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Pharmacotherapy with 17β-estradiol and progesterone prevents development of mouse experimental autoimmune encephalomyelitis

Laura Garay[1,2], Maria Claudia Gonzalez Deniselle[1,2], Lobke Gierman[3], Analia Lima[1], Paulina Roig[1] and Alejandro F. De Nicola[1,2,4,*]

[1] Laboratory of Neuroendocrine Biochemistry, Instituto de Biologia y Medicina Experimental, Buenos Aires, Argentina
[2] Department of Human Biochemistry, Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina
[3] Biomedical Sciences, Radboud University, Nijmejen, The Netherlands
[4] Instituto Universitario de Ciencias de la Salud, Fundación Barceló, Buenos Aires, Argentina

Abstract

Background: Pregnant women with multiple sclerosis (MS) show disease remission in the third trimester concomitant with high circulating levels of sex steroids. Rodent experimental autoimmune encephalomyelitis (EAE) is an accepted model for MS. Previous studies have shown that monotherapy with estrogens or progesterone exert beneficial effects on EAE. The aim of the present study was to determine if estrogen and progesterone cotherapy of C57BL/6 female mice provided substantial protection from EAE.
Methods: A group of mice received single pellets of progesterone (100 mg) and 17 β-estradiol (2.5 mg) subcutaneously 1 week before EAE induction, whereas another group were untreated before EAE induction. On day 16 we compared the two EAE groups and control mice in terms of clinical scores, spinal cord demyelination, expression of myelin basic protein and proteolipid protein, macrophage cell infiltration, neuronal expression of brain-derived neurotrophic factor mRNA and protein, and the number of glial fribrillary acidic protein (GFAP)-immunopositive astrocytes.
Results: Clinical signs of EAE were substantially attenuated by estrogen and progesterone treatment. Steroid cotherapy prevented spinal cord demyelination, infiltration of inflammatory cells and GFAP$^+$ astrogliocytes to a great extent. In motoneurons, expression of BDNF mRNA and protein was highly stimulated, indicating concomitant beneficial effects of the steroid on neuronal and glial cells.
Conclusions: Cotherapy with estrogen and progesterone inhibits the development of major neurochemical abnormalities and clinical signs of EAE. We suggest that a combination of neuroprotective, promyelinating and immunosuppressive mechanisms are involved in these beneficial effects.

Keywords: estradiol; experimental autoimmune encephalomyelitis; myelination; neuroprotection; progesterone.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease that affects the central nervous system. MS is most frequently diagnosed during early adulthood and has a gender bias towards females (approx. 2:1) (1). Although MS presents in several forms, in approximately 80% of cases it shows a relapsing-remitting course. Importantly, it is widely recognized that a significant decline in the rate of MS relapse occurs during the third trimester of pregnancy, whereas a significant increase develops during the 3 months post partum (2–5). The absence of MS relapses during pregnancy is believed to be due to the protective and anti-inflammatory effects of sex steroid hormones circulating in pregnant women (3, 6). Therefore, the potential therapeutic benefit of sex steroid hormones for MS patients has been taken into consideration (7).

A common model used to study MS is induced experimental autoimmune encephalomyelitis (EAE) in rodents (8, 9). Neuropathology of EAE spinal cord includes inflammatory cell infiltration, demyelination with oligodendrocyte loss, microglial activation, axonal loss, astrocytosis and neuronal dysfunction (10–14). Clinical correlates of spinal cord pathology include loss of tail tonicity, limb paralysis and even death (9, 10, 15).

*Corresponding author: Dr. Alejandro F. De Nicola, Laboratory of Neuroendocrine Biochemistry, Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires, Argentina
Phone: +54-11-47832869, Fax: +54-11-47862564,
E-mail: denicola@dna.uba.ar
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A number of treatments have been used to prevent the development or halt the progression of EAE. Steroids have been used because they show immunosuppressive, neuroprotective and promyelinating effects in diseases of the nervous system involving different etiopathogenic factors (16–18). Estrogens have been the most widely studied compounds for EAE treatment. When given before EAE induction, estrogens decrease the clinical manifestations and neurochemical abnormalities of the spinal cord (19–23). Progesterone has mixed effects on EAE, ranging from a lack of activity to increased vulnerability to disease improvement (13, 14, 24). However, the synthetic progestin medroxyprogesterone has clear protective effects in EAE (25). Although the effects of combined estrogen and progesterone treatment have not been studied in EAE models, this approach led to greater therapeutic improvement over single therapy in cuprizone-induced demyelination and experimental autoimmune rheumatoid arthritis (26–28).

