiveness to block the expression of several proinflammatory molecules, including tumor necrosis factor alpha (TNFα) and its receptor type 1, CD11b a marker of activated microglia, the toll-like receptor 4 (TLR4), the inducible form of nitric oxide synthase (iNOS) and interleukins (Roy et al., 2006; Brown and Neher, 2010; Mc Guire et al., 2011; Okun et al., 2011). These proinflammatory factors contribute to pathology of the oligodendrocytes and produce demyelination (Mc Guire et al., 2011; Okun et al., 2011). We assumed that progesterone antagonism of local inflammation counteracts damage to myelin-producing cells and their progenitors, allowing the transcription of the central myelin proteins and transcription factors involved in oligodendrocyte precursor proliferation, differentiation and remyelination (Qi et al., 2001; Fancy et al., 2004; Ligon et al., 2006). The observed reduction of proinflammatory mediators and increased transcription of myelin proteins and transcription factors, supports that progesterone may be a useful therapeutic strategy for spinal cord demyelinating diseases.

EXPERIMENTAL PROCEDURES

Experimental animals and treatments

Ten-week-old female C57BL/6 mice were used for all the experiments. Mice obtained from the Veterinary Faculty, University of La Plata, Argentina were housed in our animal facility under controlled temperature of 22 °C and fed mice chow *ad libitum*. Mice were randomly assigned to one of 3 experimental groups: control, EAE and EAE + progesterone. After anesthesia with isofluorane, the last group received sc a pellet of 100 mg of progesterone (Sigma–Aldrich Inc, USA) one week before EAE induction. All EAE mice received a sc injection on each flank of 200 μg MOG_{40-54} (Peptides International, Louisville, USA) (Sun et al., 2003; Garay et al., 2007, 2009), per mouse emulsified in complete Freund's adjuvant CFA (Sigma–Aldrich) containing 0.6 mg *Mycobacterium tuberculosis* (Instituto Malbran, Argentina).

Mice received injections of *Pertussis* toxin ip (400 ng) (Sigma–Aldrich) immediately after immunization and another boost on the day after. Controls receiving CFA and *Pertussis* toxin without MOG did not develop signs of EAE. Mice were monitored daily for weight loss and neurological signs of EAE. Disease severity was scored as previously published for EAE mice (Garay et al., 2007):grade 0, no signs; grade 1, partial loss of tail tonicity; grade 2, loss of tail tonicity, difficulty in righting; grade 3, unsteady gait and mild paralysis; grade 4, hind-limb paralysis and incontinence and grade 5: moribund or death. EAE symptoms started from day 12 and mice were sacrificed on day 17 when the disease was still in an acute phase. This period of time was chosen because previous studies demonstrated the ability of progesterone to enhance faulty neurochemical parameters and to improve clinical signs of EAE mice (Garay et al., 2007). Accumulative score was calculated as the sum of all scores from disease onset to day 17. Animal procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH Guide, Institute's Assurance Certificate: A5072-01) and were approved by the institute's Animal Care and Use Committee.

Determination of mRNA of myelin proteins MBP and PLP, oligodendrocyte transcription factors Olig1, Olig2 and NKx2.2, and proinflammatory markers by real time PCR

After decapitation under isofluorane anesthesia, mice spinal cords were carefully removed. Cervical and lumbar spinal cords were frozen in dry ice and immediately stored at −80 °C until used for real time PCR studies. Tissues were homogenized with a Polytron homogenizer and total RNA was then 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. Sequences of primers for amplification are indicated in Table 1. Cyclophilin b was chosen as a housekeeping gene based on the similarity of mRNA expression across all sample 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 determined 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 each amplification, 10 ng of cDNA was used and PCR 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 0.2–0.4 μM. We used four animals in the control group and 10 mice in the EAE and EAE + progesterone groups.

Table 1. Sequence of primers employed for real time PCR

Gene	Gene Bank Accession Number	Forward Primer (5′-3′)	Reverse Primer (5′–3′)
Olig 1	NM_016968	ACCAACGTTTGAGCTTGCTT	GGTTAAGGACCAGCCTGTGA
Olig 2	NM_016967	AGCAATGGGAGCATTTGAAG	CAGGAAGTTCCAGGGATGAA
Nkx2.2	NM_010919	CGGGCTGAGAAAGGTATGGA	TGTGCTGTCGGGTACTGG G
MBP	NM_001025100	ATCCAAGTACCTGGCCACAG	CCTGTCACCGCTAAAGAAGC
PLP	NM_199478	CTGGCTGAGGGCTTCTACAC	GACTGACAGGTGGTCCAGGT
TNFα	NM_013693	GAAAAGCAAGCAGCCAACCA	CGGATCATGCTTTCTGTGCTC
TNFR1	NM_011609	GCTGACCCTCTGCTCTACGAA	GCCATCCACCACAGCATACA
TLR4	NM_021297	GGCTCCTGGCTAGGACTCTGA	TCTGATCCATGCATTGGTAGGT
MyD88	NM_010851	AGGCGATGAAGAAGGACTTTCC	TCAGTCTCATCTTCCCCTCTGC
IkappaB	NM_020529	TTGGTCAGGTGAAGGGAGAC	GTCTCGGAGCTCAGGATCAC
iNOS	NM_010927	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
IL-6	NM_031168	GAAACCGCTATGAAGTTCCTCTCTG	TGTTGGGAGTGGTATCCTCTGTGA
IP-10	NM_021274	CAGTGAGAATGAGGGCCATAGG	CGGATTCAGACATCTCTGCTCAT
INF_{γ}	NM_008337	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
Cyclophilin b	NM_001025612	GTGGCAAGATCGAAGTGGAGAAAC	TAAAAATCAGGCCTGTGGAATGTG

Determination of Iba1+ microglial cells and CC1+ oligodendrocytes by immunohistochemistry

