Efficacy of the selective progesterone receptor agonist Nestorone for chronic experimental autoimmune encephalomyelitis

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ABSTRACT

Progesterone plays a protective role in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS). Besides spinal cord neuropathology, MS patients present a dysfunctional hippocampus. In this work we studied the therapeutic effects of the progestin Nestorone in the brain of mice with chronic EAE. Nestorone decreased clinical grade and enhanced motor behavior. In addition, it increased cell proliferation and doublecortin positive neuroblasts in the hippocampus, increased GABAergic interneurons and attenuated the number of Iba1+ microglia/macrophages, events possibly linked to enhancement of neurogenesis. Therefore, Nestorone protected against hippocampus abnormalities and improved functional outcomes of EAE mice, suggesting its potential value for MS.

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1. Introduction

The most common inflammatory/demyelinating disease of the central nervous system is multiple sclerosis (MS). Considered an inflammatory disease of autoimmune origin, there are also indications that primary cytodegeneration may be the initial event in some clinical forms of MS (McQuilter and Bernard, 2007; Trapp and Nave, 2008; Stys et al., 2012; Ellwardt and Zipp, 2014). In this regard, there might be intrinsic defects of the oligodendrocytes, with release of myelin proteins and debris that provokes a secondary reaction of the immune system (Matute and Perez-Cerda, 2005; Stys et al., 2012).

A role of steroid hormones in MS has been substantiated by its gender difference (women to men ratio about 2.3) and by the decline in relapse rate during pregnancy. The last effect could be due to the

protective and anti-inflammatory effects of high levels of estrogens and progesterone circulating in pregnancy, whereas the reappearance of post-partum relapses is ascribed to declining sex steroid levels (Confavreux et al., 1998; El-Etr et al., 2005). The steroid hypothesis has recently received support from the finding of low levels of steroidogenic enzymes and the neurosteroid allopregnanolone in the brains of patients with MS and mice with experimental autoimmune encephalomyelitis (EAE), a commonly used model of MS (Noorbakhsh et al., 2011).

Progesterone protective effects have been shown for the spinal cord of EAE rodents. Thus, progesterone pretreatment of EAE mice reduces inflammatory cell infiltration, demyelination, microglial activation, axonal loss, astrocytosis, decreases several proinflammatory mediators, prevents neuronal dysfunction and enhances recovery of motor functions (Garay et al., 2007, 2009, 2012). In our experience, steroid administration at the time of EAE induction or in animals with established disease is not effective. Another report has shown that progesterone given at the time of EAE induction reduces clinical scores, decreases proinflammatory and increases anti-inflammatory chemokines (Yates et al., 2010). When given at the onset of neurological deficits in rats with EAE, progesterone induces nuclear sublocalization of the Olig1 transcription factor involved in remyelination followed by clinical benefit (Yu et al., 2010). Whereas the mentioned studies have used animals in the acute phase of EAE, progesterone also prevents spinal cord

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damage in Dark Agouti rats with chronic EAE (Giatti et al., 2012). Moreover, in cotherapy with estrogens, progesterone counteracts not only EAE-induced spinal cord demyelination (Garay et al., 2008) but also cuprizone-induced demyelination of the corpus callosum (Acs et al., 2009) symptoms which are not circumscribed to the spinal cord. Brain atrophy and gray matter lesions are observed at early disease onset (Ellwardt and Zipp, 2014), whereas cell loss in CA1 and CA3 pyramidal cells of the hippocampus have been described by Papadopoulos et al. (2009). The hippocampus abnormalities are closely bound to the memory impairment and cognitive decline of MS patients (Koenig et al., 2013; Sumowski and Leavitt, 2013). Memory, cognition and learning are key hippocampal functions linked to dexterus gyrus neurogenesis (Kempermann, 2002; Gould, 2007; Galea et al., 2006). In this regard, neurodegenerative changes and altered neurogenesis have been discovered in EAE mice, including a decrease in CA1 pyramidal layer volume, reduction of cell proliferation (Puchino et al., 2008; Guo et al., 2010; Zielh et al., 2010), or enhanced cell proliferation but reduced capacity to generate neuroblasts and mature neurons (Giannakopoulou et al., 2013; Huehnchen et al., 2011). These reports have studied animals in the acute phase of EAE or 20 days afterwards. In most cases, EAE develops around day 12 after injection of a myelin peptide in experimental animals.

