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Effects of Progesterone on Oligodendrocyte Progenitors, Oligodendrocyte Transcription Factors, and Myelin Proteins Following Spinal Cord Injury

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KEY WORDS
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ABSTRACT
Progesterone is emerging as a myelinizing factor for central nervous system injury. Successful remyelination requires proliferation and differentiation of oligodendrocyte precursor cells (OPC) into myelinating oligodendrocytes, but this process is incomplete following injury. To study progesterone actions on remyelination, we administered progesterone (16 mg/kg/day) to rats with complete spinal cord injury. Rats were euthanized 3 or 21 days after steroid treatment. Short progesterone treatment (a) increased the number of OPC without effect on the injury-induced reduction of mature oligodendrocytes, (b) increased mRNA and protein expression for the myelin basic protein (MBP) without effects on proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG), and (c) increased the mRNA for Olig2 and Nkx2.2 transcription factors involved in specification and differentiation of the oligodendrocyte lineage. Furthermore, long progesterone treatment (a) reduced OPC with a concomitant increase of oligodendrocytes; (b) promoted differentiation of cells that incorporated bromodeoxyuridine, early after injury, into mature oligodendrocytes; (c) increased mRNA and protein expression of PLP without effects on MBP or MOG; and (d) increased mRNA for the Olig1 transcription factor involved in myelin repair. These results suggest that early progesterone treatment enhanced the density of OPC and induced their differentiation into mature oligodendrocytes by increasing the expression of Olig2 and Nkx2.2. Twenty-one days after injury, progesterone favors remyelination by increasing Olig1 (involved in repair of demyelinated lesions), PLP expression, and enhancing oligodendrocytes maturation. Thus, progesterone effects on oligodendrogenesis and myelin proteins may constitute fundamental steps for repairing traumatic injury inflicted to the spinal cord.

INTRODUCTION

Spinal cord injury (SCI) affects all cell types populating the spinal cord. Ventral horn motoneurons show early degeneration and chromatolysis (Gonzalez et al., 2005; Nacimiento et al., 1995; Price and Porter, 1972), with death occurring by necrosis or apoptosis depending on the severity of the lesion. Astroglial cells become activated, with increased expression of the intermediate filament glial fibrillary acidic protein (GFAP). Oligodendrocytes show apoptotic changes and axons are markedly demyelinated (Grossman et al., 2001; Li et al., 1999). Lastly, microglia becomes activated, increasing their mobility, secretion of cytokines, and mounting an inflammatory response (Bareyre and Schwab, 2003). Responsible etiopathogenic mechanisms include glutamate excitotoxicity, increased calcium entry, release of reactive oxygen species with oxidative stress, activation of proteases, membrane lipid peroxidation, inhibition of the mitochondrial respiratory chain, and excess production of nitric oxide (Beattie et al., 2000).

Mature oligodendrocytes are the source of myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (Baumann and Pham-Dinh, 2001). Demyelination due to oligodendrocyte loss follows spinal cord contusion or transection (Grossman et al., 2001; Siegenthaler et al., 2007; Zai and Wrathall, 2005). However, mature oligodendrocytes cannot repair myelin after injury-induced demyelination, and remyelination derives from recruitment of an endogenous population of oligodendrocyte precursor cells (OPC) (Levine et al., 2001; McTigue et al., 2001; Rosenberg et al., 2005; Zai and Wrathall, 2005). Whereas some authors sustain that successful remyelination after SCI is aborted if proliferated OPC cannot convert into myelin-producing cells, others have demonstrated partial remyelination (Horky et al., 2006; Ishii et al., 2001; Rabchevsky et al., 2007; Siegenthaler et al., 2007; Suyama et al., 2007; Zai and Wrathall, 2005). The oligodendrocyte lineage traverses several steps, from OPC expressing the proteoglycan NG2, to mature oligodendrocytes that express the MBP, PLP, and MOG genes

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and their product proteins. Regulation of the oligodendrocyte pathway is multifactorial, and the transcription factors Olig1, Olig2, and Nkx2.2 play specific roles during OPC differentiation and remyelination (Fancy et al., 2004; Ligon et al., 2006; Lin et al., 2006; Qi et al., 2001). Therefore, analysis of agents influencing remyelination would require the study of their effects on OPC proliferation, expression of myelin genes, and transcription factors that control oligodendrogenesis (Nicolay et al., 2007).

Progesterone (PROG) is a recognized promyelinating and neuroprotective factor (Schumacher et al., 2007). In the PNS, PROG promotes synthesis of the myelin proteins Po and PMP22 and increases the transcription factors Krox-20 and Sox-10 by Schwann cells (Desarnaud et al., 1998; Guennoun et al., 2001; Koenig et al., 1995; Magnaghi et al., 2006, 2007). PROG and its metabolites also prevent myelin alterations of diabetic neuropathy and maintain integrity of peripheral nerves during neurodegeneration and aging (Azcoitia et al., 2003; Leonelli et al., 2006; Veiga et al., 2006). In the CNS, PROG increases the expression of MBP and the mature oligodendrocyte marker cyclic nucleotide 3′-phosphodieterase (CNPase) in cultures of mixed glial cells and organotypic slice cultures of cerebellum (Ghoumari et al., 2003; Jung-Testas et al., 1996); besides, PROG induces remyelination after toxin-induced lesions of the cerebellar peduncle of aging rats (Ibanez et al., 2004). Additionally, a modulatory effect of endogenous PROG and their reduced derivatives on the oligodendrocyte cell lineage has been already demonstrated under *in vitro* conditions, suggesting that PROG may contribute to OPC proliferation and differentiation (Gago et al., 2004; Ghoumari et al., 2005).

The spinal cord is a suitable target to study PROG effects on myelination. Classical as well as membrane receptors for PROG exist in neurons, and possibly oligodendrocytes (Labombarda et al., 2000a, 2003). After SCI in rats, PROG treatment for 3 days enhances the expression of MBP protein and mRNA, highly stimulates the number of NG2+ cells, and stimulates several neuronal parameters (Gonzalez et al., 2004; Labombarda et al., 2002, 2006a). The present work demonstrated that short PROG treatment influenced proliferation and differentiation of OPC into mature oligodendrocytes by increasing the expression of transcription factors directed toward differentiation (Olig2 and Nkx2.2). Prolonged PROG treatment for 21 days postinjury increased Olig1, involved in repair of demyelinated lesions, stimulated oligodendrocyte maturation and upregulated the major central myelin protein PLP. These effects of PROG on myelin parameters may contribute to repairing the spinal cord from the severe damage inflicted by trauma.

MATERIALS AND METHODS

Male Sprague–Dawley rats (250–300 g) were anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). SCI was performed by transecting the spinal cord at the thoracic level (T10) (Gonzalez et al., 2004; Labombarda et al., 2006a). Although ketamine shows some neuroprotective properties (Wang et al., 2008), it did not prevent the pronounced neuropathology developing in the injured spinal cord of steroid-free animals (Gonzalez et al., 2004; Labombarda et al., 2006a). Furthermore, ketamine shows a fast clearance (Rischke et al., 1992) suggesting that it did not represent a major confounding factor for hormone treatment, which was given at later times (see below).

We verified the completeness of the transection by passing the sharp edge of a 25G needle through the lesion site. Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI) application controlled bleeding, and body temperature was maintained at 37°C. Urinary bladders were manually expressed twice a day, and infections were prevented by administration of cefalexine (20 mg/kg daily) starting immediately before surgery. In sham-controls (CTL), the spinal cord was not lesioned. Half of the animals in the CTL and SCI groups received sc. oil or PROG (Proluton, Schering, Argentina) at 16 mg/kg/day for 3 or 21 days. PROG was given to awaken animals 3 h after injury. The Schering preparation contains natural progesterone and is not a progestin, since others have shown that progestins do not always exert the same effects as PROG itself (Brinton et al., 2008). Thus, four groups of animals were prepared per time period: CTL, CTL + PROG, SCI, and SCI + PROG. The dose of PROG chosen prevents oedema, secondary neuronal loss and improves cognitive responses following brain contusion (Stein, 2001, 2008). Animals were killed 3 or 21 days after SCI or sham operation. The 3-day treatment was selected because OPC proliferation peaks at this time period (Horky et al., 2006; Ishii et al., 2001), whereas 21 days are sufficient for OPC differentiation and maturation (Horky et al., 2006; Rosenberg et al., 2005; Zai and Wrathall, 2005).

Myelin parameters and cell populations were analyzed at the thoracic level immediately caudal to the lesion site, where proliferation of OPC and remyelination have been previously studied by McTigue et al. (2001) and Horky et al. (2006). PROG effects were studied in the dorsal (DT), lateral (LT), and ventral (VT) tracts to have representative regions of white matter. The Animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals (Assurance Certificate N A5072-01 to Instituto de Biología y Medicina Experimental) and received approval of the Institute's Animal Care and Use Committee. Efforts were made to keep the number of lesioned animals to a minimum. Separate sets of animals comprising CTL, CTL + PROG, SCI, and SCI + PROG groups were used for immunocytochemistry and real time PCR, as each procedure required different tissue handling.