Thoracic spinal cords obtained after decapitation of anesthetized mice were postfixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2 for 2.5 h and processed for routine paraffin embedding. Five micrometer sections were deparaffinised, heated for 10 min at 120 °C to retrieve antigens in 10-mM sodium citrate buffer pH 6.0 containing 0.05% Tween 20. Afterward, sections were treated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Sections were rinsed in PBS and incubated with 5% BSA for 10 min at 37 °C. Specific primary antibodies were prepared in 5% BSA, 0.1% Triton X-100 in PBS. Immunohistochemistry was carried out using a 1:1500 dilution of rabbit anti Iba1 antibody (Wako, Japan) and 1:100 dilution of a mouse adenomatus polyposis coli (CC1) antibody (anti-APC AB-7, Cat. OP80, Calbiochem, USA). After overnight incubation at 4 °C for CC1 and 4 days incubation for Iba1, sections were washed with PBS and incubated with 1/200 dilution of goat anti-rabbit IgG or horse anti-mouse IgG secondary antibodies (Vector Labs, CA, USA) prepared in 5% BSA, 0.1% Triton X-100 in PBS for 1 h at 22 °C. After application of an avidin–biotin–peroxidase (ABC) complex for 30 min (ABC kit Vector Labs), the reaction was finally revealed with 0.5 mg/ml diaminobenzidine tetrachloride (Sigma–Aldrich) in the presence of 0.01% H_2O_2 for 6 min in the dark. The sections were given a final rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

Colocalization studies

Anti-GFAP and anti-OX42 antibodies were used to detect the colocalization of TNFα with astrocytes and microglia respectively. Thirty-micrometer frozen sections were blocked with a 3% donkey serum-PBS solution and incubated with a 1/200 dilution of a goat anti-TNFα (Santa Cruz Biotechnology, USA) prepared in 2% donkey serum 0.1% Triton X-100 in PBS. After an overnight incubation at 4 °C, sections were washed and incubated with a 1/200 dilution of a cy3-labeled anti-goat antibody prepared in the same incubation buffer for 1 h at room temperature. To study if TNFα colocalized with astrocytes, spinal cord sections were incubated with 1/250 dilution of the rabbit anti-GFAP (Sigma–Aldrich) overnight. Sections were washed again and incubated for one hour with anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes) secondary antibody for 1 h at RT. To study if TNFα colocalized with microglia, a Mouse-on-Mouse kit was used (Vector Labs.). After incubating with a blocking solution, a 1/100 dilution of a mouse anti OX-42 primary antibody (Millipore, CA, USA) was applied overnight at 4 °C. Sections were rinsed in PBS and incubated with an antimouse IgG antibody conjugated to Alexa 488 for 1 h. All sections were rinsed in PBS and coverslipped with Fluoromont-G (Southern Biotech, Birmingham, AL, USA). Photographs were taken under a Nikon Eclipse E 800 confocal laser microscope equipped with Nikon 11691 photographic equipment.

Quantification of CC1 and Iba 1 immunopositive cells

A computerized image analyzer (Bioscan Optimas VI, Edmonton, WY, USA) equipped with a Panasonic GPKR222 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; Gonzalez Deniselle et al., 2007). To asses the extension of the microgliosis, Iba1 + cells were counted in white matter ventral funiculus, ventrolateral funiculus and gray matter ventral horn in anatomically matched sections of the spinal cord. Cell counting comprised at least 4 sections per mice. Number of mice was 4 for the control group and 10 mice for the EAE and EAE + progesterone groups. CC1 + cells were assessed in the white matter region located between the dorsal horns. This white matter region was analyzed because of its abundant infiltration of inflammatory cells and demyelination foci in EAE (Garay et al., 2007). In both cases, cell density was calculated and data were expressed as the mean number of labeled cells ± S.E.M. per unit area (μm^2).

Statistical analysis

Differences in accumulative scores and progression curves of the EAE and EAE + progesterone groups were analyzed by the Mann–Whitney U non parametric test. Significance was set at $p < 0.05$. Group differences for real time PCR studies and immunostaining of Iba1 and CC1 + cells were determined by one-way ANOVA, followed by post hoc comparisons with the Newman–Keuls test.

RESULTS

Fig. 1 shows the time course of EAE development in C57Bl6 female mice with or without progesterone treatment. Steroid-untreated mice showed a progressive

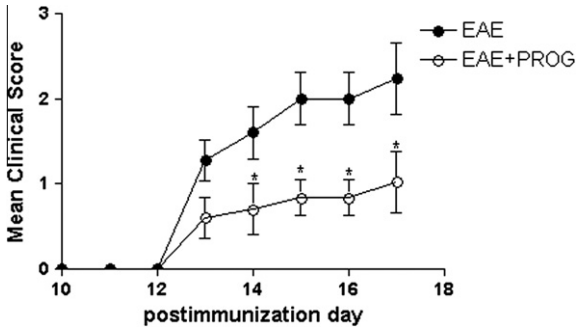

Fig. 1. Progression of clinical signs in EAE mice with or without progesterone pretreatment. Clinical signs appeared on day 12 after EAE induction and tissues were collected on day 17 at the peak of the disease. Progesterone-treated EAE mice (o), remained at grade 1, i.e. partial loss of tail tonicity. EAE without progesterone (•) reached grade 2.2, i.e. complete loss of tail tonicity, difficulty in righting, with some mice reaching grade 3. Thus, progesterone attenuated the clinical course of EAE in C57Bl6 mice. $^{*}p < 0.05$ vs. EAE (non-parametric Mann–Whitney *U* test).

clinical impairment from day 12 until day 17 post-immunization; instead, progesterone-receiving EAE mice presented an attenuation of the clinical signs, as they never exceeded grade 1 (partial loss of tail tonicity). As also shown in Fig. 1, the curves for the EAE and EAE + progesterone groups were different using a non-parametric test from day 14 until day 17. In addition, the accumulative score on day 17 reached 15.42 ± 3.08 for EAE and 6.93 ± 2.18 for EAE + PROG ($p < 0.05$).