Therefore, based on the hypothetical role that a high level of sex steroids (estrogens and progesterone) could play in remission of MS in pregnant women and pregnant mice (3, 4, 29), experiments were designed to clarify if estrogen and progesterone cotherapy of C57BL/6 female mice provides substantial protection from induced EAE. To this end, clinical signs, expression of myelin proteins, inflammatory cell infiltration, astrogliosis and neurotrophic factor expression were compared between steroid-treated and untreated EAE mice.

Materials and methods

For steroid treatment, C57BL/6 female mice (9–11 weeks old) received two subcutaneous pellets 1 week before EAE induction, the first containing 100 mg of progesterone (PROG) and a second 2.5 mg of 17β-estradiol (E2) (Sigma, St. Louis, MO, USA). EAE mice without steroid treatment received equivalent cholesterol pellets. EAE was induced using a peptide corresponding to the published sequence of rodent myelin oligodendrocyte glycoprotein MOG_{40-54} (YRSPFSRVVHLYRNG) (9). This peptide was synthesized by Dr. Clara Peña (Faculty of Pharmacy and Biochemistry, University of Buenos Aires). Experimental mice received a subcutaneous injection on each flank of 200 μg of MOG_{40-54} emulsified in complete Freund's adjuvant (CFA) (Sigma) containing 0.6 mg of *Mycobacterium tuberculosis* (Instituto Malbran, Argentina). The animals received intraperitoneal injections of *Pertussis* toxin (400 ng; Sigma) immediately after immunization and another boost on the day after. Some animals received CFA and *Pertussis* toxin without MOG, but none of them developed 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 (9, 13): 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 in hormone-free animals developed on approximately day 10 and they were sacrificed on day 16 when the disease was still in an acute phase. 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.

Infiltration area and total myelin

Animals were deeply anesthetized with ketamine (50 mg/100 g body weight) and transcardially perfused with 0.9% NaCl prepared in diethylpirocarbonate-treated water, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Cervical and lumbar spinal cords were carefully removed, postfixed in the same fixative for 2.5 h at 4°C and processed for routine paraffin embedding. Other batches of spinal cords were cryoprotected by immersion in 20% sucrose overnight and kept frozen at −70°C until use. Paraffin sections were stained with hematoxylin and eosin (H&E) to assess immune cell infiltration and with luxol fast blue (LFB) to mark the area of demyelination. LFB staining was carried out according to Kim et al. (19). The LFB-negative area, representing white matter demyelination, was determined and expressed as described below for myelin basic protein (MBP) and proteolipid protein (PLP) immunostaining. The area of infiltrated immune cells was determined as a percentage of the total white matter area (mean ± SEM for 10 EAE mice and 8 EAE + E_2 + PROG).

Myelin immunohistochemistry

Sections (5 μm) of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Immunocytochemistry was carried out as previously described (13) using a 1:500 dilution of rabbit anti-MBP primary antibody (Cytomation; Dako, Glostrup, Denmark) or a 1:100 dilution of the AA3 rat anti-PLP antibody (kindly provided by Dr. S.R. Winkler, Department of Neuroscience, University of Connecticut School of Medicine, Farmington, CT, USA). Focal areas showing negative immunostaining for MPB or PLP were delimited at the white matter by computerized image analysis using Optimas VI software (30, 31). The surface area of these regions was added up and demyelination was expressed as a percentage of the total surface area of white matter sampled, as previously reported (12, 13, 32). These experiments comprised three spinal cord sections per animal for 8–9 animals per group.

In situ hybridization for BDNF mRNA

Cryostat sections of the spinal cord were fixed in 2% paraformaldehyde, washed in 0.5× sodium citrate/sodium chloride buffer (SCC; 1× SCC, 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), dried and acetylated with acetic anhydride. To detect BDNF mRNA, we used a 50-mer synthetic oligonucleotide probe (Oligos Etc, Wilsonville, OR, USA) complementary to bp 650–699 of the coding region of

mouse BDNF Exon VIII (5′-AGT TCC AGT GCC TTT TGT CTA TGC CCC TGC AGC CTT CCT TGG TGT AAC CC-3′) (33). The probe was end labeled with (^{35}S)dATP using terminal transferase (Boehringer-Mannheim, Mannheim, Germany). Hybridization was carried out using 10^6 c.p.m. of ^{35}S-labeled probe in 100 μL of hybridization cocktail containing: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50% formamide, 3× SCC buffer, 10 mM dithiothreitol, 0.1 mg/mL salmon sperm DNA, 1 mM EDTA, 4 μg/mL heparin, 0.4 mg/mL tRNA and 10% dextran sulfate. After overnight hybridization at 42°C, sections were washed several times in SCC, dried, dipped into Kodak NTB-2 emulsion and exposed in the dark for 4 days. Sections were then developed with Dektol (Kodak, 1:2 dilution with water), fixed in Ektaflo fixer, counterstained with cresyl violet and mounted in Permount.