Immunocytochemistry of NG2 Cells, Mature Oligodendrocyte, and Central Myelin Proteins (MBP, PLP)

Methods for the immunocytochemical staining of NG2 cells, mature oligodendrocytes, and MBP and PLP have

been previously reported (Labombarda et al., 2006a). Rats were perfused with 4% paraformaldehyde (PFA), the spinal cords extracted, postfixed in this fixative for 4 h, embedded into gelatine and left overnight in 4% PFA. Sixty-micrometer vibratome sections were exposed to 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase, washed and incubated with specific antibodies. For NG2-immunodetection (Labombarda et al., 2006a), we used a 1/500 dilution of the rabbit NG2 polyclonal antibody (Dr. Robert Stallcup, the Burnham Institute, La Jolla, CA). Mature oligodendrocytes were stained with a 1/100 dilution of the adenomatus polyposis coli (CC1) antibody from Calbiochem prepared in 0.5% Triton X100 in PBS. For myelin protein staining, sections were exposed to a 1/500 dilution of rabbit anti-MBP antibody (Dako, Cytomation) in PBS containing 1% goat serum, or a 1/100 dilution of the AA3 rat anti-PLP antibody (Dr. S.R. Winkler, Dept. of Neuroscience, University of Connecticut School of Medicine, Farmington, CT) in PBS containing 1% goat serum. After several washes with PBS, sections were incubated with their respective anti-rabbit, mouse, or rat biotinylated secondary antibodies (1/200 dilution, 60 min), then with ABC complex for 30 min (ABC kit, Vector Labs, CA) and finally revealed with diaminobenzidine tetrachloride (0.50 mg/mL, Sigma, St. Louis, MO) in the presence of 0.01% H_2O_2 for 7 min in the dark. The sections were given a final rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

Bromodeoxyuridine (BdrU) Administration and Immunofluorescence for Detection of BrdU-CC1; NG2-OX42 and CC1-GFAP Double-Labeled Cells

The CC1 antibody recognizes antigens of mature oligodendrocytes; however weak labeling of astrocytes was reported (Horky et al., 2006; McTigue et al., 2001). To verify the specificity of CC1 staining a double immunofluorescence procedure was adopted, using the CC1 antibody (1/100) followed by a polyclonal GFAP antibody (1/400, marker of mature astrocytes, Sigma). This method failed to detect double-labeled CC1+/GFAP+ cells, indicating that CC1+ cells were not astrocytes. It was considered that single-labeled cells (CC1+/GFAP-) represented oligodendrocytes. In addiction, NG2 labeling in injury spinal cord is not only restricted to OPC population, but also includes a subset of macrophages. Thus, the phenotype of NG2+ cells was determined using a double labeling immunofluorescence protocol, in which sections were exposed to the polyclonal NG2 antibody (1/400, Dr. William Stallcup), and the monoclonal OX42 antibody (1/200, marker of macrophages/microglia, Chemicon). It was considered that single-labeled cells (NG2+/OX42−) were indicative of OPC. For double-labeling with NG2-OX42 or CC1-GFAP, sections were blocked with a 10% goat serum-TBS (Tris-buffered saline) solutions and incubated with the respective primary antibodies in 1% goat serum containing 0.5% Triton X100 solution in TBS at 4°C overnight.

To determine whether cells dividing at 2 and 3 days after injury subsequently differentiate into mature oligodendrocytes, four pulses of BrdU (50 mg/kg ip) were given each day at 48 and 72 h after lesion. These animals were euthanized and processed for double immunofluorescent labeling (BrdU-CC1) 18 days after the last BrdU injection (i.e., 21 days after injury). Sixty-micrometer vibratome sections were pretreated with 50% formamide in 2× SSC for 10 min at 65°C followed by 30 min in 2 N HCl at 37°C, 10 min in 0.1 M borate buffer pH 8.5, and three 5-min rinses in TBS, pH 7.5. Nonspecific labeling was blocked with TBS 0.5% Triton X-100 and 3% normal goat serum for 30 min. The tissue was incubated with a rat antibody against BrdU (1/200, Accurate Chemicals) and a monoclonal antibody against CC1 (1/100, Calbiochem, San Diego) for 2 days at 4°C in TBS 0.5% Triton X-100 and 3% normal goat serum. It was considered that double-labeled cells (BrdU+/CC1+) were indicative of mature, differentiated oligodendrocytes derived from progenitor proliferation.

In all double-labeling procedures, incubations with the primary antibodies were rinsed three times in TBS 0.1% Triton X-100 for 15 min before application of the secondary antibodies: goat anti-rat IgG conjugated to FITC (1/250, Sigma); goat anti-mouse IgG conjugated to Alexa 555 (1/1,000, Molecular probes); goat anti-mouse IgG conjugated to Alexa 488 (1/1,000, Molecular probes); and goat anti-rabbit IgG conjugated to TRITC (1/250, Sigma). Incubation with secondary antibodies was followed by three rinses in TBS. Sections were mounted with Fluoromont G and kept in the dark at 4°C until analysis by confocal microscopy.

Quantitative Analysis

To examine immunopositive cells and immunoreaction intensity in white matter regions of the spinal cord, 60-μm-thick serial coronal sections were prepared from 1-mm-long spinal cord stumps immediately below the lesion. Among these serial sections, four representative sections at least 240-μm apart form each other, were subjected to immunostaining with different antibodies. Cross sections were examined under a light microscope, at 400× magnification, equipped with a digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope. Double-labeled cells were examined under a Nikon Eclipse E 800 confocal scanning laser microscope. Images were acquired sequentially in a line-scanning mode through an optical section of 1 μm in the *z*-axis, and merged using Nikon EZC1 version 2.1 software. Image analysis was performed using Bioscan Optimas II software.

Determination of the Density of Immunopositive Cells

A modified procedure from Horner et al. (2000) was adopted. A template was overlaid onto the coronal sections, dividing the spinal cord into concentric circles (Fig. 1A) and quadrants (Fig. 1B). When superimposed,

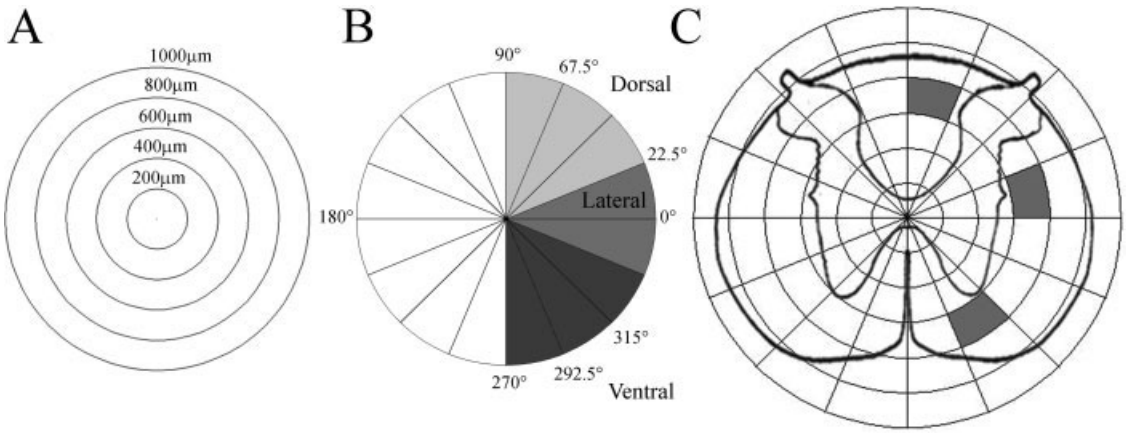

Fig. 1. Microanatomy-based approach for counting immunopositive cells. Electronic templates that divided the spinal cord into circles (**A**) and radial quadrants (**B**) were overlaid onto stained coronal sections (**C**). Cells were counted in sectors shown in gray (C) of white matter measuring 50,000 μm^2 of the dorsal, lateral and ventral tracts (DT, LT, VT). These areas were localized between the third (600 μm from the central canal) and the fourth circle (800 μm from the central canal) and the quadrants formed by the angle of 67.5° and 90° for DT, 0° and 22.5° for LT, and 292.5° and 315° for VT.

this procedure constructs a counting grid dividing the surface of the spinal cord into defined sectors, which can be identified by the angle and distance from the central canal (Fig. 1C). Immunopositive cells were counted in sectors comprising a defined area measuring 5×10^4 μm^2 of DT, LT, and VT of the white matter. These areas were located between the third (600 μm from the central canal) and fourth circle (800 μm from the central canal) and the quadrants formed by the angle of 67.5° and 90° for the DT, 0° and 22.5° for LT, and 292.5° and 315° for VT. Cell profiles containing a visible nucleus and located within the chosen sector was counted as positive. Cell quantification was carried out in four sections per animal, averaged and expressed as number of cells per 5×10^4 μm^2 for each tract and for each animal. The *n* used for statistical analysis was the number of rats per group. For NG2, CC1, and double-labeled NG2/Ox42 experiments, five rats were used in CTL and CTL + PROG and six rats in the SCI and SCI + PROG groups. For BdrU experiments, six animals per group were used.

Determination of MBP and PLP-Immunoreactive Areas

The procedure was already reported to demonstrate PROG effects on MBP immunoreactivity in rats with or without SCI (Labombarda et al., 2006a). Staining intensity of MBP and PLP was determined in areas (5×10^4 μm^2) covering three white matter regions: DT, LT, and VT, anatomically delimited by the method described in the previous paragraph. Computer-assisted image analysis (Labombarda et al., 2002) was used to transform differences in color intensity of immunopositive areas into gray differences, and results were expressed as the mean ± SEM inverse logarithm of grain intensity per unit area (μm^2) (ILIGV/area = LIGV) (Ferrini et al., 1995; Labombarda et al., 2002). Sections were processed simultaneously under identical light beam, wavelength and gray-scale threshold throughout the experiment. Data corresponding to four sections per animal were combined to give an independent mean for each rat (n = 5 rats per CTL and CTL + PROG and 6 per SCI and SCI + PROG). In all cases, the number of rats served as the n number.

