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Regular Article

# Progesterone attenuates astro- and microgliosis and enhances oligodendrocyte differentiation following spinal cord injury

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ABSTRACT

Reactive gliosis, demyelination and proliferation of NG2+ oligodendrocyte precursor cells (OPC) are common responses to spinal cord injury (SCI). We previously reported that short-term progesterone treatment stimulates OPC proliferation whereas chronic treatment enhances OPC differentiation after SCI. Presently, we further studied the proliferation/differentiation of glial cells involved in inflammation and remyelination in male rats with SCI subjected to acute (3 days) or chronic (21 days) progesterone administration. Rats received several pulses of bromodeoyuridine (BrdU) 48 and 72 h post-SCI, and sacrificed 3 or 21 days post-SCI. Double colocalization of BrdU and specific cell markers showed that 3 days of SCI induced a strong proliferation of S100β+ astrocytes, OX-42+ microglia/macrophages and NG2+ cells. At this stage, the intense GFAP+ astrogliosis was BrdU negative. Twenty one days of SCI enhanced maturation of S100β+ cells into GFAP+ astrocytes, but decreased the number of CC1+ oligodendrocytes. Progesterone treatment inhibited astrocyte and microglia /macrophage proliferation and activation in the 3-day SCI group, and inhibited activation in the 21-day SCI group. BrdU/NG2 double labeled cells were increased by progesterone at 3 days, indicating a proliferation stimulus, but decreased them at 21 days. However, progesterone-enhancement of CC1+/BrdU+ oligodendrocyte density, suggest differentiation of OPC into mature oligondendrocytes. We conclude that progesterone effects after SCI involves: a) inhibition of astrocyte proliferation and activation; b) anti-inflammatory effects by preventing microglial activation and proliferation, and c) early proliferation of NG2+ progenitors and late remyelination. Thus, progesterone behaves as a glioactive factor favoring remyelination and inhibiting reactive gliosis.



## Introduction

Spinal cord injury (SCI) produces functional and morphological alterations of gray and white matter cells within the injured area and surrounding tissue. Injured motoneurons suffer chromatolytic changes and alter the expression of essential molecules including brain-derived neurotrophic factor (BDNF), Na, K-ATPase, microtubule-associated protein 2, growth-associated protein GAP-43 and choline acetyltransferase (Gonzalez et al., 2004, 2009; Labombarda et al., 2002).

SCI also motorizes a reaction of glial cells. This reaction is amplified in astrocytes and their precursors, that migrate, alter their phenotype and show hypertrophic and proliferative changes (Wang and Bordey, 2008). This peak of astrocyte activation occurs within 3 days after injury (Morin-Richaud et al., 1998). Several molecular triggers of astrogliosis are known and have been recently reviewed (Sofroniew, 2009). Astrogliosis is commonly assessed by measuring the up-regulation of the cytoskeletal protein glial fibrillary acidic protein (GFAP), vimentin and the calcium-binding protein S100β (Wang and Bordey, 2008). S100β stimulates astrocyte proliferation and is preferentially expressed by dividing astrocytes although it also labels mature forms. GFAP is strongly expressed in reactive astrocytes. Reactive astrocytes change their gene expression, release proinflammatory mediators that attract macrophages and microglia and induce their local and distal proliferation. The gain of detrimental effects by reactive astrocytes is incompletely understood but might result from specific signaling cascades targeting microglia and other inflammatory cells (Bezzi and Volterra, 2001). However, the significance of injury-induced astrogliosis for the recovery of spinal cord function is controversial. Whereas some beneficial functions have been recognized for astroglial scar formation (Sofroniew, 2009), there are also conclusive data showing that in spinal cord injured rats and dogs, scar formation constitutes an impediment for axonal regeneration (Hu et al., 2010).

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Oligodendrocytes, the myelinating cells of the CNS, are extremely labile to the effects of SCI. SCI causes oligodendrocyte loss followed by axonal demyelination and impaired nerve conduction (Grossman et al., 2001; Siegenthaler et al., 2007; Zai and Wrathall 2005). However, spared oligodendrocytes cannot repair myelin, because remyelination occurs from recruitment of an endogenous population of oligodendrocyte precursor cells (OPC) that express the NG2 surface proteoglycan and rapidly proliferate in response to SCI (Levine et al., 2001; McTigue et al., 2001; Rosenberg et al., 2005; Suyama et al., 2007; Zai and Wrathall 2005). However, injury-induced proliferating NG2 cells are poorly differentiated into myelin-forming oligodendrocytes (Horky et al., 2006; Rabchevsky et al., 2007; Siegenthaler et al., 2007) but they extensively maturate if properly stimulated by myelin transcription factors or hormonal treatments ( Ghoumari et al., 2003; Labombarda et al., 2009).