For semiquantitative evaluation of BDNF mRNA, the number of silver grains per cell was determined over motoneurons from the spinal cord ventral horn showing a clear nuclear profile. Grain counting was performed by computer-assisted image analysis (Optimas VI; Bioscan, Edmonton, WA, USA) and calculated after background subtraction (31). Results are expressed as the mean±SEM grain density (number of grains per unit area of soma, grains/μm^2). Data for 10 neurons per animal (for 5 control, 10 EAE and 8 EAE+E_2+PROG) were combined to obtain a mean value per animal, with animals used as independent variables. Images were acquired at the same magnification using a Panasonic GP-KR222 digital camera connected to an Olympus BH2 microscope and the image analysis software Optimas VI.

Detection of BDNF protein by immunofluorescence

For BDNF immunoflourescence, 30-μm cryostat sections were preincubated with 10% goat serum for 10 min at 37°C, followed by overnight incubation at 4°C with a 1:1000 dilution of the BDNF polyclonal antibody (Chemicon International, Temecula, CA, USA) prepared in 2% goat serum, 1% Triton-X100 in phosphate-buffered saline. After washing, sections were incubated with a 1:200 dilution of a goat-anti rabbit IgG coupled to fluorescein isothiocyanate in 2% goat serum, 1% Triton X-100 for 1 h at room temperature. Sections were washed, mounted with Vectashield (Vector Labs, Burlingame, CA, USA) and examined under a Nikon Eclipse E 800 confocal scanning laser microscope equipped with Nikon 11691 photographic equipment. Photographs were taken at magnification of 200×. Confocal images were saved and further analyzed by computerized image analysis using Optimas VI software. The number of neurons/mm^2 showing BDNF immunofluorescence (mean±SEM) was statistically compared in 8–10 animals per group.

GFAP$^+$ astrocytes

For GFAP immunostaining (34), spinal cords were post-fixed in 4% paraformaldehyde and embedded in paraffin and 5-μm-thick sections were treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidases. Sections were then incubated overnight at 4°C with a 1:250 dilution of the primary GFAP rabbit polyclonal antibody (G-9269, Sigma). Sections were exposed to a goat anti-rabbit secondary antibody (1:200 dilution, 60 min) and further processed according to the ABC kit instructions (Vector Labs). In all immunoreactions, the peroxidase activity was determined using diaminobenzidine tetrachloride (0.25 mg/mL, Sigma) as substrate in the presence of 0.01% H_2O_2 for 7 min in the dark.

An Optimas VI image analysis system equipped with a Panasonic GP-KR222 video camera was used for quantitative analysis. Digitized images of tissue sections containing the spinal cord were displayed on the video screen under identical lighting conditions. Using this program we set a threshold for positive cell area and nucleated cells exhibiting GFAP labeling were considered within these limits (34). Data are expressed as the mean±SEM number of labeled cells per unit area of gray matter. Cell counting was carried out for at least four anterior horns per animal (5 control, 10 EAE and 8 EAE+E_2+PROG).

Radioimmunoassay of steroid levels in serum

Serum 17β-estradiol and progestin levels were determined at the time of sacrifice using a Coat-A-Count Progesterone RIA Kit (Diagnostic Product Corporation, Los Angeles, CA, USA). Intra- and interassay coefficients of variation were 3.6% and 5.6%, respectively.

Statistical analysis

Differences in peak disease score were analyzed by Student's t-test according to Bebo et al. (23) and Palaszynski et al. (35), who used a similar statistical method for comparison. Significance was set at $p<0.05$. Group differences for LFB staining, MBP and PLP immunostaining and in situ hybridization for BDNF mRNA and protein were determined by one-way ANOVA, followed by post hoc comparisons with the Newman-Keuls test.

Results

Clinical scores for EAE mice were considerably modified by steroid treatment. Although disease onset was not different between EAE (10.8±1.4 days) and EAE+E_2+PROG mice (10.5±1.5 days), maximum clinical scores were 2.9-fold higher for EAE mice (2.17±0.31 vs. 0.75±0.25; $p<0.05$). These lower clinical scores were positively correlated to higher circulating steroid levels. Serum progesterone levels in EAE+E_2+PROG mice were 67.4±12.8 ng/mL (n=7) compared to 12.9±2.1 ng/mL (n=4) in EAE mice, whereas estradiol levels were 937±134 pg/mL (n=7) and 9.1±2.9 pg/mL, respectively. The progesterone levels in steroid-treated EAE mice are similar to those in pregnant mouse serum, whereas the estradiol levels are pharmacological (36, 37).