Determination of mRNA of Myelin Proteins and Oligodendrocyte Transcription Factors Olig1, Olig2, and Nkx2.2 by Real Time RT-PCR

Spinal cord tissue localized below the lesion site was homogenized with a Polytron homogenizer. Total RNA was then extracted using Trizol reagent (Life Technologies, Invitrogen). 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, and their quality was verified by gel electrophoresis. Total RNA was subjected to Dnase 1 (InvitroGen) treatment (2 U for 10 min at room temperature) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 μg of total RNA using a SuperScript II Rnase H reverse transcriptase kit (InvitroGen) for 60 min at 42°C in the presence of random hexamer primers. Primers for real-time RT-PCR were designed using Oligo Primer Analysis Software version 6.54 (Molecular Biology Insights, Cascade, CO). Primer sequences used for amplification are indicated in Table 1. β actin was chosen as a housekeeping gene based on the similarity of mRNA expression across all samples templates.

The relative gene expression for the mRNAs of MBP, PLP, MOG, Olig1, Olig2, and Nkx2.2 was determined using the ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA), which combines PCR, cycle-fluorescence detection, and analysis software for high throughput quantification of nucleic acid

TABLE 1. Real Time RT-PCR Primers and Validation

Primer	Accession no.	Sequences (5′ to 3′)	EX%	*r*
β actin	EF_156276	F: CCG CAT CCT CTT CCT CCC T R: GAA CCG CTC ATT GCC GAT A	96.41	0.999
PLP	NM_030990	F: CAC TTA CAG CAG GTG ATT AGA GG R: AAA CAA GAG ATA AAC AAC TGG GA	94.65	0.992
MBP	BC_094522	F: AGT CGC AGA GGA CCC AAG AT R: GAC AGG CCT CTC CCC TTT C	96.02	0.998
MOG	NM_022668	F: GTC TAT CGG CGA GGG AAA GGT R: CAC GGC GGC TTC TTC TTG GT	92.00	0.996
Olig 1	AF_151367	F: GCC CAG GCC ACG AGT ACA AA R: TCC ACT CCG AAA CCC AAC GA	90.91	0.990
Olig 2	XM_001073459	F: GAA ATG GAA TAA TCC CGA ACT ACT R: CCC CTC CCA AAT AAC TCA AAC	91.99	0.991
Nkx 2.2	XM_345446	F:CGG GCT GAG AAA GGT ATG GA R: TGT GCT GTC GGG TAC TGG G	93.99	0.990

F, Forward; R, Reverse; r, correlation coefficient; Ex%, PCR efficiency defined as $Ex = (10^{-1/\text{slope}}) - 1 \times 100$.

TABLE 2. Effects of PROG on the Number of NG2+ cells/50,000 μm² in DT, LT, and VT in Controls (CTL), Rats With Spinal Cord Injury (SCI), and Steroid-Treated Lesioned Rats (SCI + PROG) in the 3 and 21 Day Experiments

	3 days			21 days		
	DT	LT	VT	DT	LT	VT
CTL	8.7 ± 0.61	7.43 ± 0.63	8.86 ± 0.73	4.87 ± 0.51	4.57 ± 0.71	5.71 ± 1.35
SCI	22.33 ± 1.8***	25.29 ± 1.92***	22.20 ± 2.04***	17.10 ± 2.13++	21.29 ± 2.26++	19.86 ± 2.61++
SCI + PROG	30.5 ± 2.6***	36.87 ± 1.65**	31.67 ± 2.38**	10.30 ± 0.96+	16.09 ± 1.49+	14.50 ± 1.37+

Results represent the mean number of NG2+ cells per 50,000 μm² (± S.E.M.; 5 rats per CTL and 6 animals per SCI and SCI + PROG). Statistical comparison between groups was performed by two-way ANOVA followed by *posthoc* Tukey *posthoc* test. The density of NG2+ cells increased after 3 and 21 days of injury in all tracts (***$P < 0.001$ vs. CTL 3 days; ++$P < 0.01$ vs. CTL 21 days). PROG treatment for 3 days significantly stimulated NG2+ cell number above levels of rats with SCI (**$P < 0.01$ in LT and VT vs. SCI 3 days; ***$P < 0.001$ in DT vs. SCI 3 days), but steroid treatment for 21 days decreased NG2+ cells compared to SCI (+$P < 0.05$ vs. SCI 21 days).

sequences. Reactions are characterized by the cycle number at which amplification of a PCR product is first detected (threshold cycle, C_T). The higher the copy number of the nucleic acid target, the sooner a significant increase in SYBR I green fluorescence is observed. Relative gene expression data were calculated using the $2^{-\Delta\Delta ct.}$ method (Livak and Schmittgen, 2001), and it was determined for each gene as fold induction with respect to its respective control. Specificity of PCR amplification and the absence of dimers were confirmed by melting curve analysis. In addition, PCR products were controlled with high-resolution gel electrophoresis. All PCR amplifications lead to a single and specific product of the predicted size. Linearity and efficiency of PCR amplification were validated before quantification (Table 1). Both parameters were assessed using standard curves generated by increasing amounts of cDNA using five points, diluted to over a 10-fold range. Relationship between the C_T and the logarithm of the cDNA concentration were studied according to (1) the correlation coefficient (r) and (2) the amplification efficiency. Correlation coefficients confirm the linearity and PCR efficiency (Ex) was calculated using the equation $Ex = (10^{-1/\text{slope}}) - 1 \times 100$ (Peinnequin et al., 2004). The linearity and efficiency of amplification of PCR assays among different templates allowed an accurate quantification of the different genes (Table 1). For each amplification, 2 ng cDNA/μL of reaction was used and PCR was performed in triplicate 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 were between 0.2 and 0.8 μM. We used 10 rats per CTL and CTL + PROG and 12 rats per SCI and SCI + PROG groups.

Statistical Analysis

Comparison between group means was carried out by two-way ANOVA followed by the Tukey *posthoc* test. A $P < 0.05$ was considered to be significant.

RESULTS

Effects of PROG on Oligodendrocyte Progenitors (OPC) and Mature Oligodendrocytes

A previous report showed that a 3-day course of PROG treatment amplifies the SCI-induced stimulation of cells showing NG2 immunostaining (Labombarda et al., 2006a). Here, we compared PROG effects on NG2+ cells in rats killed either 3 or 21 days after SCI using immunohistochemistry and a DAB-staining method. As shown in Table 2, PROG treatment during 3 days significantly increased the number of NG2+ cells in the DT, LT, and VT over levels of rats with SCI, while decreased them after 21 days. To discard the contribution of macrophages and activated microglia to NG2 measurement (Bareyre and Schwab, 2003; McTigue et al., 2001), NG2+/Ox42− cells were counted in a double immunofluorescence assay in which staining for NG2 was followed by anti-Ox42 staining of the same sections. It was considered that single-labeled cells (NG2+/OX42−) were indicative of OPC. The number of NG2+/Ox42− cells per 5×10^4 μm² was identified by confocal microscopy and determined in DT, LT, and VT of the white matter below the lesion site (see Fig. 2). Quantitative data showed a low density in CTL rats (see Fig. 2). In agreement with others (Hubbard, 2003; Labombarda