As shown in Fig. 2A–D mRNAs of MBP and PLP and the myelin transcription factors NKx2.2 and Olig1 showed a different expression pattern according to the experimental condition. Thus, a significant depletion of the mRNA for MBP (Fig. 2A) and PLP (Fig. 2B) occurred in EAE vs. control female mice. The EAE-induced decrease of myelin protein mRNAs was significantly attenuated by progesterone treatment, which significantly increased the transcription to near control levels for MBP mRNA (F (2,26) = 6.82; $p < 0.01$) and PLP mRNA (F (2,25) = 7.99; $p < 0.01$) (Fig. 2A, B). Similar to the response of myelin proteins, mRNAs for NKx2.2 (Fig. 2C) and Olig1 (Fig. 2D) were significantly reduced in EAE mice and recovered by progesterone treatment (NKx2.2: F (2.19) = 5.06; $p = 0.01$; Olig1: F (2,19) = 10.15; $p = 0.001$). A similar trend was obtained for the Olig2 transcription factor. However, although Olig2 mRNA was reduced in EAE mice and increased by progesterone, results did not reach significance (fold increase controls: 1.0 ± 0.59; EAE: 0.56 ± 0.82; EAE + progesterone: 0.80 ± 0.18). Thus, the response of myelin protein and transcription factor mRNA suggest a promyelinating and remyelination drive in the spinal cord of EAE mice receiving progesterone.

The positive change in myelin and transcription factor mRNA was confirmed by measuring the number of CC1 + myelin-forming oligodendrocytes. The microscope image of Fig. 3A shows a reduction of CC1 + cells in steroid-untreated EAE compared to

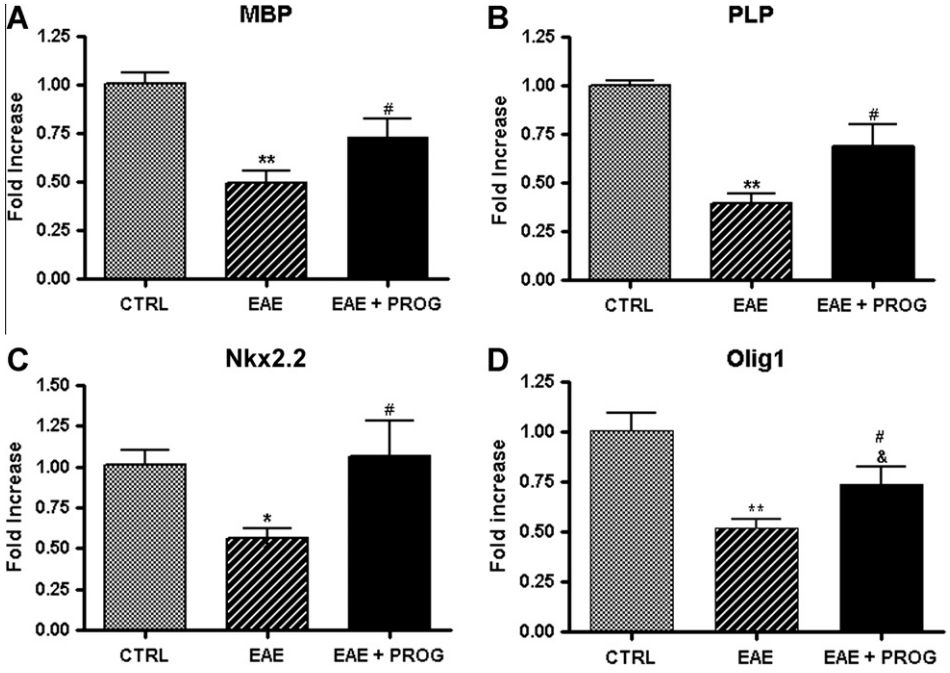

Fig. 2. Quantitative analysis of the effects of progesterone on myelin proteins and myelin transcription factors mRNA determined by real time PCR. The graphs represent MBP (A), PLP (B), NKx2.2 (C) and Olig1 (D) mRNA. Statistical comparison by ANOVA showed down regulation of myelin proteins and transcription factor mRNAs in EAE compared to control mice (CTRL): $^{**}p < 0.01$ for MBP, $^{**}p < 0.01$ for PLP, $^{*}p < 0.05$ for NKx2.2 and $^{**}p < 0.01$ for Olig1. Progesterone treatment increased mRNA levels of MBP (A, $^{\#}p < 0.05$ vs. EAE) PLP (B, $^{\#}p < 0.05$ vs. EAE), NKx2.2 (C, $^{\#}p < 0.05$ vs. EAE) and Olig1 (D, $^{\#}p < 0.05$ vs. EAE). No significant differences were found between EAE + progesterone and control groups for MBP, PLP and Nkx2.2. For Olig1, the EAE + progesterone was lower than its control group ($^{\&}p < 0.05$). Results represent the mean ± S.E.M. of $n = 4$ control mice and $n = 10$ mice for EAE and EAE + progesterone groups.