The area covered by F4/80-immunopositive cells, i.e., cells belonging to a macrophage/microglia lineage, amounted to 14±3.7% of the total white matter area (n=9 mice); estradiol and progesterone cotreatment decreased this area to 0.94±0.39% ($p<0.001$). These data indicate a strong immunosuppressive

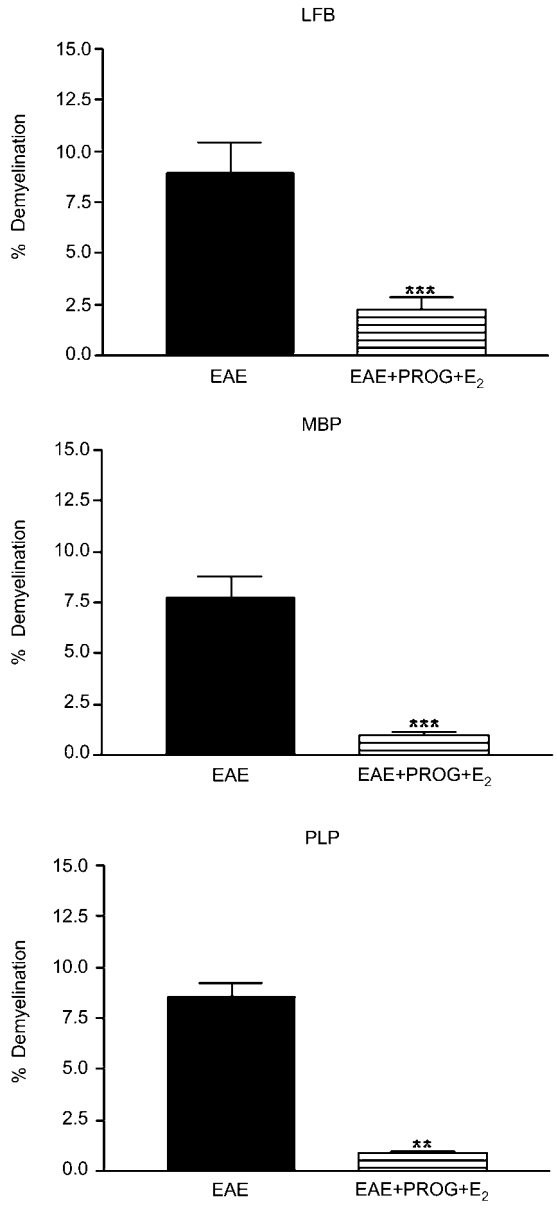

Figure 1 Quantitative data for demyelination as a percentage of total white matter area according to luxol fast blue staining (LFB, upper graph) and immunoreaction for myelin basic protein (MBP, middle graph) and proteolipid protein (PLP, lower graph).
The three graphs show data for EAE (dark columns) and EAE + E_2 + PROG (horizontal line columns) groups. **$p < 0.01$ vs. EAE; ***$p < 0.001$ vs. EAE. Results represent the mean ± SEM for three spinal cord sections per animal for a total of 8–9 animals per group.

effect of steroid cotreatment in the spinal cord of EAE mice.

Induction of EAE produced focal demyelination of the spinal cord, as previously shown (13, 14). Figure 1 shows demyelination as a percentage of total white matter area as measured by LFB staining and immunohistochemistry for the two central myelin proteins MBP and PLP. Demyelination amounted to nearly 10% for total myelin (LFB staining) and was slightly lower using specific MBP or PLP immunostaining (7.6% and 8.5%, respectively). Estradiol and progesterone cotreatment led to a dramatic decrease in EAE-induced demyelination ($p < 0.001$ for LFB and MBP and $p < 0.01$ for PLP, Figure 1).

Because spinal cord astrocytosis follows EAE induction (38, 39), we determined the number of $GFAP^+$ astrocytes in the gray matter of EAE mice with or without steroid cotreatment. As shown in Figure 2, the number of reactive astrocytes was ~500 /mm^2 in EAE gray matter, substantially higher than for control mice (~130 /mm^2) and EAE + E_2 + PROG mice (180 /mm^2). Estradiol and progesterone cotreatment led to a similar astrocyte number in control and EAE + E_2 + PROG mice ($p > 0.05$).