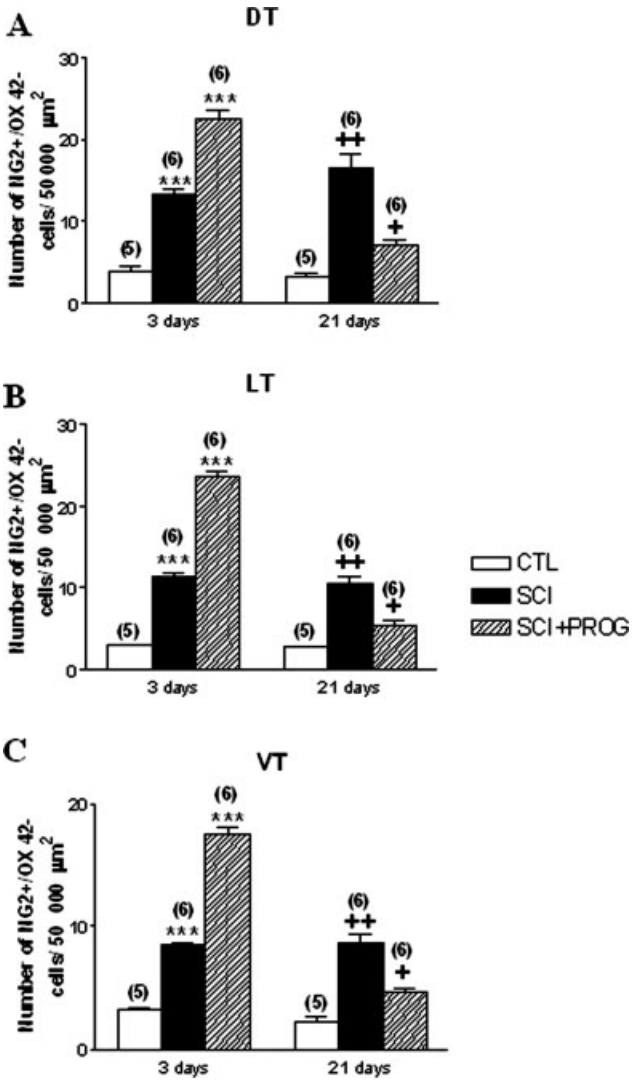

Fig. 2. Effects of PROG on the number of NG2+/OX42− cells in DT (**A**), LT (**B**), and VT (**C**) in controls (CTL), rats with spinal cord injury (SCI), and steroid-treated lesioned rats (SCI + PROG) in the 3- and 21-day experiments. Results represent the mean number of NG2+/OX42− cells per 50,000 μm^2 (± S.E.M.; five rats per CTL and six animals per SCI and SCI + PROG). The number of rats used is indicated between parentheses. Statistical comparison between groups was performed by two-way ANOVA followed by Tukey *posthoc* test. The density of NG2+/OX42− cells increased after 3 and 21 days of injury in all tracts (***$P < 0.001$ vs. CTL 3 days; ++$P < 0.01$ vs. CTL 21 days). PROG treatment for 3 days significantly stimulated NG2+/OX42− cell number above levels of rats with SCI (***$P < 0.001$ vs. SCI 3 days), but steroid treatment for 21 days decreased NG2+/OX42− cells compared with SCI (+$P < 0.05$ vs. SCI 21 days).

et al., 2006a; Levine et al., 2001; Nishiyama et al., 1999), SCI stimulated the proliferation of NG2+/Ox42− cells in rats killed 3 or 21 days after injury. A 3-day treatment of injured rats with PROG further increased NG2+/OX42− cell number in DT, LT, and VT vs. the SCI group (see Fig. 2). In contrast, after 21 days of PROG treatment, density of NG2+/Ox42− cells was significantly reduced in DT, LT, and VT respect of the SCI group. Furthermore, the number of NG2+/OX42− obtained after 3 days of PROG treatment was 3–5 times higher than the value at 21 days (***$P < 0.001$, SCI + PROG 3 days vs. SCI + PROG 21 days), showing time-dependent PROG effects. PROG *per se* had no influence on the population of NG2+/Ox42− cells in CTL (Labombarda et al., 2006a). It is interesting to note that the pattern of PROG effects on NG2+ cells was similar, independently on whether conventional immunocytochemistry with DAB staining or double immunofluorescence was employed. Differences in values between both measurements may depend on cross-reaction with other cell types in the immunocytochemistry studies, which could dilute true OPC cell counts. In fact, differences were more marked between the experimental groups when counting NG2+/Ox42− cells, that is, "pure" OPC.

Figure 3 shows confocal microscopy images of the experiment described above. Whereas in control groups killed after 3 (D) or 21 days (G) cell staining was almost missing, single-labeled cells bearing the NG2+/Ox42− phenotype (OPC) appeared following SCI (E: 3 days; H: 21 days). These cells further increased in the PROG-treated SCI group at 3 days (F), but decreased in the 21-day group receiving steroid treatment (I). That these cells truly represented OPC was provided by the fact that NG2+/Ox42− cells selected for counting (arrows pointing to red cells in Fig. 3A,B), did not bear the green label shown by OX42+ macrophages/microglia (arrowheads in Fig. 3A,C), and the very few cells colocalizing both markers (asterisk in Figs. 3A–C) were discarded in our counting procedure. Another observation was the high density of Ox42+ cells following SCI (E, H) that almost disappear in the PROG-treated groups (F, I), suggesting anti-inflammatory effects of PROG (Stein, 2008).

Data so far suggested that in the short run, PROG increased the number of NG2+ cells but prolonged PROG exposure had the opposite effect, which may be due to enhancement of OPC differentiation at the expense of the progenitor population. To test this hypothesis, mature oligodendrocytes labeled with the CC1+ antibody were counted at both time periods after PROG treatment. In spite of the fact that the CC1 antibody shows specificity toward mature oligodendrocytes, weak labeling of astrocytes may occur (McTigue et al., 2001). However, cross-reaction of the CC1 antibody with astrocytes was discarded by double immunostaining with anti-GFAP and anti-CC1 antibodies following analysis by confocal microcopy. This method failed to detect double-labeled CC1+/GFAP+ cells, indicating that under our experimental conditions CC1+ cells were not astrocytes. Figure 4 shows in quantitative form that SCI significantly reduced CC1+ cells in DT, LT, and VT at 3 and 21 days following surgery, compared with the CTL and CTL + PROG groups, respectively. In contrast to stimulation of NG2+/Ox42− labeled OPC exerted by PROG at 3 days (see Fig. 2), this treatment length did not change oligodendrocyte number in DT, LT, or VT. However, 21 days after injury, PROG therapy effectively stimulated CC1+ cell density in the three white matter regions (see Fig. 4). The effect of PROG on CC1 cell number was also time-dependent being twofold higher at 21 days than 3 days (**$P < 0.01$, SCI + PROG 21 days vs. SCI + PROG 3 days). Photomicrographs sup-

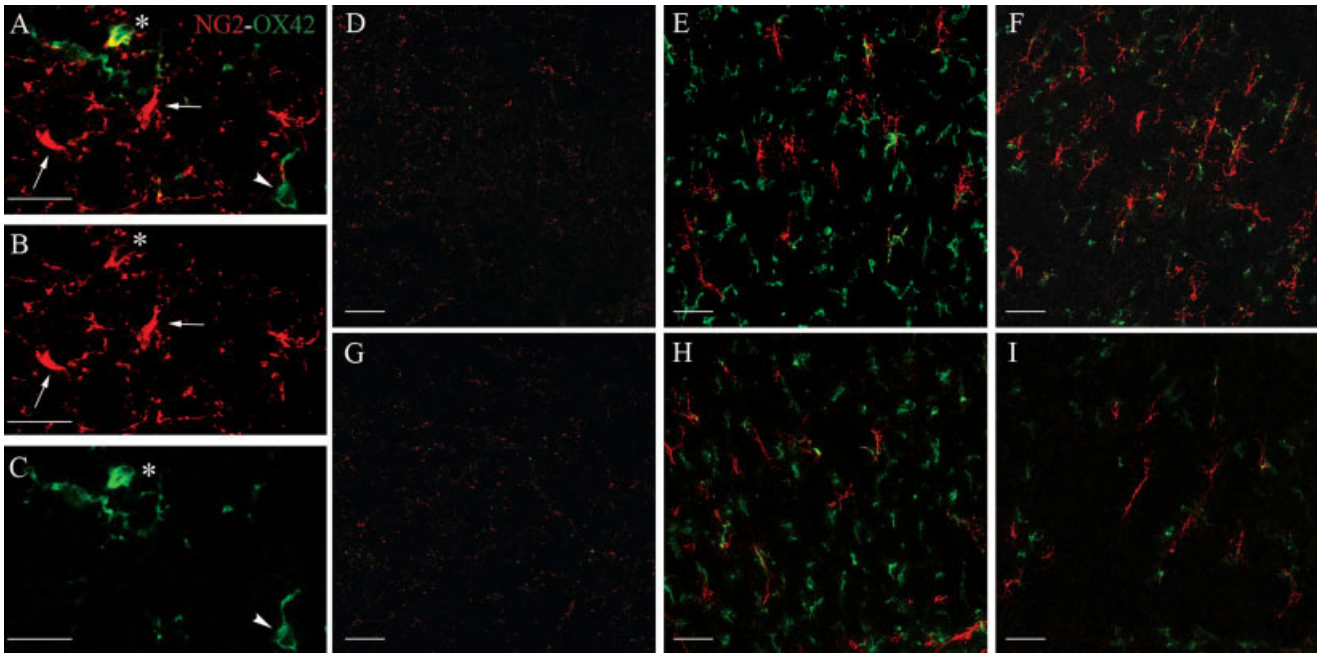

Fig. 3. Confocal photomicrographs showing OPC in DT of the spinal cord below the lesion site in rats killed 3 (**D–F**) or 21 days (**G–I**) postinjury. Single-labeled cells bearing the NG2+/OX42− phenotype indicate OPC (**A**, **B**, arrows pointing to red cells). Double-labeled NG2+/OX42+ cells (A–C, yellow cell with asterisk) and single-labeled cells NG2−/OX42+ (A, **C** arrowheads pointing to green cells) indicate macrophages/activated microglia. Group labeling as described in the legend to Figure 2. CTL (D, G), SCI (E, H), and SCI + PROG (F, I). In CTL groups (D,G) weak or no staining for NG2+/OX42− cells was observed, whereas after SCI, NG2+/OX42− cells acquired a more reactive phenotype (E for 3 days and H for 21 days). PROG increased the density of NG2+/OX42− cells after 3 days of injury (F) but reduced their number after 21 days (I). Scale bar: A–C = 10 μm; D–I = 20 μm.

porting quantitative data are shown in Fig. 5. Here, graphs C and G show the decreased density of CC1+ cells at 3 and 21 days after SCI respectively, compared with their respective CTL (A, E) and CTL + PROG groups (B, F). Convincing evidence for a higher number of CC1+ cells in the SCI group receiving PROG for 21 days is shown in H, in contrast to the lack of effect of PROG in the 3-day group (D).