SCI also inflicts a strong inflammatory reaction characterized by microglial activation and infiltration of neutrophils, monocytes/macrophages and dendritic cells. Activated microglial cells show an ameboid phenotype with thick, short processes, as opposed to the highly branched morphology of resting microglia (Batchelor et al., 2008). Activated and resting microglia/macrophages are usually identified by their strong staining with the OX-42 antibody recognizing the type 3 complement receptor CR3 in mononuclear phagocytes (Ling et al., 1990). The exaggerated inflammatory response that follows SCI is highly detrimental. Activated immune cells release proinflammatory cytokines, reactive oxygen species and toxic levels of nitric oxide, causing the necrotic or apoptotic death of neurons and OPC in addition to axonal damage and demyelination (Arevalo et al., 2009; Trivedi et al., 2006). Thus, overshadowing the immune response may be a useful strategy to avoid secondary damage to the lesioned spinal cord.

Progesterone is currently under scrutiny as an emerging neuroprotective factor for trauma and diseases of the central and peripheral nervous system. In peripheral nerve lesions and diabetic neuropathy, progesterone and reduced derivatives promote remyelination, axonal regeneration and the recovery of function (Koenig et al., 1995; Leonelli et al., 2007; Roglio et al., 2008). In experimental brain trauma, progesterone has the ability to reduce edema and inflammatory cytokines, prevents neuronal loss and mitochondrial dysfunction and improves functional outcomes (Pettus et al., 2005; Robertson et al., 2006; Stein, 2008). Two recent Phase II clinical trials carried out in the USA and China have shown a significant improvement in the level of disability among patients with brain injury receiving progesterone (Wright et al., 2007; Xiao et al., 2008). Progesterone neuroprotective effects have been also observed following kainic acid damage to the hippocampus, in focal brain ischemia in rats, in motoneurons exposed to glutamate in vitro or axotomized in vivo and in the spinal cord of mouse models of neurodegeneration and inflammation ( Ciriza et al., 2004; Garay et al., 2008; Gonzalez Deniselle et al., 2007; Ibanez et al., 2004; Ogata et al., 1993; Robertson et al., 2006; Stein , 2005; Stein and Wright, 2010). In addition, promyelinating effects of progesterone have been described in toxin-induced demyelination in aging rats (Ibanez et al., 2004) and in organotypic cultures of neonatal cerebellum (Ghoumari et al., 2005).

Prior studies in rats with SCI demonstrated the beneficial effects of progesterone treatment. In motoneurons; progesterone restores the expression levels of several molecular markers and subsides chromatolysis. In glial cells, progesterone enhances differentiation of oligodendrocyte progenitors, and modulates transcription factors needed to repair injury-induced demyelination (Labombarda et al., 2006a, 2009, 2010a). With this background on hand, the present study was designed to analyze at two different times of SCI and progesterone administration (3 and 21 days), steroid effects on astrocytes, NG2+ cells, mature oligodendrocytes and microglia, employing immunostaining with specific markers for cell identification. Additionally, the role of proliferation/differentiation in the response to injury and progesterone administration was assessed by double immunofluorescence colocalizaton of bromodeoxyuridine (BrdU) with the astrocyte proteins GFAP and S100β, NG2+ oligodendrocyte precursors, CC1+ oligodendrocytes and OX-42+ microglia/macrophages.