We next examined the effect of EAE and steroid cotreatment on neuronal expression of BDNF, a neurotrophin involved in spinal cord regeneration, recovery of motor function and modulation of neuronal excitability (40–42). The morphological appearance of the probe hydridized to BDNF mRNA is shown in Figure 3 (upper microphotograph). Bright-field microphotographs of individual motoneurons obtained at high magnification clearly indicated lower BDNF mRNA expression in EAE mice compared to control and EAE + E_2 + PROG mice. Quantitative analysis of the number of grains representing probe hybridized to mouse BDNF mRNA is shown in Figure 3 (lower graph). BDNF mRNA expression in motoneurons from

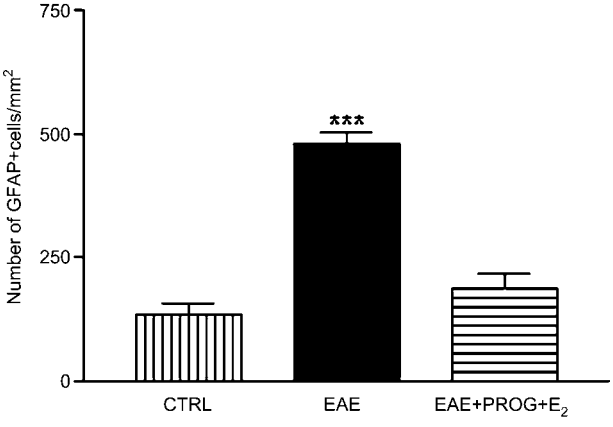

Figure 2 Number of GFAP-immunopositive astrocytes in the anterior horn of the spinal cord for control C57BL/6 female mice (CTL, vertical line column) and EAE (dark column) and EAE + E_2 + PROG mice (horizontal line column).
***EAE group significantly different from control and EAE + E_2 + PROG groups (both $p < 0.001$). Control vs. EAE + E_2 + PROG, no significant difference ($p > 0.05$). Results represent the mean ± SEM number of labeled cells per unit area. Cell counting was carried out for four anterior horns per animal (5 control, 10 EAE and 8 EAE + E_2 + PROG mice).