In contrast with available data reporting progesterone effects in the spinal cord of EAE mice, results regarding the effects of progestins on neurogenesis or other hippocampus parameters in the EAE model are lacking. Novelties of the present study include: first, the use of a synthetic progestin instead of the natural hormone, and second, the analysis of biological parameters, clinical grade and motor function in the chronic phase (60 days) of EAE. The synthetic progestin Nestorone (16-methylene-17alpha-acetoxy-19-norpregn-4-ene-3,20-dione) a 19-nor progesterone derivative shows 70% higher affinity for the progesterone receptor than the natural ligand, shows null estrogenic, and androgenic or glucocorticoid activity and is not bound to plasma proteins (Sitruk-Ware et al., 2003; Kumar et al., 2000). In addition to these unique pharmacological properties, Nestorone brings strong neuroprotection against experimental ischemic brain damage and increases neurogenesis as well as myelin repair in a chemical demyelination model (Liu et al., 2012; Hussain et al., 2011). Therefore, evidence gathered in vivo after brain ischemia and in vitro with demyelinated cerebellar cultures, encouraged us to investigate if Nestorone could also become a therapeutic tool for the brain of mice with chronic EAE.

2. Materials and methods

2.1. Experimental animals

Nine-week-old female C57BL/6 mice purchased from the Faculty of Veterinary Sciences, University of La Plata, Argentina, were used for all the experiments. Although sex differences in disease outcome and spinal cord pathology have been reported in rodents with EAE (Voskuhl et al., 1996; Massella et al., 2012), female mice were used to compare present with past results obtained with progesterone in this gender (Garay et al., 2007, 2008, 2009, 2012). Mice were kept under pathogen-free conditions under a 12:12 h light/dark cycle (lights on 07.00 h), with controlled humidity and temperature (22 °C), and fed standard mice chow. EAE was induced by immunizing with a sc injection of 200 μg of MOG40–51 (Peptides International, Louisville, USA), emulsified in complete Freund’s adjuvant CFA (Sigma-Aldrich) containing 0.6 mg of Mycobacterium tuberculosis (Instituto Malbran, Argentina). Ip. injections of pertussis toxin (400 ng) (Sigma-Aldrich) were administered 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. Animals received daily sc injections of Nestorone (provided by The Population Council, New York, USA) at the dose of 0.4 mg/kg dissolved in vegetable oil or vehicle only from the first day of clinical manifestations and continued for ten days. This dose was chosen as clinically more effective based on pilot studies using higher doses of Nestorone (0.8 mg/kg). Treatment was then suspended and mice were euthanized on day 60 post induction of EAE, this period of time being considered the chronic phase of the disease. At the time of killing, female mice were 4.5 months old (adult age).

Animal procedures were carried out in accordance with 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.

2.2. Immunohistochemistry for doublecortin (DCX) and Ki67 in brain

Mice were deeply anesthetized with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg), and transcardially perfused with 25 ml of 0.9% NaCl solution followed by 25 ml 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PBS) pH 7.2. Brains were removed, postfixed for 2 h at 4 °C in the same fixative and cryoprotected with a solution made of ethylene glycol-glycerol-0.1 M sodium phosphate buffer pH 7.4 (0.6:0.5:1.0) overnight. After a change of the same solution, brains were kept frozen at −20 °C until used. To label proliferating neuroblasts and immature neurons, we used a DCX antibody. To this end, 60 μm coronal brain sections were obtained using a vibrating microtome. Sections were exposed to methanol: H2O2 (100:1) for 10 min at room temperature (RT) 25 °C, 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 Labs) followed by biotinylated anti-goat IgG made in rabbit (1:1200, Sigma). After processing following the ABC kit (Vector Laboratories, Burlingame, CA, USA) instructions, sections were developed with diaminobenzidine chloride 0.25 mg/ml (Sigma) and 0.05% H2O2 at RT. The tissue was finally dried, dehydrated and mounted with Permount (Fischer Scientific, Pittsburgh, PA, USA). Non-specific staining was assessed in the absence of primary antibody. DCX-positive cells were counted on every 6th section, throughout the rostrocaudal extension of the DG corresponding to Plates 41–51 from the stereotaxic atlas of the mouse brain (Paxinos and Watson, 2009), according to the optical dissector method. Data corresponded to six–nine animals per experimental group. All positive cells were counted with a 40× objective under a Zeiss Axioplan microscope (Germany). Cell counts were restricted to the subgranular cell layer of the dentate gyrus. The number of DCX-immunoreactive cells was multiplied by 6 to estimate the total number of DCX-labeled cells in the dentate gyrus.