Therefore, we assumed that the differential effects obtained for PROG on NG2+ and CC1+ cells indicated an early PROG influence on OPC density followed by differentiation and maturation of these cells into oligodendrocytes. To validate this assumption, BrdU was administered during 2 and 3 days following SCI and the number of BrdU+/CC1+ cells was counted after 21 days postinjury. Figure 6 shows the colocalization of CC1 with BrdU in oligodendrocytes of the DT analyzed by confocal microscopy. In CTL, most CC1+ cells did not show BrdU staining (D, arrows), whereas after SCI without PROG treatment, differentiated cells bearing BrdU staining did not colocalize with CC1 (E, arrows). In contrast, a strong colocalization of both markers was obtained in the group SCI + PROG (F, arrows), suggesting that PROG therapy, 21 days after SCI, differentiated the early proliferating progenitors into mature, CC1+ cells. Further validation was provided by the quantitative data of Table 3, which shows that in SCI + PROG animals, CC1+/BrdU+ cells represented almost half of the total CC1+ cells, in contrast to low percentages (6–11%) found in the SCI or CTL groups.

Effects of PROG on Oligodendrocyte Transcription Factors

Transcription factors modulate oligodendrocyte function under physiological and pathological circumstances (Fancy et al., 2004; Ligon et al., 2006; Lin et al., 2006). Their measurement, therefore, could provide an index of the early and late steps modulated by PROG during remyelination. Figure 7 shows the change in the mRNA expression of the transcription factors Olig2 (upper graph), Nkx2.2 (middle graph), and Olig1 (lower graph) in CTL and SCI rats receiving vehicle or PROG treatment for either 3 or 21 days. In the 3-day period, SCI triggered a significant reduction of Olig2 and Olig1 mRNA (Fig. 7A,C) without changes in levels of Nkx2.2 (B), whereas after 21 days, SCI also reduced Nkx2.2, Olig1, and Olig2 mRNA respect of their respective CTL groups (A–C). The decreased expression of transcription factors was unevenly counterbalanced by PROG treatment; whereas short PROG treatment upregulated Olig2 and Nkx2.2, the steroid was inactive at 21 days for Olig2 and Nkx2.2. This result coincided with the stimulation of OPC cell density (see Fig. 2) and was expected as both transcription factors are co-expressed

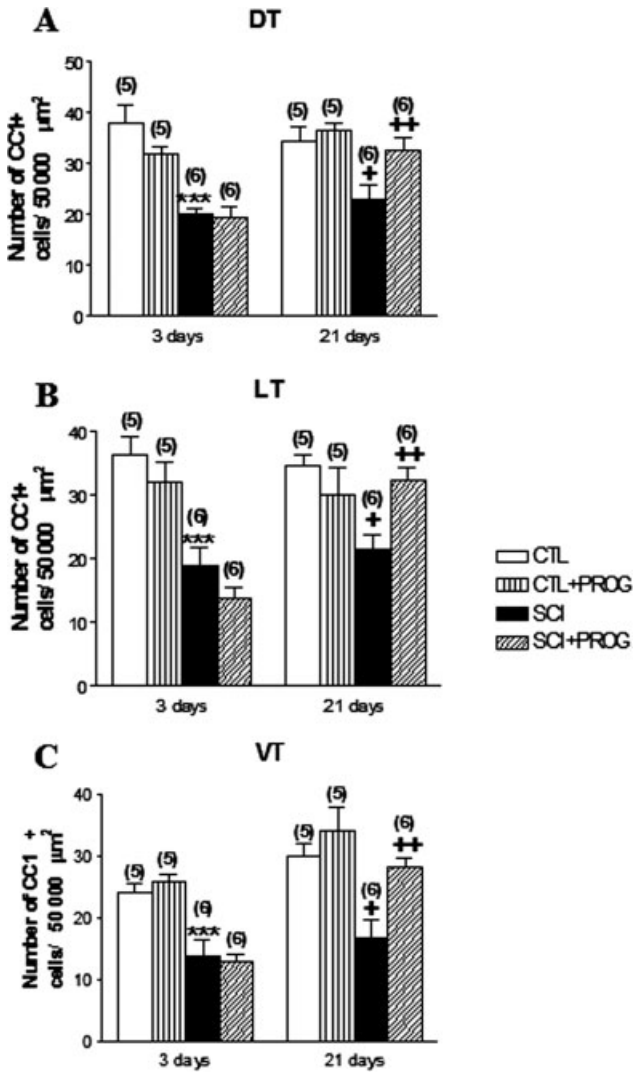

Fig. 4. Effects of PROG on the number of CC1+ cells in DT (**A**), LT (**B**), and VT (**C**) in controls (CTL), controls + PROG (CTL + PROG), rats with SCI, and steroid-treated lesioned rats (SCI + PROG) in the 3 and 21 day experiments. Results represent the mean number of CC1+ cells per 50,000 μm^2 ($\pm$ S.E.M; five rats per CTL and CTL + PROG and six rats per SCI and SCI + PROG). The number of rats used is indicated between parentheses. The three white matter tracts responded equally to experimental treatments. Three and twenty-one days of SCI downregulated the number of CC1+ cells respect of CTL (***$P < 0.001$ vs. CTL 3 days, +$P < 0.05$ vs. CTL 21 days). PROG treatment for 3 days did not change the density of positive cells (SCI + PROG 3 days vs. SCI 3 days: NS), however, CC1 cell number was stimulated over the SCI group after 21 days of PROG treatment (++$P < 0.01$ vs. SCI 21 days).

by proliferating cells (Fancy et al., 2004). However, 21 days after injury, PROG treatment upregulated Olig1 mRNA solely (Fig. 7C). Since Olig1 is involved in remyelination, this response may be connected to the enhanced number of CC1+ cells appearing under these conditions (see Fig. 4).

Effects of PROG on Myelin Proteins: Real Time RT-PCR and Immunocytochemical Studies

MBP and PLP are components of central myelin responding to PROG treatment (Garay et al., 2007; Ghoumari et al., 2003; Ibanez et al., 2004; Jung-Testas et al., 1996), whereas data for MOG are lacking. We determined the mRNA levels for these three proteins following 3 and 21 days of SCI. Figure 8A–C shows that SCI significantly depleted by more than half the expression of MBP, PLP, and MOG mRNA at both 3 and 21 days, suggesting an ongoing demyelination. Under this circumstance, PROG had differential effects on the mRNA of these proteins. Three days of steroid treatment increased the mRNA for MBP but not PLP or MOG, whereas 21 days after injury PROG treatment significantly stimulated PLP mRNA alone (Fig. 8A–C). A nonsignificant trend existed, however, for upregulation of MBP mRNA at 21 days.

PROG effects on MBP and PLP was also reflected at the protein level. We carried out immunocytochemical analysis for MBP and PLP but not MOG, since PROG had no effect on MOG mRNA. Figure 9 demonstrated that changes of MBP (left-hand graph) and PLP immunoreactivity (right-hand graph) closely followed changes of their mRNA levels. Thus, injury-induced demyelination, according to the measurement of the two central myelin proteins, was present early (3 days) and persisted at 21 days following SCI. This response was marked in the DT, LT, and VT (A, B, D–F) but spared the VT in the case of the 3-day group for MBP (C). Furthermore, PROG reestablished MBP protein immunoreaction intensity in the DT and LT within 3 days but not after 21 days of treatment. PLP immunoreactivity decreased following SCI at both time periods (D–F), but PROG restored PLP staining in the white matter comprising the DT, LT, and VT in the 21-day group only (Fig. 9D–F). Therefore, the effects of PROG on myelin protein expression were time-dependent, with early modulation of MBP and late effects on PLP.

DISCUSSION

The present study revealed that PROG modulates myelin parameters after injury-induced demyelination. Thus, short PROG treatment increased the number of OPC, increased the expression of MBP, and enhanced the expression of the Olig2 and Nkx2.2 transcription factors involved in specification and differentiation of the oligodendrocyte lineage. More prolonged PROG treatment enhanced maturation and differentiation of oligodendrocytes, increased PLP, and increased the Olig1 transcription factor involved in myelin repair. These data suggest that following injury, PROG could be a major differentiation factor for oligodendrocytes *in vivo*.