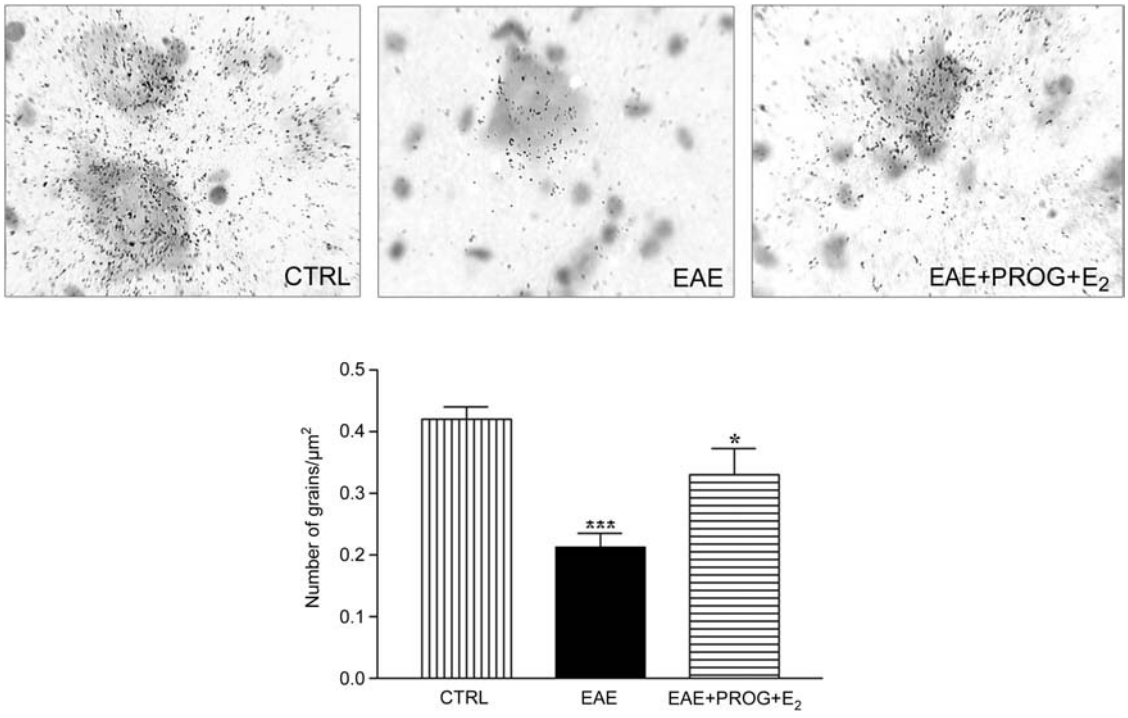

Figure 3 In situ hybridization for BDNF mRNA.
The upper bright-field photomicrographs show abundant grains over motoneurons in a control mouse (CTRL) and grain depletion in EAE and EAE+E2+P mice. Grain counting (lower graph) confirmed that BDNF mRNA was lower in EAE vs. control (***$p<0.001$) and EAE vs. EAE+E_2+PROG (*$p<0.05$) but not between CTRL and EAE+E_2+PROG. Data were obtained for 10 neurons per animal for control ($n=5$), EAE ($n=10$) and EAE+E_2+PROG ($n=8$) mice.

EAE mice was half of that in control mice ($p<0.001$) and was significantly higher in EAE+E_2+PROG compared to EAE mice ($p<0.05$) but not compared to control animals ($p>0.05$).

In the next step we investigated if steroid pretreatment modulated BDNF protein as well as mRNA levels. Because of a technical problem, data for control mice were lost; thus, only motoneurons from EAE and EAE+E_2+PROG mice were available for BDNF immunofluorescence analysis. The upper photomicrograph in Figure 4 shows marked differences in BDNF immunofluorescent neurons between the groups and quantitative analysis of BDNF protein revealed significant differences (Figure 4, upper graph). Thus, the BDNF-immunopositive neuronal density, which was 42.4 ± 9.4 cells/mm^2 in EAE mice, increased two-fold on estradiol and progesterone cotreatment (86.4 ± 16.7 cells/mm^2; $p<0.05$).

Discussion

The present data conclusively demonstrate that combined treatment with estradiol and progesterone modified the course of MOG_{40-45}-induced EAE in female C57BL/6 mice. Combined steroid exposure not only prevented major spinal cord neuropathology, but also strongly reduced the clinical signs of EAE. Our treatment paradigm followed previously used steroid pretreatments resulting in reduction of clinical signs, neuroprotection, increased myelination and diminished immune responses of EAE mice receiving estrogens or progesterone singly (13, 19, 21–23). The question remains as to whether combined estrogen and progesterone treatment after disease onset results in comparable, lower or higher effectiveness.

Regarding EAE clinical evolution, EAE+E_2+PROG mice reached grade 1 only, with partial loss of tail tonicity, in contrast to EAE mice, for which grade 2 or higher clinical scores were observed. EAE mice showed complete loss of tail tonicity, difficulty in righting and some degree of limb paralysis on day 16 after immunization. To analyze the neurochemical changes underlying the abnormal clinical signs of EAE and the benefits of combined estrogen and progesterone therapy, we carried out histochemical and immunohistochemical staining to reveal myelin status in the spinal cord. In agreement with previous reports (13, 14), staining for total myelin and immunostaining for PLP and MBP revealed larger focal areas of demyelination in EAE compared to EAE+E_2+PROG. Dominant foci of demyelination