To determine cell proliferation, we employed Ki67 immunocytochemistry. For this purpose, 60 μm sections were washed three times in TBS, pH 7.4, incubated in 0.01 M citrate buffer pH 6.0 at 90 °C for 40 min, left to cool and further incubated with H2O2 (100:1.5) in TBS during 15 min at RT. After washes in TBS, sections were blocked for 30 min in TBS containing 2% nonfat milk at RT and incubated overnight at 4 °C with a polyclonal anti Ki67 antibody made in rabbit (1:1000, Novocastra, Leica Biosystems Newcastle Ltd, United Kingdom) diluted in 0.5% Triton X-100 TBS, 1% goat serum. After 3 washes in TBS, sections were incubated with a biotinylated anti-rabbit IgG made in goat (1:200, Sigma-Aldrich) in 0.5% Triton X-100 PBS, 1% goat serum for 90 min in a shaker at RT. After 3 washes in TBS, they were processed following the ABC kit and developed using 0.5 mg/ml DAB, 2.5% nickel, 0.05% H2O2. Sections were dehydrated and mounted as specified before. Non-specific staining was assessed in the absence of primary antibody. The method of counting of KI67 + cells was identical to DCX + cells. Five–six animals per group were analyzed.
2.3. Immunofluorescence staining of microglia/macrophages in brain

Microglial/macrophage cells were immunostained with Iba1 antibody. For this purpose, free-floating sections were rinsed in PBS, incubated with 5% BSA for 10 min at 37 °C followed by a rabbit anti-Iba1 antibody (1:2000, Wako, Japan) prepared in 5% BSA, 0.1% Triton X-100 in PBS. After two overnight incubations at 4 °C, sections were washed with PBS and incubated with 1/1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 488 secondary antibody (Invitrogen, Molecular Probes, Eugene, OR, USA) prepared in 5% BSA, 0.1% Triton X-100 in PBS for 1 h at RT. After several washes, sections were mounted with Fluoromount G (Southern Biotech, Birmingham, Alabama, USA) and examined under a Nikon Eclipse E 800 confocal scanning laser microscope. Photographs were saved and further analyzed by a computerized image analysis system equipped with Optimas VI software. The number of Iba1+ cells with nuclei counterstained with propidium iodide was counted in a constant area of white matter comprising the stratum oriens and stratum radiatum of the hippocampus. Results were expressed as the number of cells per mm² and were obtained from seven–nine animals per group. In addition to the total number of Iba1+ cells, we determined two different phenotypes of Iba1+ cells based on their morphology (Kreutzberg, 1996). For this purpose, confocal images at 600× magnification were taken near the outer molecular layer of the dentate gyrus region in which individual Iba1+ microglia/macrophages and their processes were easily identified without the interference of nearby immunoreactive cells. A mean of 10 z-stack images of 1 mm thick was acquired to individualize each cell. Volume visualization of the z stack images using Free Viewer EZ-C1 3.70 enabled the counting of the total number of processes per cell. Data were expressed as % of cells in each category: Group 1: less than three processes and Group 2: four or more processes. Nine to eleven animals per group were evaluated.

2.4. Double immunofluorescence for neuronal nuclei antigen (NeuN) and GABA

The phenotype of interneurones of dorsal hippocampus was investigated using double immunostaining for NeuN and GABA. Free-floating sections were incubated with the monoclonal NeuN antibody (anti-Neuronal Nuclei MAB 2377, Chemicon-Millipore, Billerica, CA, USA) at a 1:1000 dilution and with the polyclonal rabbit anti-GABA antibody (anti-GABA #9 made in rabbit; a kind gift from Dr. Peter Somogyi, Anatomical Neuropharmacology Unit, Oxford, UK) at a 1:750 dilution. After an overnight incubation with the primary antibodies, the slices were rinsed three times in TBS 0.1% Triton X-100 for 15 min before application of the second antibodies: goat anti-mouse IgG conjugated to Alexa Fluor 555 and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (dilution 1:1000, Invitrogen, Molecular Probes, Eugene, OR, USA). Incubation with second antibodies was followed by three rinses in TBS. Sections were mounted with Fluoromont G (Southern Biotech, Birmingham, AL, USA Alabama, USA) and kept in the dark at 4 °C until analysis by confocal microscopy. Double-labeled cells were examined under a Nikon Eclipse E 800 confocal scanning laser microscope equipped with Nikon 11691 photographic equipment. Photographs were saved and further analyzed by a computerized image analysis system equipped with Optimas VI software. The number of double labeled GABA+/NeuN+ cells was counted in a constant area of the stratum lacunosum moleculare of the hippocampus and was expressed per mm². Three sections per animal were examined at a similar level of the hippocampus from six animals per group.