These effects are in agreement with previous observations that PROG stimulates remyelination of the lesioned and aging peripheral nerves, after toxin-induced demyelination, in glial cell cultures and in diabetic neurodegeneration (Azcoitia et al., 2003; Gago et al., 2001; Ibanez et al., 2004; Jung-Testas et al., 1996; Koenig et al., 1995; Leonelli et al., 2006). The effects on

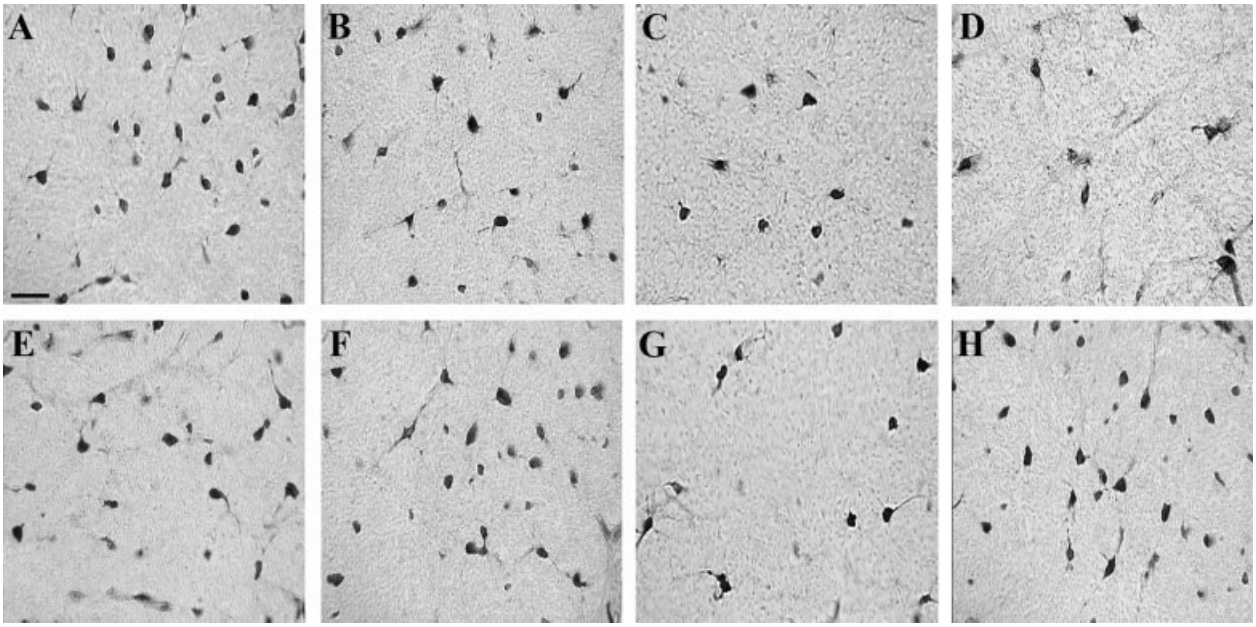

Fig. 5. Photomicrographs showing CC1 cells in DT of the spinal cord in rats killed 3 (**A**–**D**) or 21 days (**E**–**H**) postinjury. Group labeling as described in the legend to Figure 4. CTL (A, E), CTL + PROG (B, F), SCI (C, G), and SCI + PROG (D, H). CC1 cell density was decreased 3 and 21 days after injury, (C for 3 days and G for 21 days). PROG treatment increased CC1+ cells after 21 days of injury (H), but this parameter did not exceed the SCI level after 3 days of steroid treatment (D). Scale bar = 20 μm.

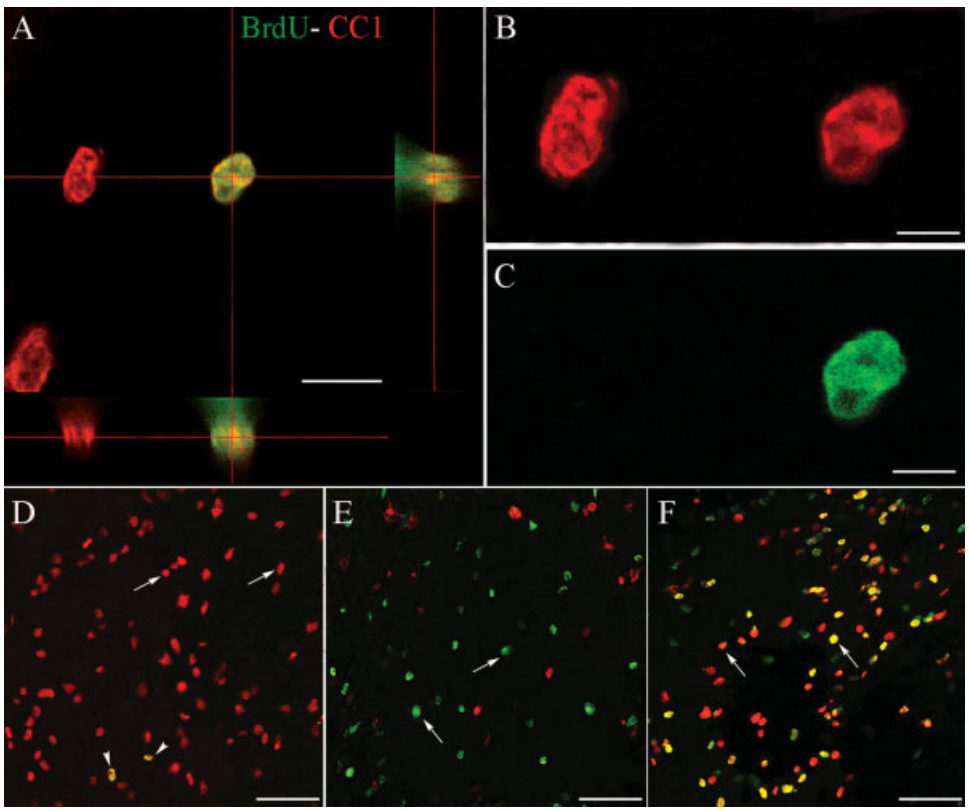

Fig. 6. Effects of PROG on the colocalization of CC1 (marker of mature oligodendrocytes) with BrdU in rats killed 21 days after injury. BrdU was given at 2 and 3 days immediately after injury. Thus, double-labeled CC1+/BdrU+ cells indicated early dividing cells that differentiated into mature oligodendrocytes after 21 days (**A**: SCI + PROG group, 12 μm confocal stack with orthogonal views taken at the center of one cell; **B**, **C**: 0.3 μm, single channel confocal images of BrdU (green) and CC1(red) channel respectively). Most CC1+ cells of CTL rats did not colabel with BdrU (**D**, arrows), since only a small percentage of CC1+ cells were BdrU immunoreactive (D, arrowheads). In injured rats, BrdU+ cells did not express the oligodendrocyte marker, CC1 (**E**, arrows). However, CC1 and BrdU colocalization (**F**, yellow and orange) was increased after treatment (F, arrows), suggesting that PROG induced oligodendrocyte differentiation after 21 days of injury. Scale bars = 10 μm in A; 5 μm in B and C; 50 μm in D–F.

TABLE 3. Effects of Spinal Cord Injury and PROG Treatment on the Colocalization of the Mature Oligodendrocyte Marker CC1 With Bromodeoxyuridine (BrdU)

	Total CC1+ cells			% of CC1+/BrdU+		
	DT	LT	VT	DT	LT	VT
CTL	37.54 ± 4.39	65.75 ± 10.63	71.60 ± 1.89	6.11 ± 1.97	3.54 ± 1.08	9.19 ± 2.25
SCI	19.83 ± 3.58**	9.25 ± 1.88**	29.75 ± 9.81**	8.01 ± 1.20	11.25 ± 6.57	6.68 ± 1.86
SCI + PROG	39.30 ± 3.56***	62.00 ± 5.65***	55.71 ± 5.47*	44.52 ± 2.81+++	41.29 ± 0.91++	50.23 ± 4.46+++

Results represent the number of cells per 50,000 μm^2 and the percentage of double-labeled cells/total CC1 cells (mean ± SEM of 4 sections per animal, 6 animals per group). Group labeling and statistical analysis as detailed in the Legend to Figure 2. Statistical significance: For Total CC1+ cells: *$P < 0.05$ vs. SCI; **$P < 0.01$ vs. CTL; ***$P < 0.001$ vs. SCI. For % of CC1+/BrdU+ cells: ++$P < 0.01$ vs. SCI and CTL; +++$P < 0.001$ vs. SCI and CTL.

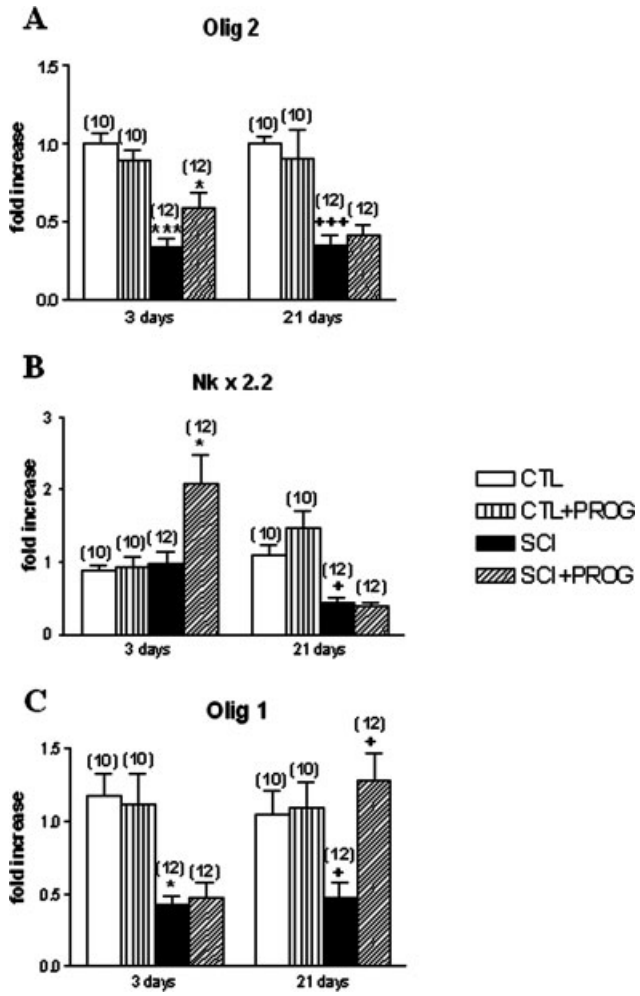

Fig. 7. Effects of PROG on Olig2 (**A**), Nkx2.2 (**B**), and Olig 1 (**C**) mRNA expression in the 3 and 21 day experiments. Group labeling as described in the legend to Figure 4. Data represent the mean ± SEM (n = 10 rats per CTL and CTL + PROG and 12 rats per SCI and SCI + PROG) of fold induction respect of CTL levels. The number of rats used is indicated between parentheses. Statistical comparison showed a downregulation of mRNAs in SCI compared with CTL (for Olig2: ***$P < 0.001$ vs. CTL 3 days and +++$P < 0.001$ vs. CTL 21 days; for Nkx 2.2: +$P < 0.05$ vs. CTL 21 days and for Olig1: *$P < 0.05$ vs. CTL 3 days and +$P < 0.05$ vs. CTL 21 days). In the 3 days experiment, PROG treatment increased Olig 2 (*$P < 0.05$ vs. SCI 3 days) and Nkx 2.2 mRNAs (*$P < 0.05$ vs. SCI 3 days). In the 21 days experiment, PROG upregulated Olig1 mRNA levels (+$P < 0.05$ vs. SCI 21 days) but had no effect on Olig2 or Nkx2.2 mRNAs.