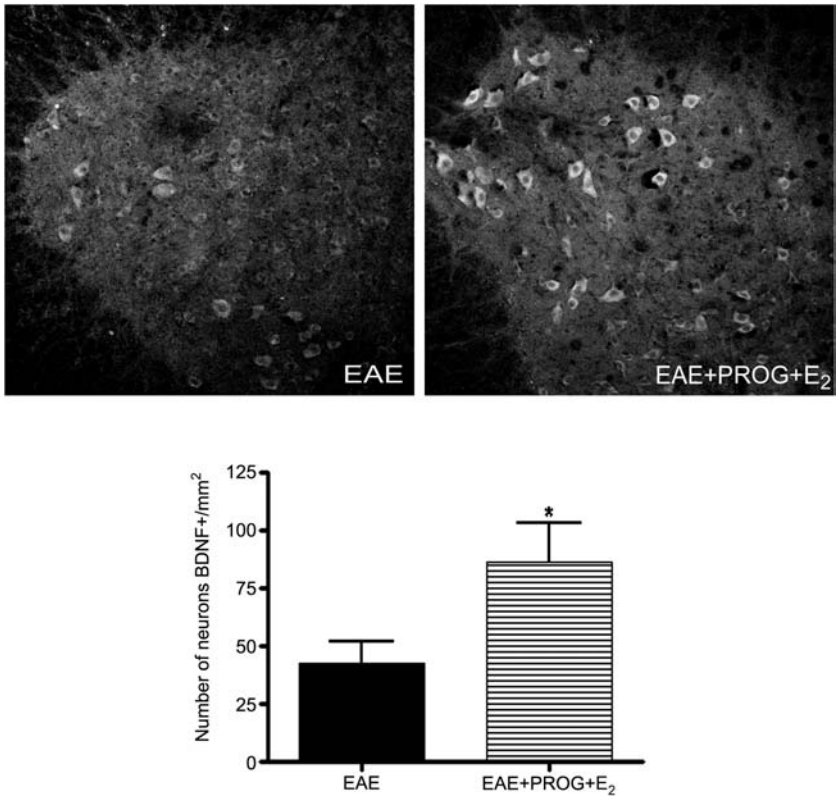

Figure 4 BDNF immunofluorescence
Upper graph: BDNF immunofluorescence images showing BDNF-immunopositive neurons in the right anterior horn of a representative spinal cord section from an EAE mouse (left-hand graph) and an EAE + E_2 + PROG mouse (right-hand graph). The number of fluorescent motoneurons is substantially higher in the steroid-treated mouse. Lower graph: Quantitation of the number of $BDNF^+$ immunofluorescent motoneurons/mm^2 ventral horn above an established intensity threshold. The number of neurons was significantly higher in EAE + E_2 + PROG than in EAE mice (*$p < 0.05$; $n = 8–10$ mice per group).

were more frequently observed in the ventral and dorsal funiculus. Clearly, demyelination of the corticospinal tract with the dorsal funiculus and motor axons within ventral funiculus (43) can explain motor impairment and paralysis.

The higher degree of myelin protein staining found in EAE + E_2 + PROG mice suggests that estradiol and progesterone either induced remyelination or prevented demyelination of the dorsal and ventral funiculus. Evidence from other animal models has conclusively demonstrated that both progesterone and estradiol are promyelinating factors for diseases of the central and peripheral nervous system, including EAE (16–18). Although progesterone can target mature oligodendrocytes, it preferentially stimulates proliferation of oligodendrocyte precursors (44). In oligodendroglial cell cultures the presence of progesterone receptors in oligodendrocytes and enhancement of MBP-positive cells after progesterone treatment suggest direct effects on this cell type (44, 45). Estrogens protect oligodendrocytes from cytotoxicity (46) and after spinal cord injury they exert antioxidant and anti-inflammatory effects that maintain myelin (47). The estrogen mechanisms leading to increased myelination may directly affect oligodendrocytes, because these cells can express nuclear and membrane forms of the estrogen receptor (48, 49).

In addition to demyelination, EAE mice showed infiltration of cells of a predominant macrophage/microglial lineage according to F4/80 immunocytochemical staining and confirmed by HE staining. This infiltration was significantly reduced in EAE + E_2 + PROG mice, indicating marked attenuation of the immune response. The immune-related changes in EAE suggest a strong pro-inflammatory Th1 response in EAE mice. Although Th1 responses are involved in cellular immunity, graft rejection, delayed hypersensitivity and production of the pro-inflammatory cytokines IL-2, IL-12, IFN-γ and TNF-α, Th2 responses are involved in humoral immunity and production of the anti-inflammatory cytokines IL-10 and TGF-β (4, 50). Regarding the effect of steroid hormones on these processes, estradiol doses typical of pregnancy levels significantly enhance IL-10 secretion from PLP-specific T-cell clones isolated from MS patients. In vitro effects of estradiol and progesterone on T-cell clone

stimulation include Th2 shifts in cytokine secretion (51, 52). Although cytokine parameters were not determined in the present experiments, existing data suggest a similarity in the effects of pregnancy, estradiol and progesterone, since all change a Th1 proinflammatory response to a Th2 anti-inflammatory response, explaining the decrease in inflammatory cells in the spinal cord of EAE + E_2 + PROG compared to EAE mice.

The observation of reactive astrocytes showing strong GFAP immunostaining also indicates that inflammatory factors play a role in the spinal cord of EAE mice. Reactive astroglia accumulate in MS lesions and are a prominent feature of the EAE spinal cord (38). Under normal conditions, astrocytes play a protective role and maintain the neurometabolic coupling necessary for neuronal function (53). In EAE, however, it has been shown that astrocytes (as well as microglia) are a source of TNF-α, other proinflammatory cytokines and excess nitric oxide (54). These factors damage oligodendrocytes and neurons and impair migration of oligodendrocyte precursors into areas of demyelination (54). The astroglial response might be secondary to neuronal suffering, since neuronal abnormalities are a constant feature of MS and EAE. Because estradiol and progesterone protect neurons from a variety of insults, including those of a traumatic, degenerative, oxidative or inflammatory nature (17, 18), inhibition of $GFAP^+$ astrocyte numbers can be ascribed in part to maintenance of neuronal function. In EAE rodents, estrogen or progesterone neuroprotection is widely evidenced by changes in expression of BDNF, as discussed below, and other markers, including increases in Na,K-ATPase expression and preservation of axonal numbers and neuronal integrity (13, 55).