2.5. Immunocytochemistry for glial fibrillary acidic protein (GFAP) and myelin basic protein

The number of GFAP immunopositive cells was assessed in the stratum oriens and stratum radiatum of the hippocampus. Free-floating sections were exposed to 1/250 dilution of rabbit anti-GFAP polyclonal antibody (C-9269, Sigma) and developed with a goat anti-rabbit IgG conjugated to Alexa Green 488 (dilution 1:1000) (Invitrogen, Molecular Probes, Eugene, OR, USA). Sections were counterstained with propidium iodide and only double stained cells were counted. Images were obtained and processed as detailed for Iba1 in both stratum radiatum and stratum oriens. The number of GFAP+ cells was expressed per mm² obtained from seven–ten animals per group.

To determine MBP immunoreactivity, free-floating sections were incubated with a 1/600 dilution of a rabbit anti-MBP polyclonal antibody (a kind gift from Dr. A. Camagnoni, UCLA) and revealed using a biotinylated goat anti-rabbit complex (Vectastain ABC Elite Kit) followed by incubation with diaminobenzidine chloride 0.25 mg/ml (Sigma) and 0.05% H₂O₂ at RT. Images were taken with an Olympus BH2 microscope and analyzed with the image analysis software Bioscan Optimas VI. The percentage of immunoreactive area was assessed in a constant area of corpus callosum considering four animals per group.

2.6. Motor behavior on the rotarod

A rotarod apparatus (Model 755 Stoelting, IL) was used to evaluate motor coordination, balance and learning during the chronic stable face of EAE. Mice were placed on an accelerating rod (1 to 20 rpm, ramp speed 200 s) over a period of 300 s. Mice remaining steady on the rod after 300 s were removed and the rotarod time scored as 300. Animals were trained every day during a week and underwent six consecutive trials during the following two weeks before sacrifice. The length of time that the mouse remained on the rod (latency to fall) was recorded by triplicates. The three measurements were averaged to report a single value for each mouse per day. We used nine–ten animals per experimental group.

2.7. Statistical analysis

All results were expressed as the mean ± SEM. Immunohistochemistry data were analyzed by one way ANOVA followed by a post-hoc Newman–Keuls test. Clinical scores were analyzed by Mann–Whitney U test and rotarod scores were determined by Repeated Measures ANOVA followed by post-hoc Newman–Keuls multiple comparison tests. Statistical analysis was performed with PRISM 4 (GraphPad Prism software, San Diego, CA, USA). The level of significance was set at p < 0.05.

3. Results

3.1. Effects of Nestorone on clinical scores and motor behavior of EAE mice

Fig. 1A shows the clinical grades reached by EAE mice receiving vehicle or Nestorone treatment once a day for 10 consecutive days, starting on the onset of EAE signs. Measurements of scores were initiated on day 7 after induction and continued until the time of killing (day 60). As previously observed using a similar induction protocol, first signs of EAE appeared around days 10–12 (Garay et al., 2007, 2012). However, a marked difference was registered for both groups, in that Nestorone-receiving EAE mice showed a significantly lower clinical grade, with a mean score of about 1, compared to the vehicle-receiving EAE mice, which showed a mean score of around 2 (p = 0.001, Mann–Whitney U test). None of the control mice receiving pertussis toxin plus M. tuberculosis developed motor abnormalities.

ANOVA showed that motor behavior in the rotarod was also significantly different between groups (F(2,35) = 30.60; p < 0.001). Thus, after taking 6 sessions on the rotarod during the last 2 weeks before killing, control mice ran for at least 250 s (Fig. 1B). In contrast, steroid-naïve EAE mice showed a lower performance, remaining on the rod for about 150 s before falling down (post-hoc test: vehicle EAE vs. control: p < 0.001). Instead, Nestorone treated EAE mice remained running for
Neurogenesis were analyzed using the marker of immature neurons and mitosis. As shown qualitatively in the images of Fig. 2A and in quantitative form in Fig. 2B, differences in the number of Ki67+ progenitors were found between controls, EAE and the EAE + Nestorone groups (**p < 0.001 for both EAE cases vs. control). Still, mice in the EAE + Nestorone group tested better than the EAE group (**p < 0.05).