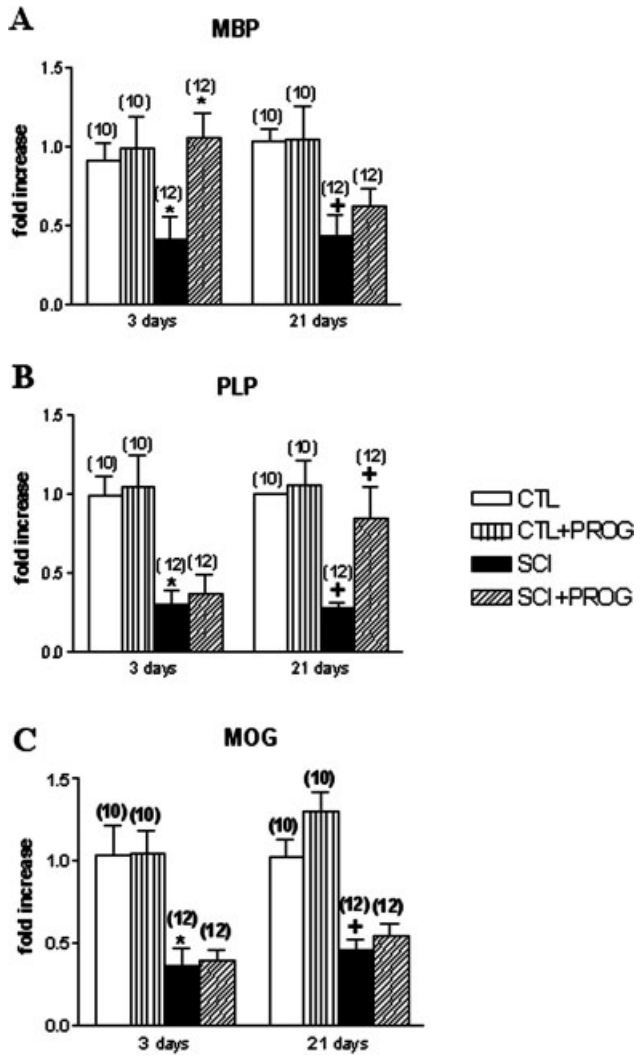

Fig. 8. Effects of PROG on MBP (**A**), PLP (**B**), and MOG (**C**) mRNA expression in the 3 and 21 days experiment. Group labeling as described in the legend to Figure 4. Data represent the mean ± SEM (n = 10 rats per CTL and CTL + PROG and 12 rats per SCI and SCI + PROG) of fold induction respect of CTL levels. The number of rats used is indicated between parentheses. Three and twenty-one days after SCI myelin protein mRNAs were downregulated (*$P < 0.05$ vs. CTL 3 days, +$P < 0.05$ vs. CTL 21 days). PROG treatment for 3 days increased the expression of MBP mRNA (*$P < 0.05$ vs. SCI 3 days), whereas 21 days after injury PROG upregulated PLP mRNA expression (++$P < 0.05$ vs. SCI 21 days). MOG mRNA levels were not modified by PROG either at 3 or 21 days.

myelin-producing cells occur in parallel with effects on neurons, astrocytes, and inflammatory cells in the course of CNS injury, neurodegeneration and neuroinflammation (De Nicola et al., 2006; Garay et al., 2007; Ibanez et al., 2004; Labombarda et al., 2000b; Schumacher et al., 2007; Stein, 2008).

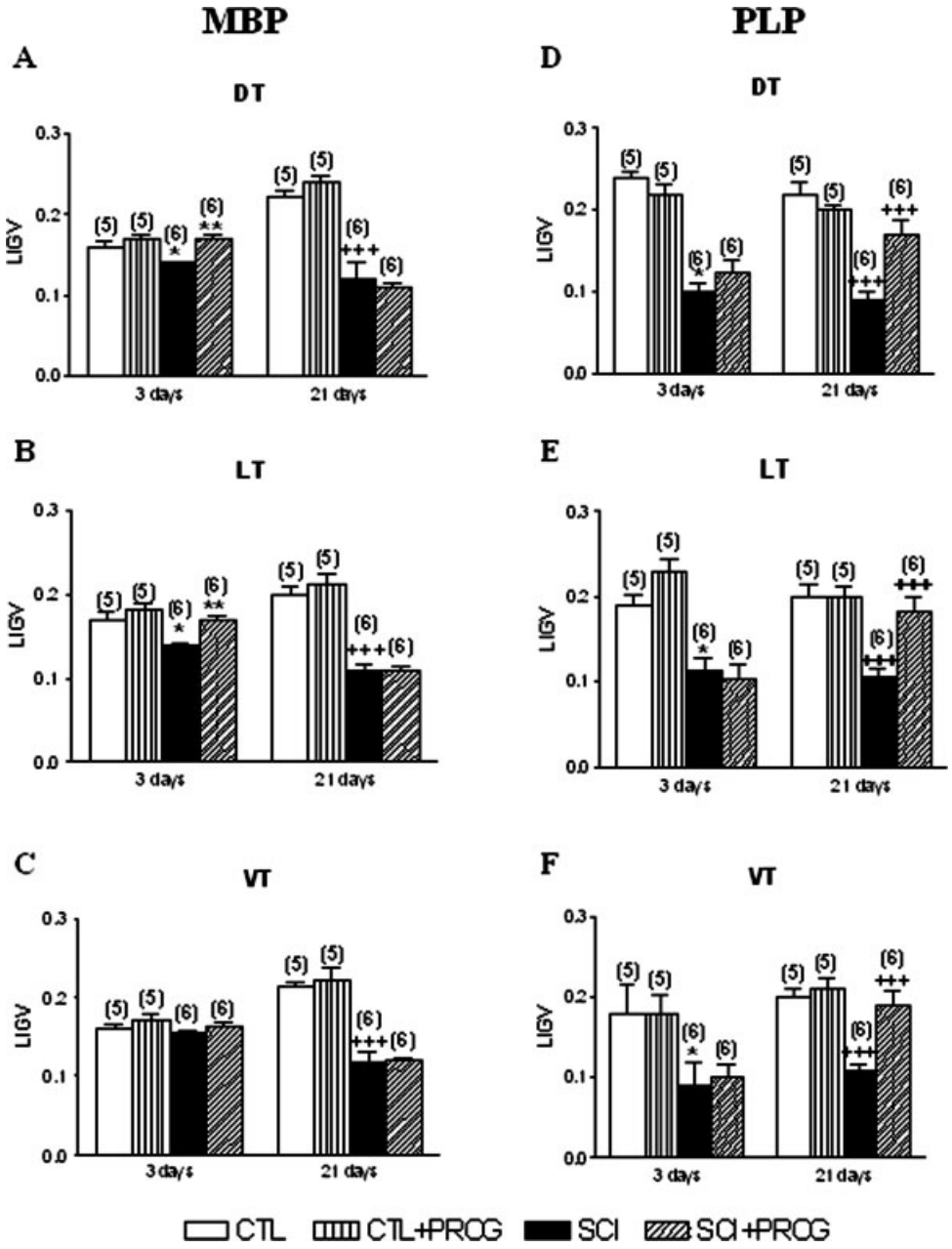

Fig. 9. Effects of PROG on MBP (**A–C**) and PLP (**D–F**) immunostaining intensity in DT (A, D), LT (B, E), and VT (C, F) of the white matter in the 3 or 21 days experiments. LIGV was defined as the inverse logarithm of grain intensity per unit area (μm^2). Group labeling and statistical comparison as described in the legend to Figure 4. Results represent the mean ± S.E. M of four sections per animal (n = 5 rats per CTL and CTL + PROG and 6 animals per SCI and SCI + PROG). The number of rats used is indicated between parentheses. Steroid-naive rats with SCI showed reduced staining for MBP and PLP after 3 or 21 days (*$P < 0.05$ vs. CTL 3 days; +++$P < 0.001$ vs. CTL 21 days). PROG treatment for 3 days after SCI restored only staining intensity of MBP (**$P < 0.01$ vs. SCI 3 days). On the contrary, after 21 days, PROG restored only PLP immunostaining (+++$P < 0.001$ vs. SCI 21 days). MBP expression in VF was not significantly modified by experimental conditions or steroid treatment after 3 days.