Our study also demonstrated a steroid effect on BDNF mRNA and protein expression. BDNF is a protective and trophic factor for the spinal cord, and several studies have shown that BDNF administration rescues injured or degenerating neurons and induces axonal outgrowth. BDNF has beneficial effects in neurodegenerative diseases (56). Increased BDNF mRNA was observed in motor neurons, and immunofluorescence assays revealed a significant increase in the amount of $BDNF^+$ neurons in EAE + E_2 + PROG compared to EAE mice. Recovery of BDNF indicates a healthier motoneuron status in steroid-treated EAE mice. The effects of estradiol and progesterone on BDNF could be due to transcriptional mechanisms. Detection of intracellular progesterone receptors (PRs) in the spinal cord suggests a role for classical receptor-mediated events (16, 17). For estrogens, hormone response elements are present in the BDNF promoter (57) and probably drive positive regulation of BDNF gene transcription under estrogen influence, as reported here for EAE mice. A PR responsive element has not been yet reported. We have hypothesized that in a permissive environment imposed by injury or neurodegeneration, progesterone increases BDNF expression in the spinal cord (58, 59). A tentative hypothesis is that estrogen and progesterone enhancement of endogenous neuronal BDNF could provide a trophic environment within the spinal cord and might be part of the steroid-activated pathways that provide neuroprotection.

Several reports have dealt with the effects of sex steroids in EAE. For example, single therapy with estradiol offers protection against development of EAE when given prior to disease induction, an effect involving estrogen receptor alpha (ERα) (22). The involvement of ER isoforms in EAE was further elucidated using ER-selective ligands and ERα and ERβ selective agonists had differential effects on EAE development (60). Both compounds were tested prior to disease induction. The synthetic estrogen ethynyl estradiol and fluasterone (a synthetic androstene derivative with estrogen-like properties) are also effective when given at the onset of EAE. Bebo et al. (23) showed that low-dose treatment with estradiol producing diestrous circulating levels can significantly reduce clinical manifestations of EAE in male and female mice. Estriol, an estrogen metabolite exhibiting low estrogenic potency, ameliorates EAE in mice provided serum estriol levels similar to those in late pregnancy are achieved (23). It is possible that estrogen effects in EAE are due to their potent immunomodulatory properties, but, as already discussed, estrogen might also have direct effects on oligodendrocytes (48, 49).

The effects reported for single treatment with progesterone in EAE are more variable than those found for estrogen. Although one study found no positive effect of progesterone (24), we found that pretreatment with progesterone before EAE induction – producing circulating progesterone levels within the range observed in mouse pregnancy – attenuated clinical signs of EAE and mice exhibited less pronounced motor impairment, lower levels of inflammatory cell infiltration and restoration of myelin protein expression (13, 14). The effects of progesterone on myelin in EAE are in accordance with its promyelinating actions in diseases of the peripheral and central nervous system (16–18). Therefore, estrogen and progesterone should be combined to maintain myelination in EAE mice, a possibility of interest to MS patients, who suffer from a primary demyelinating disease. Therapeutic improvement on combined estrogen and progesterone treatment may not be exclusive to EAE. It has recently been shown that greater beneficial effects are achieved for combined steroid therapy in mice with cuprizone-induced demy-

elination (26). Moreover, in collagen-induced arthritis, an immune-mediated disease, cotreatment with estrogen and progesterone in physiological doses induced more pronounced suppression of experimental arthritis than that for estradiol treatment alone (27, 28). Thus, it is possible that pharmacotherapy with estradiol and progesterone has therapeutic advantages for immune-mediated diseases.

Interestingly, dual steroid treatment is used in a randomized, placebo-controlled and double-blind clinical trial for MS being carried out in Europe at present. The aim of the Popart-Mus trial is to prevent MS relapses related to the post partum condition by administering high doses of progestin in combination with estradiol (2, 3, 6). In addition to the beneficial effect both hormones may have on spinal cord remyelination, neuroprotection and immunosuppression, progestin addition can avoid the undesirable side effects caused by monotherapy with estradiol.

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