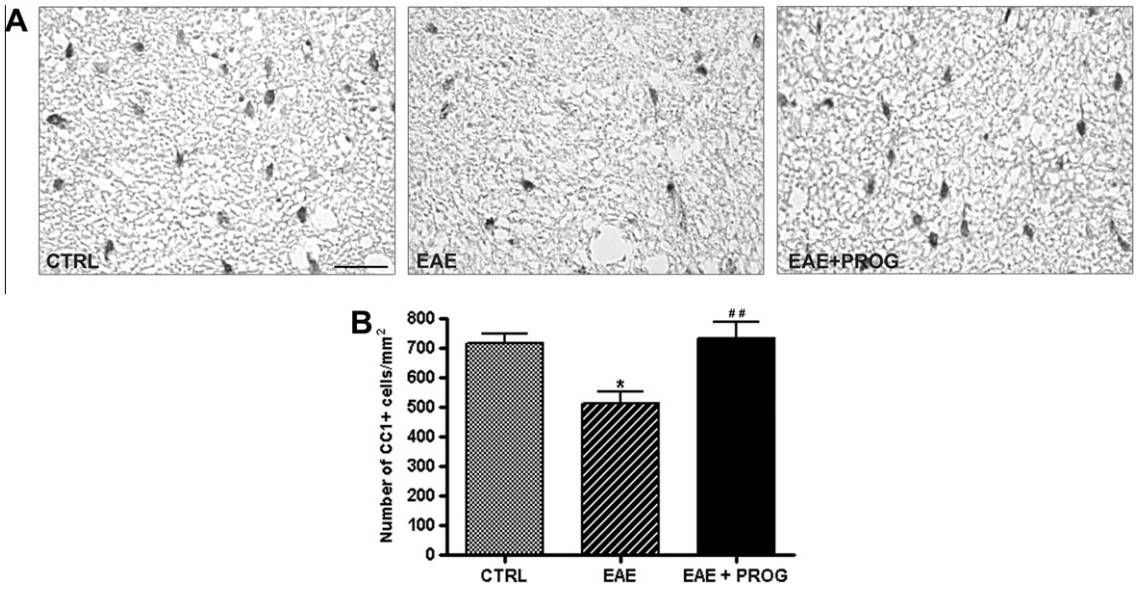

Fig. 3. (A) Representative photomicrographs of the dorsal funiculus of the spinal cord showing changes in the number of CC1 + cells (mature oligodendrocytes) in control (CTL), EAE and EAE + progesterone-pretreated mice. EAE induction reduced CC1 + density whereas progesterone treatment of EAE mice reconstituted the population of CC1 + cells. Inset bar = 20 μm. (B) Quantitative analysis of the number of CC1 + cells in the dorsal white matter funiculus of the spinal cord showed that EAE significantly decreased CC1 + cell density vs. controls ($^{*}p < 0.05$), whereas progesterone pretreatment of EAE significantly increased CC1 + cells over the EAE group ($^{\#\#}p < 0.01$). No differences were found between the EAE + progesterone and the control groups. Results represent the mean density (± S.E.M) of CC1 + cells per mm^2 from $n = 4$ mice per control group, $n = 10$ per EAE and EAE plus progesterone groups.

control (CTRL), whereas progesterone maintained CC1 + cell density (EAE + PROG). Quantitative analysis (Fig. 3B) confirmed that fewer CC1 + cells were present in EAE mice vs. CTRL ($^{*}p < 0.05$) and that progesterone significantly increased CC1 + cells compared to EAE ($^{\#\#}p < 0.01$). (F (2,20) = 7.37; $p < 0.01$). Thus, the stimulatory effects of progesterone on myelin protein and myelin transcription factor mRNA translated into the appearance of more mature oligodendrocytes.

Measurement of mRNAs of the proinflammatory mediators, a microglial/macrophage marker and iNOS are shown in Fig. 4. In control mice (CTRL), baseline mRNA levels were measured for the microglial marker CD11b (A), TLR4 (B), iNOS (C), TNFα (D) and TNFR1 (E). In EAE mice, CD11b (A) and TLR4 mRNA (B) experienced a four- to five-fold increase, indicating their relevance for the inflammatory response. However, a highly responsive gene was TNFα, which increased 25-fold in the spinal cord of EAE mice (D). iNOS mRNA showed a substantial elevation in EAE. However, this elevation was of borderline significance in the post hoc test due to considerable inter-animal variation. Progesterone treatment of EAE mice exerted a pronounced attenuation of the proinflammatory markers. Thus, a significant down-regulation of CD11b (F (2,26) = 5.0; $p = 0.01$), TLR4 (F (2.21) = 4.73; $p < 0.05$), TNFα (F(2,21) = 6.03; $p < 0.01$) and TNFR1 (F(2,18) = 6.29; $p < 0.01$) mRNAs was induced by progesterone. Regarding iNOS mRNA, a non-significant decrease was observed in the post hoc test, although results of the ANOVA analysis reached significance when the controls, EAE and EAE + progesterone groups were compared ($p < 0.05$). Other mRNA molecules related to spinal cord inflammation registered a significant fold increase in the spinal cord of EAE mice vs. controls, including gamma interferon (fold increase in EAE: 23.8 ± 7.5, $p < 0.05$), IP-10 (6.5 ± 1.3, $p < 0.05$), and MyD88 (2.2 ± 0.23, $p < 0.05$). A non-significant increase was found for IL-6 (19.5 ± 6.5). IκB mRNA showed a decrease in EAE (0.53 ± 0.03, $p < 0.001$). None of the above-mentioned molecules, however, was significantly altered by progesterone treatment.

Immunostaining with the specific microglial cell marker Iba1 showed that control spinal cord (Fig. 5A, CTRL) barely contained Iba1 + cells, in contrast to the intense microglial reaction observed in EAE mice spinal cord (EAE, middle image). As shown on the right hand image of Fig. 5A, progesterone blocked the microgliotic reaction. When microglial cell density was quantitatively analyzed, results in control mice showed less than 100 Iba1 + cells per mm^2. This figure is in contrast to the increased density of Iba1 + cells observed in the gray matter (GM), white matter ventral funiculus (VM-VF) and white matter ventrolateral funiculus (WM-VLF) of EAE mice (Fig. 5B, cross-hatched columns). EAE-induced microgliosis was markedly and significantly diminished by progesterone treatment in the three spinal cord areas analyzed (GM: F (2,29) = 5.57; $p = 0.01$); WM-VF: F (2,19) = 5.24; $p = 0.01$); WM-VLF: F (2,18) = 6.89; $p < 0.01$) (Fig. 5, dark columns). The increased microglial cell density in EAE was correlated with increased transcription of proinflammatory mediators and the microglial cell marker CD11b, whereas progesterone-induced Iba1 + cell depletion was associated with a significant decreased expression of