3.2. Effects of Nestorone on hippocampus neurogenesis

Neurogenesis is a multiple step process, starting with the proliferation of progenitors. The progenitors stain positive for Ki67, a marker protein expressed during all active phases of the cell cycle: G1, S, G2, and mitosis. As shown qualitatively in the images of Fig. 2A and in quantitative form in Fig. 2B, differences in the number of Ki67+ progenitors were found between controls, EAE and the EAE + Nestorone groups. ANOVA demonstrated significant group differences (F(2,9) = 22.62; p < 0.001). The post-hoc test showed that the number of Ki67+ cells in the dentate gyrus was increased 1.36 times in vehicle-treated EAE mice compared to control mice, although the difference was not significant (p > 0.05). Instead, Nestorone treatment down-regulated Iba1+ cell number of EAE mice (b vs. c). The ANOVA test demonstrated significant group differences (F(2,17) = 6.33; p < 0.01) (Fig. 4B). Quantitative analysis and the post-hoc test demonstrated a 2.18-fold increase of Iba1+ cells in vehicle-receiving EAE mice compared to control mice (p < 0.05), whereas Nestorone treatment decreased by ~30% the Iba1+ microglial/macrophage cells compared to steroid-naive EAE mice (p < 0.05).

The morphology phenotype of Iba1+ cells was also determined to discern if changes of cell number were accompanied by changes from a resting to an activated microglial stage. As shown in Fig. 5A, the hippocampus from EAE mice (a and b) were characterized by highly ramified Iba1+ cells, whereas those from EAE + Nestorone-treated mice (c and d) changed this profile into a less ramified, bipolar or round phenotype. Fig. 5B shows the % Iba1+ cells divided according to their morphological phenotype into groups 1 and 2, as described in the Materials and Methods section.Steroid naïve EAE mice showed a prevalence of group 2 Iba1+ cells (>4 processes per cell), in contrast to Nestorone-treated mice, in which a higher proportion of Iba1+ cells belonged to group 1 (<3 processes per cell). Statistical comparison using Student’s “t” test, showed that % group 1 cells were significantly higher in EAE + vehicle vs. EAE + Nestorone (p < 0.05), whereas % group 2 cells were significantly higher in EAE + Nestorone vs. EAE + vehicle (p < 0.01). In summary, Nestorone treatment of EAE mice decreased the number of Iba1+ cells, whereas the reactive phenotype of these cells seems considerably attenuated.

3.4. Nestorone treatment increased GABA immunopositive interneurons but did not change GFAP + astrocytes or myelin basic protein (MBP) immunoreactivity in corpus callosum

Excitatory GABAergic input shows a positive regulation of dentate gyrus neurogenesis (Ge et al., 2007; Tozuka et al., 2005). Because progesterone increases GABA + neurons in the hippocampus (Meyer et al., 2013), we investigated if Nestorone modulated the same parameter in chronic EAE mice. Table 1 shows that Nestorone given to EAE mice resembled the progestosterone effect, because the number of GABA + cells was significantly increased in the EAE + Nestorone group compared with steroid naïve-EAE mice (p < 0.05).

Astrocyte activation is a prominent feature of acute EAE. Reactive astrocytes are a source of proinflammatory factors which contribute to EAE impairment (Brambilla et al., 2014). In contrast to previous findings in the spinal cord of mice in the acute phase of EAE, astrogliosis was absent from white matter hippocampus regions (stratum radiatum and stratum oriens), and the hilus of the dentate gyrus of chronic EAE mice (Table 1). Nestorone treatment did not change the astrocyte number in these mice.

Staining for MBP in the corpus callosum of chronic EAE mice was not different from control staining (Table 1), suggesting that remyelination demonstrated significant differences in DCX + cell numbers between the three groups (ANOVA: F(2,18) = 8.23; p < 0.01). In the post-hoc test, EAE mice receiving the vehicle showed a slightly higher DCX + cell number compared to that of controls (p < 0.05), whereas treatment of EAE mice with Nestorone additionally increased the number of cells containing DCX immunostaining (EAE vs. EAE + Nestorone: p < 0.01).
Fig. 2. Effects of chronic EAE and Nestorone on cell proliferation in the dentate gyrus. A: The photomicrographs show Ki67+ cells in the dentate gyrus of control (CTRL), EAE + vehicle (VEH) and EAE + Nestorone (NES) mice. Scale bar: 50 μm. Quantitative assessment of the number of Ki67+ cells (B) shows a non-significant trend for increased cell proliferation in the EAE + vehicle group compared to control mice. Nestorone-treated EAE mice contained a significantly higher number of Ki67+ progenitors in the dentate gyrus compared to CTRL (**p < 0.001) and to EAE + VEH (**p < 0.01).

Fig. 3. Effects of chronic EAE and Nestorone on doublecortin (DCX)+ neuroblasts in the dentate gyrus. A: Microscope images of DCX+ cells in the subgranular zone of the dentate gyrus, in CTRL, EAE + VEH and EAE + NES groups. Image bar: 50 μm. For legends refer to Fig. 2. B: Quantitative assessment of the number of DCX+ cells showed higher number in EAE + VEH vs. CTRL (*p < 0.05). The EAE + NES group showed the highest number of DCX+ cells (**p < 0.01 vs. CTRL and vs. EAE + VEH).
had already occurred 60 days after EAE induction (Table 1). Nestorone had no effect on this parameter.