Effects of PROG on the Oligodendrocyte Lineage

In a similar SCI model, we have previously shown that PROG treatment for 3 days increases NG2+ cells and mRNA and protein levels of MBP (Labombarda et al., 2006a). A role for PROG is also apparent *in vitro*, because dramatic changes in the endogenous synthesis and metabolism of PROG and in neurosteroidogenesis are observed during oligodendrogenesis in culture (Gago et al., 2004). The generation of mature oligodendrocytes is a multiple-step process, in which OPC proliferate, migrate, and differentiate into mature, myelin-producing cells (Baumann and Pham-Dinh, 2001; McDonald and Belegu, 2006). Normally, OPC remain in a quiescent state that rapidly activates in response to demyelination. In a chemically induced multiple sclerosis model,

remyelination is due to differentiation and maturation of adult OPC instead of pre-existing oligodendrocytes that suffer apoptotic cell death (Suyama et al., 2007). In injury-induced demyelination, OPC proliferation stops before differentiation (Ishii et al., 2001; Levine et al., 2001; McTigue et al., 2001; Suyama et al., 2007) although some remyelinating capacity persists (Horky et al., 2006; Rabchevsky et al., 2007; Rosenberg et al., 2005; Siegenthaler et al., 2007; Zai and Wrathall, 2005). However, the fact that PROG increased the number of new oligodendrocytes (CC1+/BrdU+ cells), supports the idea that the steroid imposes a milieu favoring differentiation of proliferating progenitors into mature forms.

Effects of PROG on the Expression of Transcription Factors and Myelin Proteins

Remyelination is regulated by transcription factors such as the basic helix-loop-helix transcription factors Olig1 and Olig2 and the homeodomain transcription factor Nkx2.2. According to Lin et al. (2006), Olig 1 is required to repair demyelinated lesions, whereas Olig2 is required for OPC specification. Division and differentiation of OPC goes in parallel with a significant increase in Olig2 and Nkx.2.2 expression (Ligon et al., 2006). Not only Olig2 and Nkx2.2. need to be co-expressed by the same cells during remyelination, but it is also known that Olig2 increases the expression of Nkx2.2. Thus, to fully understand the role of PROG on remyelination, it was necessary to analyze hormone effects on OPC and also on transcription factors that may be involved in transition of OPC into myelin-producing cells.

In agreement with literature reports (Hubbard, 2003; Labombarda et al., 2006a; Levine et al., 2001; Nishiyama et al., 1999), we observed that SCI stimulated the recruitment of OPC, determined as increased NG2+/Ox42– immunostaining in DT, LT, and VT at both 3 and 21 days periods postinjury. However, this effect of SCI did not preclude demyelination, because immunostaining for the myelin proteins MBP and PLP, their mRNAs as well as the number of mature CC1+ oligodendrocytes suffer a pronounced depletion at 3 and 21 days (an exception was the lack of injury effect on MBP immunostaining of the VT at 3 days). According to Franklin and Kotter (2008), impaired remyelination is caused by defective differentiation and maturation of OPC, rather than a defective recruitment of OPC. Another question is what caused the injury-induced OPC recruitment? Evidently, this mechanism depends on factors arising after axonal damage but not on Olig1 and Olig 2, because they were downregulated at 3 days and Olig1 and Olig2 besides Nkx2.2 decreased at 21 days after SCI. Furthermore, OPC differentiation and commitment into myelin-producing cells requires upregulation and co-expression of Olig2 and Nkx2.2 by the same cells (Watanabe et al., 2004), but this event does not occur after SCI. This could explain the failure of the injured tissue to remyelinate the lesioned areas.

In contrast, PROG treatment changed this scenario into one leading to remyelination. Thus, after 3 days of PROG treatment, recruitment of OPC showing a NG2+/Ox42− phenotype and a reactive morphology was further increased over levels of SCI rats receiving vehicle. The 3-day period of PROG treatment also increased MBP mRNA and protein expression and increased Olig2 and Nkx2.2 mRNA. Nkx2.2 has consensus binding sites on the PLP and MBP promoters, allowing for a direct control of the synthesis of the major central myelin proteins. However, Nkx2.2. induces PLP gene expression but represses MBP *in vitro* (Gokhan et al., 2005; Qi et al., 2001; Wei et al., 2005). This dual action means that through regulation of transcription factors, MBP and PLP can be differentially regulated. In principle, MBP and these transcription factors should be localized in different cell types, because Nkx2.2 represses the MBP promoter (Gokhan et al., 2005; Wei et al., 2005). In a first scenario, PROG could stimulate the co-expression of Olig2 and Nkx.2.2 in OPC inducing their differentiation into mature forms (Ligon et al., 2006; Watanabe et al., 2004), and at the same time, acting on surviving oligodendrocytes, PROG could enhance MBP mRNA and protein immunostaining. Alternatively, PROG action on OPC could block Nkx2.2. repression of the MBP promoter, inducing the transcription factor Sp1 that removes Nkx2.2 inhibition (Wei et al., 2005). Along this line, it has been shown that mouse NG2 precursor cells can express mRNA for MBP (Ye et al., 2003).

Moreover, the changing pattern of oligodendrogenesis obtained after the long PROG treatment again suggests a myelination drive. Thus, the early rise in NG2+/Ox42− OPC promoted by early PROG treatment was followed by a reduction of this cell type after 21 days of steroid exposure, and a concomitant increase of mature oligodendrocytes. Double labeling for CC1-BrdU strongly confirmed that PROG early influences OPC, inducing their differentiation and maturation, which otherwise would remain in the undifferentiated state, as shown by the poor colabeling of CC1 with BrdU in untreated rats with SCI. The fact that 45% of CC1 cells in the SCI + PROG group were also BrdU+, indicated that they were newly differentiated oligodendrocytes. Thus, PROG restored the number of CC1+ cells to CTL levels, promoting the differentiation of OPC that divided at 2 or 3 days after injury. At the prolonged time period (i.e., 21 days), PROG enhanced PLP mRNA and protein expression, coupled to increased Olig1 mRNA. Olig1 is required for oligodendrogenesis and to repair demyelinated lesions during demyelination caused by cuprizone or lecithin application (Arnett et al., 2004). In support of this concept, the Olig1 KO mouse fails to induce PLP mRNA in response to chemical demyelination (Arnett et al., 2004). Thus, strong effects of PROG on Olig1 gene expression at the time that CC1+ cells and PLP were positively modulated is in agreement with reports that this factor influenced PLP gene expression and the remyelination of injured regions (Arnett et al., 2004; Xin et al., 2005). Surprisingly, MBP expression was not sensitive to PROG after 21 days. We assume that early effects of PROG on MBP mRNA and protein occur in an environment involving inflammatory mediators and

particular transcription factors, whereas after 21 days, PROG effects took place on a different tissue context involving other transcription factors.

Another interesting story develops when the role(s) of the central myelin proteins is considered. Campagnoni and Skoff (2001) have shown that besides the structural role of MBP and PLP in compact myelin, these molecules bear unexpected functions. For instance, PLP is involved in survival, migration, and intracellular transport in oligodendrocytes and it may be secreted paracrinally. In addition, MBP or its fragments translocate into the cell nuclei where may regulate the myelination program (Pedraza et al., 1997). Interestingly, the MBP gene promoter contains a glucocorticoid receptor (GR) consensus element (Kimbro et al., 1994). Since GR can share hormone responsive elements in the promoter region of target genes with the PROG receptor (PR) (Nordeen et al., 1998), the possibility exists for a direct regulation by PROG of the MBP gene located in surviving oligodendrocytes or OPC early after steroid treatment. Because GR/PR consensus sequence is absent from the 5′-flanking region of the PLP gene (Zhu et al., 1994), the delayed upregulation of PLP transcription/translation by PROG (21 days) suggests mediation by other factors. Based on the just-mentioned unexpected roles of MBP and PLP, the possibility opens that PROG regulation of myelin parameters may have further consequences for the injured spinal cord. Although our present investigation was focused on myelination, it did not answer whether PROG treatment provided functional benefits. Thomas et al. (1999) demonstrated that SCI animals receiving PROG had a better functional recovery compared with untreated injured rats. Preliminary evidences showed that PROG treatment of rats decreases in the long term the hyperactive stretch reflexes leading to injury-induced spasticity (H reflex) (unpublished). Further studies will elucidate if PROG improves functional parameters after SCI.

Mechanism of Action of PROG in the Spinal Cord

The intermediates of PROG action in the spinal cord have been the focus of several investigations. Besides the classical form of PR, several membrane PRs (mPR) have been described, including the 25 Dx molecule and three isoforms of a novel mPR (Labombarda et al., 2003; Labombarda et al., unpublished). PROG is rapidly metabolized into 3α, 5α-tetrahydroprogesterone (Labombarda et al., 2006b), which has promyelinating activity in the PNS and CNS (Azcoitia et al., 2003; Ciriza et al., 2004; Djebaili et al., 2005; Ghoumari et al., 2003) and enhances OPC proliferation *in vitro* (Gago et al., 2001, 2004). Thus, multiple mechanisms may account for PROG effects on oligodendrocyte differentiation and remyelination after SCI.

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