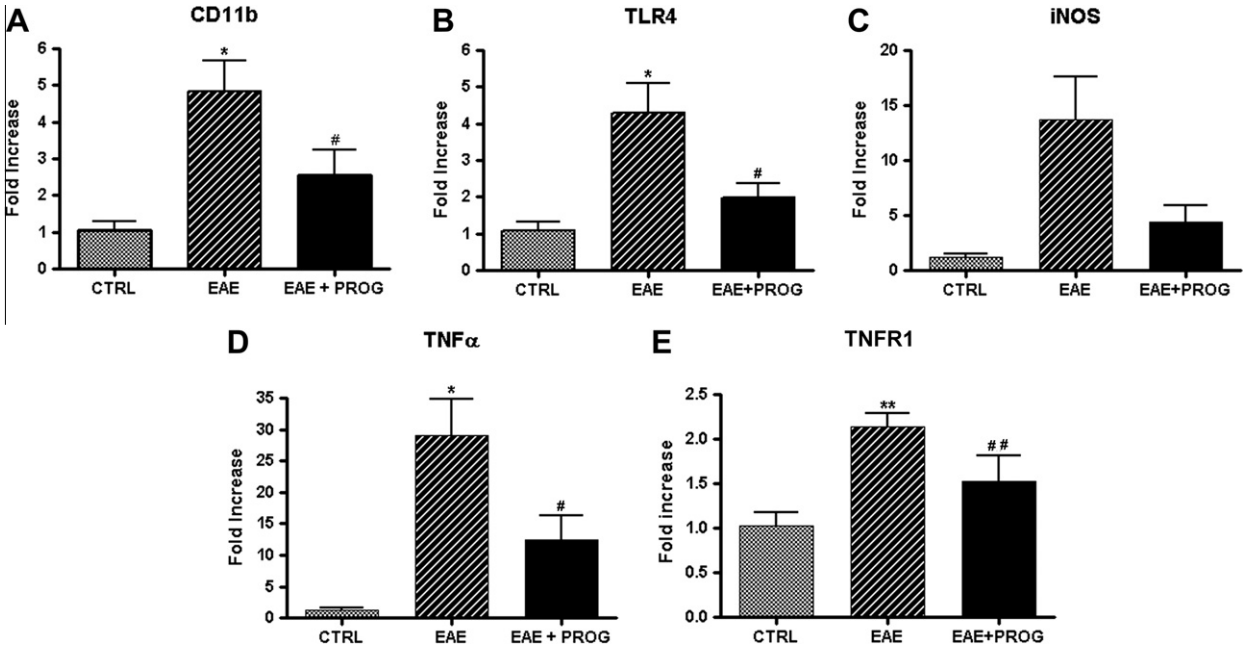

Fig. 4. Quantitative analysis of the effects of progesterone on proinflammatory mediators in the spinal cord determined by real time PCR. The graphs represent CD11b (A), TLR4 (B), iNOS (C), TNFα (D) and TNFR1 (E) mRNAs. Statistical comparison by ANOVA showed that EAE up-regulated the microglial marker CD11b, proinflammatory molecules and iNOS mRNAs compared to control mice (CTRL): $^{*}p < 0.05$ for TNFα, CD11b, and TLR4, $^{**}p < 0.01$ for TNFR1. The increase of iNOS mRNA was not significant. Progesterone treatment significantly decreased transcription of CD11b, TLR4 and TNFα (all $^{\#}p < 0.05$) and TNFR1 ($^{\#\#}p < 0.01$) vs. the EAE group. Progesterone-induced decrease of iNOS mRNA was not significant (however, see text for results of ANOVA analysis). For all markers, no differences were found between the EAE + progesterone-treated mice and their respective control groups. Results represent the mean ± S.E.M. of $n = 4$ control mice and $n = 10$ mice for EAE and EAE + progesterone groups.