4. Discussion

Preclinical and clinical evidence support that progesterone represents a good therapeutic option for trauma, ischemia, degeneration, inflammation, neuropathic pain, toxicity and diabetes affecting the central and or peripheral nervous system (Brinton et al., 2008; Schumacher et al., 2014; Stein, 2001; Sayeed and Stein, 2009; Melcangi and Panzica, 2009; De Nicola et al., 2009; Mensah-Nyagan et al., 2009). It has also been recognized that synthetic progestins derived from progesterone, and currently used for contraception or in postmenopausal hormone replacement therapies, bring additional expectations for neuroprotection (Schumacher et al., 2008). Among progestins, Nestorone is a high affinity selective ligand for the progesterone receptor (PR) (Sitruk-Ware et al., 2003; Kumar et al., 2000). Other progestone mediators include membrane progesterone receptors (α, β, γ, δ, and ε mPRs), Sigma receptors, GABAA receptors or the PGRMC1 (progesterone receptor membrane component 1). Progesterone effects on the brain, including neurogenesis, are sometimes mediated by these mediators (Liu et al., 2009; Brinton et al., 2008). However, it is presently undefined if Nestorone interacts with these molecules, and apparently is not metabolized to the PR agonist 5-alpha-dihydroprogesterone or to the GABAA receptor ligand allopregnanolone.

Although the mediators of Nestorone effects in EAE were not identified, a role of the classic PR is suggested, because signaling mechanisms other than PR have not been described for this novel progestin (Liu et al., 2012). The high affinity for PR demonstrated by Nestorone (Sitruk-Ware et al., 2003; Kumar et al., 2000) was associated with the fact that low doses provided neuroprotection, whereas considerably higher doses of progesterone are needed for protection from EAE (Garay et al., 2007, 2008, 2009, 2012). As shown in this paper, 04 mg/kg Nestorone modified clinical grades and motor behavior when given for 10 days after EAE induction in female mice. Clinically, vehicle-receiving EAE mice showed loss of tail tonicity, difficulty in righting and rear limb weakness, whereas most of the Nestorone-treated EAE mice remained in grade 1, showing only a partial or total loss of tail tonicity. Differences in motor function of the two groups were clearly distinguished in the rotarod test, where the EAE mice receiving Nestorone showed enhanced motor performance compared to the vehicle-receiving EAE mice. The rotarod measures balance, coordination and motor learning, tasks in which the cerebellar cortex seems to play a critical role (Kennard and Woodruff-Pak, 2011). In the EAE mice, Nestorone significantly increased physical performance in the rotarod during the 2 week evaluation period. However, Nestorone was given for 10 days after disease development, whereas clinical grade and motor behavior were measured repeatedly for 2 weeks before the mice were killed. Thus, in our model Nestorone effects persisted after treatment was discontinued. In the chronic phase of the disease, the C57BL/6 strain develops a monophasic EAE with a prolonged degenerative period resembling chronic MS. This time period is very important since neurological deficits are directly related to the progress in neurodegeneration. However, since MS is a chronic disease that sometimes shows remission and relapses, the C57BL/6 EAE model may not exactly resemble MS. It should be interesting to assess the effects of Nestorone reintroduction in other mice strains showing remission and exacerbation such as the SJL mouse model of EAE.