## 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.) and their spinal cords transected at the thoracic level (T10) as we previously described (Gonzalez et al., 2004; Labombarda et al., 2006a, 2009). We verified the completeness of the transection by passing the sharp edge of a 25 G needle through the lesion site. Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI, USA) 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-laminectomized controls, the spinal cord was not lesioned. Half of the animals in the SCI group received daily sc injections of vegetable oil or progesterone (Proluton, Schering, Argentina) at 16 mg/kg/day for 3 or 21 days. The first progesterone injection was given to awaken animals 3 h after injury. Thus, 3 groups of animals were prepared per time period: sham-operated controls, SCI and SCI + progesterone treated rats. We did not include a control + progesterone group because previous morphological, neurochemical and molecular analysis demonstrated the absence of steroid effects in non-injured rats (Labombarda et al., 2002, 2006a, 2009). The dose of progesterone chosen prevents edema, secondary neuronal loss and improves cognitive responses following brain contusion and stimulates NG2 cell differentiation in rats with SCI (Labombarda et al., 2009; Stein, 2001, 2008). Animals were killed 3 or 21 days after SCI or sham operation. These time periods were selected to compare previous effects of progesterone on OPC and mature oligodendrocytes with the response of other glial populations (astrocytes, microglia) (Labombarda et al., 2009).

Screening of cell phenotypes based on staining with specific markers was quantitatively analyzed at the thoracic level immediately caudal to the lesion site. The cell response to SCI and progesterone treatment was measured in representative regions of white matter (dorsal, lateral and ventral funiculus) and gray matter (dorsal and ventral horns). 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 at a minimum.

### *Experimental protocol for bromodeoxyuridine (BrdU) administration*

Two different protocols were employed for cell labeling with BrdU and phenotypic identification using specific cell markers. In protocol I (acute study), rats (n = 5 rats per group) were injected i.p. with 50 mg/kg BrdU at 8, 6, 4 and 2 h before perfusion at day 3 of SCI or sham-laminectomy. This time period is optimal to assess the peak of glial cell proliferation (Alonso, 2005; Hausmann, 2003; Morin-Richaud et al., 1998). Protocol II (chronic study) was employed to study destiny of cells dividing 48–72 h after injury. Rats (n = 5 per group) were injected four times per day with BrdU (50 mg/kg ip) at 2 h intervals on days 2 and 3 after SCI or laminectomy and perfused 21 days after surgery.

### *Single-labeling method for the immunodetection of S100β, glial fibrillary acidic protein (GFAP), OX42 and BrdU positive cells*

Rats anesthetized as described above, were perfused intracardially with 4% paraformaldehyde (PFA), the spinal cords extracted, post-fixed in this fixative for 4 h, embedded into gelatine and left overnight in 4% PFA. Sixty μm vibratome sections were exposed to 0.3% $H_2O_2$ in

methanol for 30 min to block endogenous peroxidase. Nonspecific labeling was blocked with TBS 0.5% Triton X-100 and 3% normal goat serum for 30 min. For BrdU immunohistochemistry, sections were pretreated with 50% formamide in 2X 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. For immunodetection of immature and mature astrocytes we employed a 1/800 dilution of the S100β monoclonal antibody (5G209, Santa Cruz) or a 1/250 dilution of a GFAP polyclonal antibody for reactive astrocytes (G9269, Sigma). Macrophages/microglial cells were stained with a 1/200 dilution of the OX42 monoclonal antibody (CBL 1512, Chemicon) and dividing cells with BrdU mouse monoclonal antibody (1/200, G3G4, Hybridoma Bank, IOWA). After several washes with PBS, sections were incubated with their respective anti-rabbit, mouse 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.

*Double-labeling method for the immunodetection of BrdU and different glial phenotype markers*

For double immunolabeling, a set of slices different from the ones used for single-labeling was incubated with a mouse monoclonal antibody against BrdU (1/200, G3G4, Hybridoma Bank, IOWA) or a rat monoclonal antibody (1/200, OBT 0030 Accurate Chemical) and antibodies against specific glial phenotype markers. We employed: polyclonal GFAP antibody (1/500, G9269 SIGMA), S100β monoclonal antibody (1/800, 5 G209, Santa Cruz), monoclonal Ox-42 antibody (1/200, CBL1512 Chemicon), polyclonal NG2 antibody for staining OPC (1/400, generous gift of Dr. William Stallcup) and monoclonal CC1 antibody for staining mature oligodendrocytes (1/100, OP80 Calbiochem).

In all double-labeling procedures, incubations with the primary antibodies lasted for 2 days and slices were rinsed three times in TBS 0.1% Triton X-100 for 15 min before application of the second antibodies: goat anti-rat IgG conjugated to FITC (1/250, Sigma); goat anti-mouse IgG conjugated to Alexa 555 (1/1000, Molecular Probes) goat anti-mouse IgG conjugated to Alexa 488 (1/1000, Molecular Probes), goat anti-rabbit TRITC (1/250, SIGMA), and goat anti-rabbit FITC (1/250, SIGMA). Incubation with second 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.