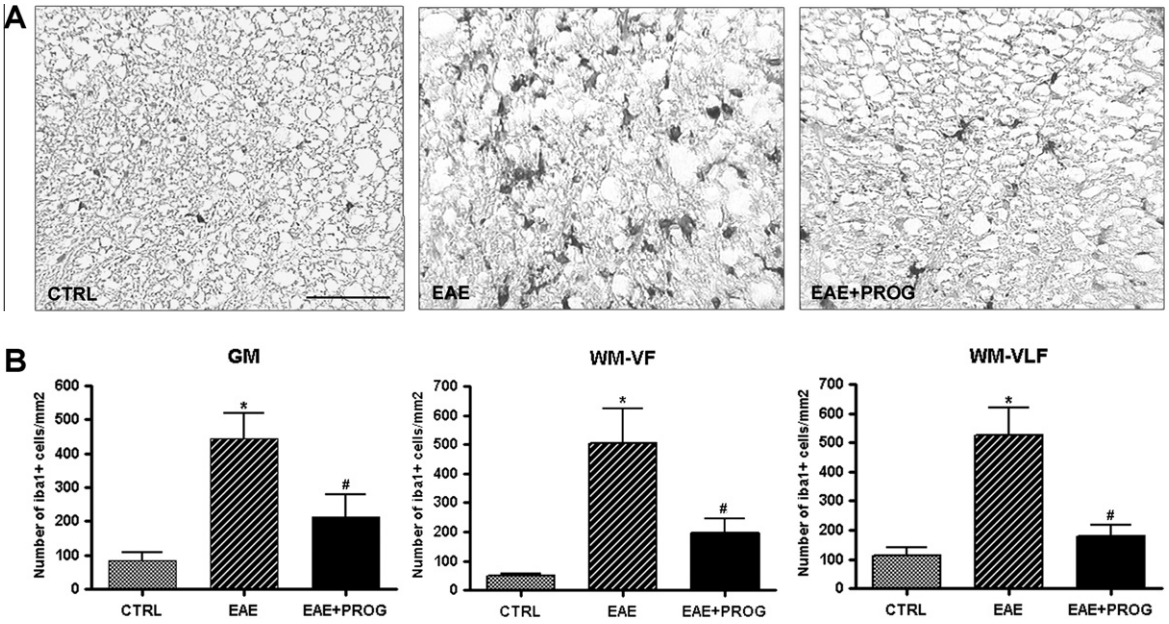

Fig. 5. (A) Representative photomicrograph of the ventral funiculus of the white matter of the thoracic spinal cord showing changes of the number of Iba1 + immunoreactive cells in a control mouse (CTRL), EAE and EAE + progesterone-pretreated mouse. Strong Iba1 + microglial cell reactivity developed in EAE, which was considerably reverted by progesterone treatment. Inset bar = 50 μm. (B) Quantitative analysis of the number of Iba1 + cells/mm^2 in the gray matter (GM), ventral funiculus of the white matter (WM-VF) and ventrolateral funiculus of the white matter (WM-VLF). Iba1 + microglial cell density of EAE mice was significantly higher in gray matter and in the two white matter regions ($^{*}p < 0.05$ vs. control). Progesterone pretreatment blocked the increased Iba1 + microglial cell density found in EAE mice ($^{\#}p < 0.05$ vs. EAE). No differences were found for Iba1 + cell density between the EAE + progesterone-treated mice and their respective control groups. Results represent $n = 4$ control mice, $n = 10$ for EAE and EAE plus progesterone groups.

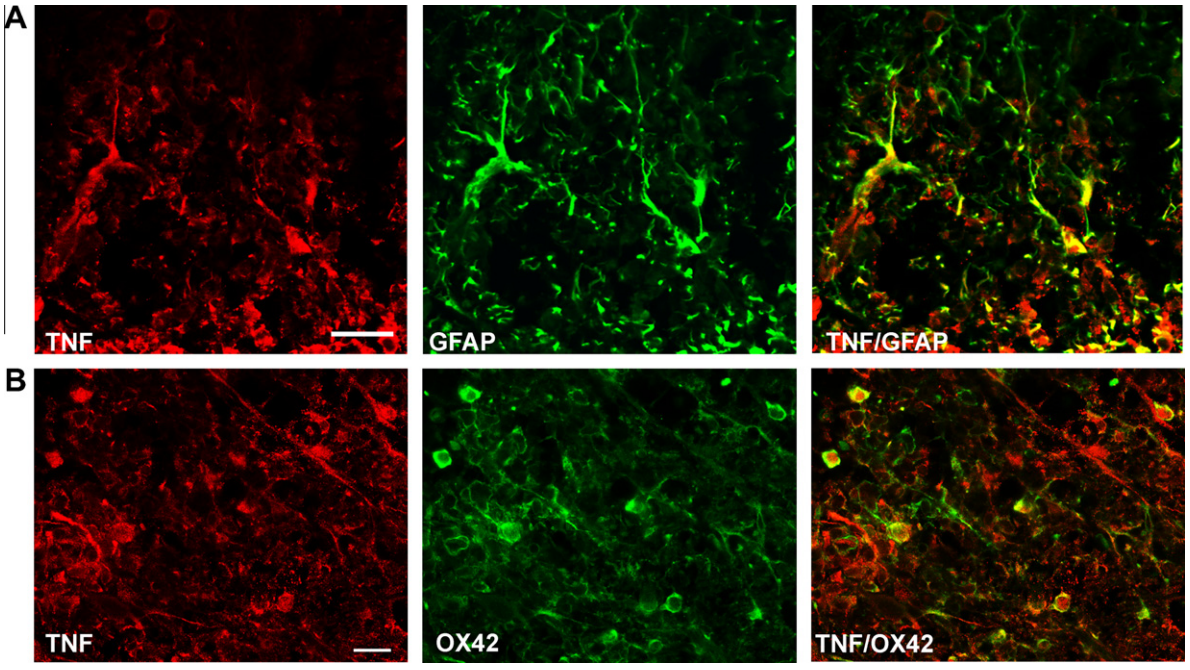

Fig. 6. Confocal laser microscope images showing the glial phenotype of TNFα immunoreactive cells in the spinal cord of EAE mice. The upper photomicrographs (A) show TNFα + cells (red, left) and GFAP + astrocytes (green, middle). Merging confirms colocalization of TNFα with GFAP + astrocytes (right image). Inset bar = 25 μm. The lower photomicrographs (B) show TNFα + cells (red, left) and OX-42 + microglial cells (green, middle). Merging confirms colocalization of TNFα with OX-42 + microglia (right image). Inset bar = 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CD11b, the proinflammatory mediators TLR4, TNFα, TNFR1, and a borderline decrease of iNOS.

Our final investigation included the phenotype identity of TNFα -producing cells, considering that this cytokine was highly stimulated in EAE, and suffered a pronounced decrease following progesterone treatment. Confocal imaging demonstrated that TNFα immunopositive cells colocalized with both GFAP, a marker of astrocytes, and with OX-42, a microglial marker (Fig. 6). These results indicated that (1) astrocytes and microglia were key players in the inflammatory response to EAE, (2) they were the likely targets of progesterone anti-inflammatory actions in the spinal cord of EAE mice.

DISCUSSION

The present study substantiates that regulation of local (spinal cord) inflammation correlates with promyelinating effects of progesterone in the acute phase of EAE. We used a protocol involving progesterone treatment in advance of EAE induction because previous work demonstrated its effectiveness to attenuate clinical signs and neuropathology of EAE mice (Garay et al., 2007, 2008, 2009, 2011). Other studies involving EAE rodents employed steroids given before disease induction (Bebo et al., 2001; Offner, 2004; Elloso et al., 2005). Recent reports, however, have shown that progesterone also decreases neuropathology when given at the time of EAE induction (Yates et al., 2010; Yu et al., 2010). The steroid pretreatment model intended to simulate the effects of high progestin levels of pregnancy on the prevention of post-partum relapses of MS (El-Etr et al., 2005; Confavreux et al., 1998). Additionally, we were unable to observe progesterone protective effects if the compound was administered on the day of EAE induction or when the disease was already established (Garay et al., unpublished results).