Clinical and behavioral changes were accompanied by changes of hippocampus neurogenesis measured by two different markers. Thus,
as reported in our current investigation, vehicle-treated EAE mice (Huehnchen et al., 2011; Giannakopoulou et al., 2013). In chronic EAE, inflammatory effects are not prevented by progesterone if treatment challenges the increased number of Iba1+ cells and modification of these cells from a reactive to a more quiescent form (Kreutzberg, 1996). Since previous reports have shown beneficial effects after administration of ex vivo activated M2 anti-inflammatory macrophages/microglia in EAE (Mikita et al., 2011; Zhang et al., 2014), we speculate that changes in the morphology with Nestorone treatment might be related to a M2 phenotype of microglia. However, specific effects after administration of ex vivo activated M2 anti-inflammatory macrophages/microglia in EAE (Mikita et al., 2011; Zhang et al., 2014), we speculate that changes in the morphology with Nestorone treatment might be related to a M2 phenotype of microglia. However, specific effects after administration of ex vivo activated M2 anti-inflammatory macrophages/microglia in EAE (Mikita et al., 2011; Zhang et al., 2014), we speculate that changes in the morphology with Nestorone treatment might be related to a M2 phenotype of microglia. However, specific effects after administration of ex vivo activated M2 anti-inflammatory macrophages/microglia in EAE (Mikita et al., 2011; Zhang et al., 2014), we speculate that changes in the morphology with Nestorone treatment might be related to a M2 phenotype of microglia. However, specific for cell proliferation we used a Ki67 antibody staining a nuclear protein expressed in all phases of the cell cycle. Ki67 resembles and enhances the expression pattern of BrdU, a marker taken up during the S phase of the cell cycle (Kee et al., 2002). For cells committed to the neuronal lineage, we used DCX staining of neuroblasts. Both markers yielded enhanced neurogenesis after administration of Nestorone. This was not surprising, because hippocampus neurogenesis is a plastic event regulated, among many other factors, by sex steroid hormones (Barha et al., 2011; Gould, 2007; Brinton et al., 2008) and neuroinflammation. Regarding the latter, changes of cell proliferation coupled to reduced newborn cell maturation have been found in mice with neuroinflammation due to acute onset EAE (Pluchino et al., 2008; Guo et al., 2010; Huehnchen et al., 2011; Giannakopoulou et al., 2013). In chronic EAE, newborn cell maturation have been found in mice with neuroinflammation due to acute onset EAE (Pluchino et al., 2008; Guo et al., 2010; Huehnchen et al., 2011; Giannakopoulou et al., 2013). In chronic EAE, as reported in our current investigation, vehicle-treated EAE mice showed slightly improved number of Ki67+ proliferating cells and DCX+ neuroblasts. A further enhancement of cell proliferation and DCX neuroblast staining was achieved in the EAE + Nestorone group. Positive effects of natural progesterone on hippocampal neurogenesis has been shown after brain trauma (Barha et al., 2011), brain ischemia (Zhao et al., 2011), aging (Frye, 2009) in normal adult mice (Zhang et al., 2010), but not in a neurodegeneration model (Meyer et al., 2013). Our data with Nestorone are in agreement with those reports showing a positive regulatory effect. The positive effect on neurogenesis could be ascribed to forced rotarod running of the animals; then, further enhancement of neurogenesis of Nestorone-treated EAE mice could be due to their improved motor behavior. In this regard, long term exercise such as running, maintains brain function and hippocampus neurogenesis (Marlatt et al., 2012), while physical skills also increase the number of surviving cells in the dentate gyrus (Curlik et al., 2013).

In terms of cognitive affection in EAE mice, Ziehn and coworkers have described hippocampal degeneration including loss of CA1 pyramidal layer volume and inhibitory interneurons with a consequent spatial learning impairment in a Barnes maze test (Ziehn et al., 2010). Since Nestorone has shown proneurogenic results in this study, we suggest these positive effects may be reflected by cognitive improvements as well. Behavioral studies will need to address the potential role of Nestorone in cognition in EAE mice.

In addition to physical exercise, other factors such as neurotransmitters may also account for the increased neurogenesis of EAE mice receiving Nestorone. In this regard, GABA plays a crucial role in regulating different steps of neurogenesis. In the adult brain, GABA+ interneurons of the hippocampus increase proliferation of neural progenitors, stimulate migration and differentiation of neuroblasts, and incite synaptic integration of the newborn neurons (Ge et al., 2007; Tozuka et al., 2005). In consonance with the reports on GABA positive regulation of neurogenesis, we found increased number of GABA+ interneurons in the white matter of the hippocampus after Nestorone treatment, suggesting a further control mechanism of hippocampus neurogenesis in chronic EAE mice by the progesterin.

Important players involved in the neuropathology of EAE mice are the microglial/macrophage cells (Jiang et al., 2009; McQualter and Bernard, 2007; Trapp and Nave, 2008). In the present study, staining of these cells with the Iba1 marker showed differences in number and phenotype between control, vehicle-treated and Nestorone-treated mice. Previous results in mice studied 16 days after EAE induction support that progesterone has potent anti-inflammatory effects, blocking the proinflammatory mediator tumor necrosis factor alpha (TNFα) and its receptor TNFR1, the microglial marker CD11b and toll-like receptor 4 (TLR4) mRNAs, and also decreased Iba1+ microglia/macrophages (Garay et al., 2012). Under our experimental protocol, however, anti-inflammatory effects are not prevented by progesterone if treatment is initiated at the time of EAE induction or in mice with clinical signs of EAE. In contrast, Nestorone treatment for 10 days effectively challenged the increased number of Iba1+ cells and modified the phenotype of these cells from a reactive to a more quiescent form (Kreutzberg, 1996). Since previous reports have shown beneficial effects after administration of ex vivo activated M2 anti-inflammatory macrophages/microglia in EAE (Mikita et al., 2011; Zhang et al., 2014), we speculate that changes in the morphology with Nestorone treatment might be related to a M2 phenotype of microglia. However, specific