We found that pretreatment with progesterone of EAE mice diminished microglial cell reactivity, according to immunocytochemistry for Iba1 and mRNA for the CD11b marker, decreased the expression of the receptor TLR4, reduced the proinflammatory cytokine TNFα and its receptor TNFR1 and showed a marginal effect on iNOS mRNA in the spinal cord. Instead, other inflammatory molecules up-regulated in EAE were steroid-insensitive. The anti-inflammatory effects of progesterone may be ascribed to direct effects on reactive astrocytes and microglia. In addition, progesterone significantly enhanced the expression of transcription factors involved in specification of the oligodendrocyte lineage and myelin repair, increased mature oligodendrocyte density and elevated the expression of mRNAs of the myelin protein MBP and PLP. Clinically, animals receiving hormone treatment showed an attenuation of neurological disability.

Effects of PROG on neuroinflammation

Several lines of evidence support the contention that progesterone has profound effects on the immune system. Progesterone favors the development of T helper cells producing Th2-type cytokines (Piccinni et al., 1995; Miyaura and Iwata, 2002). Pregnant women

with MS show a decrease in the relapse rate during the third trimester ascribed to high levels of estrogens and progesterone (Confavreux et al., 1998; Kim et al., 1999; Druckmann and Druckmann, 2005; El-Etr et al., 2005). Moreover, in splenocytes of EAE mice, progesterone treatment decreases the secretion of pro-inflammatory cytokines IL-2 and IL-17 while in models of CNS injury, it controls reactive gliosis, modifying microglial density and the expression of proinflammatory mediators (He et al., 2004; Pettus et al., 2005; Dang et al., 2011).

Our results demonstrated a generalized microgliosis in the spinal cord of EAE mice analyzed at a transcriptional level through CD11b and Iba1 immunohistochemistry. A significant attenuation of the microglial reactivity was observed in progesterone-treated EAE-treated mice probably by a direct steroid action upon these cells. Although the presence of progesterone receptors in microglia is controversial, direct antiinflammatory effects of progesterone and its derivatives on a microglial cell line have been demonstrated (Muller and Kerschbaum, 2006). Thus, impairment of microgliosis may be beneficial considering these cells represent an important source of damaging inflammatory mediators.

TLRs are a family of innate immune system receptors that initiate response to a broad spectrum of pathogens. TLR4 signaling is induced classically upon LPS (lipopolysaccharide) recognition and endogenous ligands referred to as damage-associated molecular patterns (DAMPs) (Lehnardt et al., 2008). TLR4 is highly expressed on microglia and other immune cells after trauma and inflammation of the central nervous system (Okun et al., 2011). Stimulation of this receptor can lead to activation of NFκB and mitogen-activated protein (MAP) kinases and production of inflammatory mediators including TNFα, IL-6, IL1-β, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) (Bsibsi et al., 2002; Hanke and Kielian, 2011). In addition, deregulation of this receptor could be partially responsible for EAE pathogenesis. Studies *in vivo* and *in vitro* have revealed that LPS exposure causes TLR4-dependent microglia activation associated with oligodendrocyte death, demyelination, neuronal and axonal loss; events that typically characterize EAE (Lehnardt et al., 2002, 2003). Furthermore, neuroinflammation is involved in myelin disruption and downregulation of myelin proteins (Alfonso-Loeches et al., 2010). Thus, our results showing decreased expression of TLR4 by progesterone may be beneficial to prevent the activation of proinflammatory genes, demyelination and oligodendrocyte death in EAE. This effect is in accordance with previous *in vitro* studies showing that pretreatment with pregnant levels of progesterone inhibited the up-regulation of TLR4 expression on murine macrophages exposed to LPS (Su et al., 2009). In traumatic brain injury, up-regulation of TLR4 mRNA is markedly inhibited by progesterone administration (Chen et al., 2008).

Considering TNFα a target gene downstream of TLR4 activation, it was not surprising to find higher levels of this cytokine in the spinal cord of EAE mice. In this regard, high levels of TNFα were identified in acute and chronic MS lesions suggesting its involvement in lesion formation (Selmaj et al., 1991). In EAE, inhibition of TNFα with an IgG1 monoclonal antibody effectively prevented the disease (Teuscher et al., 1990; Selmaj et al., 1991). Progesterone attenuation of TNFα mRNA hyperexpression in the spinal cord of EAE mice may lead to protection of myelinating cells. Oligodendrocytes and their progenitors are particularly vulnerable to the effects of this cytokine *in vivo* and *in vitro*. Indeed, treatment with TNFα-promoted apoptosis of mature oligodendrocytes and inhibited maturation and differentiation of their progenitors (OPC) (Cammer, 2000; Chen et al., 2011; Su et al., 2011). Colocalization studies showed that this cytokine was mainly produced by microglia and astrocytes. TNFα interaction with the TNFR1 could be responsible for the cytolytic activity of TNFα (MacEwan, 2002; Mc Guire et al., 2011). The elevation of this receptor in EAE mice was attenuated when mice received progesterone. In this way, progesterone anti-inflammatory actions were probably mediated in part by reduction of microgliosis, proinflammatory mediators and TNFR1. iNOS expression was also attenuated by progesterone, which confirms results in other systems. Thus, in a spinal cord neurodegeneration model, progesterone decreased nitric oxide production, possibly acting on the nNOS isoform (Deniselle et al., 2012). Further studies are necessary to establish the role of iNOS and the other NOS isoforms in progesterone effects in a neuroinflammatory milieu.

In addition to the inflammatory mediators reported in the present study, other cytokines have been already demonstrated to be under the control of progesterone. These include a decrease of the inflammatory interleukins IL-2 and IL-17 and an increase of the anti-inflammatory IL-10 in EAE mice (Yates et al., 2010), decreases of IL-1β and TNFα in brain-injured rats (He et al., 2004) and decreases of IL-1β, transforming growth factor (TGF)β2 mRNAs after brain ischemia (Gibson et al., 2005). Thus, suppression of the immune response seems a generalized property of progesterone in different experimental conditions.

Effects of PROG on the expression of transcription factors, oligodendrocytes and myelin proteins

The present investigation strengthened the previously reported promyelinating effects of progesterone for the central and peripheral nervous system (PNS) (Koenig et al., 1995; Melcangi et al., 1999, 2003). In the PNS, myelin protein expression was stimulated by progesterone and its derivatives (Melcangi et al., 1999). In the CNS, progesterone has shown myelinating/remyelinating effects *in vitro* in cerebellum after toxin-induced demyelination and in the lesioned spinal cord (Ibanez et al., 2004; Marin-Husstege et al., 2004; Ghoumari et al., 2005; Acs et al., 2009; Labombarda et al., 2009; Hussain et al., 2011). In response to a demyelinating lesion, the remyelination process requires the proliferation of OPC, recruitment to the

demyelination area and differentiation into myelin-forming oligodendrocytes (Chang et al., 2002; McDonald and Belegu, 2006; Franklin and Ffrench-Constant, 2008). In this relation, we have previously shown that immunostaining for the myelin proteins MBP and PLP increased in the spinal cord of progesterone-treated EAE compared to non-treated mice (Garay et al., 2007). Here, we have shown that progesterone also stimulated these proteins at the transcriptional level. Thus, progesterone promyelinating stimulus involved transcriptional events as well as an increase in myelin proteins. Considering that elevated levels of progesterone were present from the beginning of the inflammatory process, we do not discard that oligodendrocytes were spared from apoptosis. Indeed, the number of CC1 + oligodendrocytes was conserved in the EAE plus progesterone group. Taking into consideration that demyelination leads to axonal degeneration, it is not surprising that animals receiving steroid treatment had less clinical or motor function impairment.

The transcription factors NKx2.2 and Olig1 involved in oligodendrogenesis were also stimulated in EAE mice treated with progesterone. NKx2.2 is expressed by early differentiating OPC; this transcription factor also controls oligodendrocyte differentiation probably by regulation of the expression of certain myelin protein genes. In line with this possibility, a dramatic decrease in PLP and MBP expression occurs in the spinal cord of NKx2.2 null mice (Qi et al., 2001; Lee et al., 2003). Consensus NKx2.2-binding sites are found in the PLP and MBP promoters, which indicate a direct effect upon myelin genes (Qi et al., 2001). However, NKx2.2 also targets mature oligodendrocytes serving a myelin maintenance role (Cai et al., 2010). Olig1 is localized in the nucleus and may function as a transcriptional regulator for the expression of myelin oligodendrocyte glycoprotein (MOG), MBP and myelin-associated glycoprotein (MAG) (Guo et al., 2010). Interestingly, Olig1 may have a critical role in CNS repair. Indeed, a study using a toxin-induced demyelination model in Olig1−/− mice demonstrated that Olig1 function is essential for the remyelination phase of both cuprizone- and lysolecithin-induced demyelination in brain and spinal cord, respectively (Arnett et al., 2004). In line with this result, the stimulation of the expression of Olig1 and NKx2.2 with progesterone may indicate attempts to differentiate oligodendrocytes and remyelinate the affected white matter. In this regard, progesterone promotes the differentiation of OPC into myelinating oligodendrocytes in adult male rats after spinal cord injury (Labombarda et al., 2009). A recent publication described higher levels of MBP in the spinal cord of EAE rats in the chronic phase treated with progesterone compared to naïve rats (Giatti et al., 2012). Accordingly, it may be inferred that OPC reached a mature myelinating state in the presence of the steroid. In addition to having a mitogenic effect on OPC according to the progesterone regulation of Nkx2.2 and Olig1, the maintenance of CC1 + cell number (this paper) and the role of progesterone on oligodendrocyte myelin production in organotypic cultures of cerebellum (Ghoumari et al., 2005), suggest that progesterone could also protect oligodendrocytes in cases of injury. It seems also important to recognize that the protective and myelinating effects of progesterone may be exerted by the steroid itself and/or their reduced metabolites dihydroprogesterone (DHP) and tetrahydroprogesterone (THP). Genomic and non genomic actions may be involved although the classical progesterone receptor seems to have a key role, as no promyelinating effects of progesterone are observed in PR knockout mice (Hussain et al., 2011).

CONCLUSIONS

The present results in EAE mice suggest that progesterone behaves as a promyelinating and anti-inflammatory factor. Previous data using a focal demyelination model induced by lysolecithin indicates that progesterone produces promyelinating and anti-inflammatory effects at the spinal cord level (Garay et al., 2011). Therefore, progesterone may exert dual effects in EAE: first, peripheral effects by prevention of the attack of the adaptive immune system on the spinal cord, and second direct anti-inflammatory and promyelinating actions acting upon astrocytes, microglia and oligodendrocytes of the inflamed spinal cord. These possibilities expand the scope of progesterone activities. Recent theories of MS consider the disease being primarily inflammatory and then neurodegenerative, whereas others put the accent on neurodegeneration (Matute and Perez-Cerda, 2005; Trapp and Nave, 2008). It is hoped that progesterone effects described in EAE mice are reproduced in MS patients, favoring remyelination, well being and recovery of motor function.

Acknowledgements—This work was supported by grants from FONCYT (PICT 2007-01044), CONICET (PIP 112-200801-00542) and the University of Buenos Aires (20020100100089). There are no conflicts of interests.

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(Accepted 11 September 2012)
(Available online 19 September 2012)