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EAE + vehicle</th>
<th>EAE + Nestorone</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of GABA + interneurons</td>
<td>51.50 ± 5.06</td>
<td>38.7 ± 2.9</td>
<td>49.2 ± 2.0</td>
<td>*p &lt; 0.05 vs. control and EAE + Nestorone</td>
</tr>
<tr>
<td>MBP-IR corpus callosum</td>
<td>67.2 ± 2.0</td>
<td>54.4 ± 6.6</td>
<td>77.3 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>No. of GFAP+ cells stratum radiatum</td>
<td>183.0 ± 29.6</td>
<td>202.2 ± 22.1</td>
<td>240.5 ± 18.8</td>
<td>NS</td>
</tr>
<tr>
<td>No. of GFAP+ cells stratum oriens</td>
<td>190.7 ± 29.3</td>
<td>215.3 ± 27.9</td>
<td>271.6 ± 26.8</td>
<td>NS</td>
</tr>
<tr>
<td>No. of GFAP+ cells in hilus of dentate gyrus</td>
<td>570.7 ± 89.3</td>
<td>776.9 ± 148.2</td>
<td>800.6 ± 106.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Fig. 5.** A study of the phenotype of Iba1+ microglia/macrophages in the hippocampus of control, and EAE mice with and without Nestorone treatment. A fluorescence microscopy showed mostly ramified Iba1+ microglia/macrophages in mice with chronic EAE (a and b). In contrast, Iba1+ cells showed retracted processes in the EAE + Nestorone-treated mice (c and d). Scale bar: 15 μm. B: Quantitative assessment of % Iba1+ cell phenotype showed increased % group 1 (ameboid-like cells) in EAE + Nestorone mice vs. EAE (**p < 0.05) and decreased % group 2 (ramified cells) in EAE receiving Nestorone (**p < 0.01).
markers should be used together with Iba1 in order to clearly distinguish between the M1 and M2 phenotypes. Therefore, a prominent finding in chronic EAE mice was that early Nestorone treatment contributed to arrest the activation of microglia/macrophages well after therapy cessation. This is a beneficial effect for neurogenesis, because microglial activation and neuroinflammation impairs this process (Monje et al., 2003; Voloboueva and Giffard, 2011; Ekdahl, 2012; Russo et al., 2011). Nestorone may act directly on inflammatory cells, although a synergic effect with exercise should be considered. In this connection, it is known that wheel running enhances survival of new neurons, and promotes a proneurogenic phenotype in aged mice (Kohan et al., 2012). Interestingly, Nestorone-treated EAE mice resembled aging mice: in both cases rotarod running increased cell proliferation and neuroblast staining, and changed the microglial/macrophage phenotype towards a less reactive form.

In chronic EAE, we did not find the reactive astrogliosis typical of acute EAE (Garay et al., 2008, 2012), suggesting that astrogliosis subsided with elapsing time. Reactive astrocytes are a threat to neurons and oligodendrocytes during EAE-induced neuroinflammation ( Brambilla et al., 2014). The absence of astrocyte reaction in chronic EAE mice, suggests that at this stage, astrocytes are not the major contributors to nervous tissue damage. We also did not find evidence of demyelination, according to immunostaining for MBP in the corpus callosum. This finding is in contrast to the demyelination in white matter areas found in the spinal cord of acute EAE mice ( Garay et al., 2008), suggesting that remyelination in the brain already occurred 2 months after EAE induction.

In conclusion, our investigation revealed beneficial effects of Nestorone in chronic EAE. However, taking into account that the incidence and neuropathology of EAE show gender differences ( Voskuhl et al., 1996; Massella et al., 2012), the above mentioned results may be circumscribed to the females with EAE. It is not known if the study parameters (dosing, duration of treatment and outcomes) would be the same for males as for females. Thus, in female C57Bl/6 mice Nestorone decreased clinical grade and enhanced motor behavior. In addition, it increased cell proliferation and doublecortin positive neuroblasts in the hippocampus dentate gyrus, increased GABA positive interneurons and decreased the number of Iba1 + microglial/macrophagic cells, events possibly linked to enhancement of neurogenesis. Therefore, Nestorone protected against hippocampus abnormalities and also improved functional outcomes of female mice with chronic EAE, suggesting its potential value for chronic autoimmune diseases.

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