*Morphometric analysis*

To examine the total number of positive cells in the white and gray matter regions of the spinal cord mentioned above, 60 μm-thick serial coronal sections were prepared from 1 mm long spinal cord stumps located 5 mm below the lesion. Among these serial sections, 4 representative sections at least 240 μm apart from each other were subject to immunostaining with different antibodies. For single-labeled studies, 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 0.5 μm in the z-axis, and merged using Nikon EZC1 version 2.1 software. Image analysis for quantification was performed using Bioscan Optimas II software.

The number of immunopositive cells counted in dorsal, lateral and ventral funiculus, ventral horn Lamina IX of Rexed and dorsal horn Laminae II–III of Rexed (Watson et al., 2009) were pooled for each glial cell marker because no region effects were found after two-way ANOVA analysis. Cell profiles containing a visible nucleus were counted as positive. Cell quantification was carried out in four sections per animal (n = 5 rats per group), averaged and expressed as number of cells per 0.5 $mm^2$ for each animal. The *n* used for statistical analysis was the number of rats per group.

*Statistical analysis*

Comparison between groups means was carried out by one-way ANOVA followed by the Tukey post-hoc test. Data for 3 and 21 days were analyzed separately. A $p<0.05$ was considered to be significant.

## Results

*Effects of 3 and 21 days of SCI and progesterone treatment on glial cell populations*

Fig. 1 shows the effects of a 3-day and 21-day treatment with progesterone on the immunostaining for the astrocyte proteins S100β (marker of immature and mature astrocytes) and GFAP (marker of reactive astrocytes) in rats with SCI (Figs. 1A and B, respectively). In the 3-day experimental group, cell reaction to injury was similar for both markers, as the number of S100β+ and GFAP+ astrocytes per 0.5 $mm^2$ area significantly increased with respect to sham-controls. However, the astrocyte reaction to SCI was highly sensitive to progesterone treatment given at 16 mg/kg for 3 days. As shown in Figs. 1A and B, progesterone significantly reduced GFAP+ and S100β+ astrocyte density. The changes experienced by S100β and GFAP immunoreactive astrocytes after 21-day treatment with vehicle in rats with SCI (Figs. 1A and B) were remarkably similar to those yielded by these markers 3-days after SCI of rats without progesterone treatment. Thus, density of cells bearing these markers was significantly increased in respect with sham-controls. Similar to the results at 3 days, the astrocyte reaction to SCI was significantly prevented by 21 days of progesterone treatment (Figs. 1A and B).

In parallel with changes of the astrocyte population, OX-42+ cells of the microglial/macrophage lineage significantly increased after 3 days of SCI (Fig. 1C). In consonance to its effect on astrocytes, progesterone treatment for 3 days reduced by half the density of OX-42+ cells of SCI rats, which in this circumstance approached control levels. In the 21 day group OX-42+ cells also responded like astrocytes, showing again a significant increase after SCI and a significant decrease following 3 weeks of progesterone treatment (Fig. 1C). However, in the latter case OX-42+ cells did not completely return to control levels.

The role of cell proliferation in the above mentioned effects was studied by injecting BrdU four times 72 h after injury to label dividing cells. Three days following SCI, the number of BrdU+ nuclei was counted without attempting to identify the phenotype of proliferating cells. Fig. 1D shows that SCI spurred BrdU incorporation whereas progesterone treatment greatly potentiated the injury-induced BrdU+ cell density. Consequently, the pattern of response of BrdU+ cells diverged from the response of S100β, GFAP and OX-42 immunopositive cells. Whereas progesterone administration decreased cells bearing the last three markers, it increased cell proliferation. The last effect may be linked to the increased number of NG2+ cells observed after a 3 day-course of progesterone treatment (Labombarda et al., 2009, 2010a). Quantitative analysis of unclassified BrdU+ cells in rats killed 21 days after SCI demonstrated a significant stimulation respect of unlesioned controls (Fig. 1D). However, in contrast to progesterone's stimulation of cell proliferation at 3 days, the steroid reduced BrdU+ cells when given for 21 days to SCI rats.

Fig. 2 shows typical photomicrographs taken from sham-operated controls, rats with SCI and rats with SCI given progesterone for 3 days. The observation of cell morphology and immunostaining

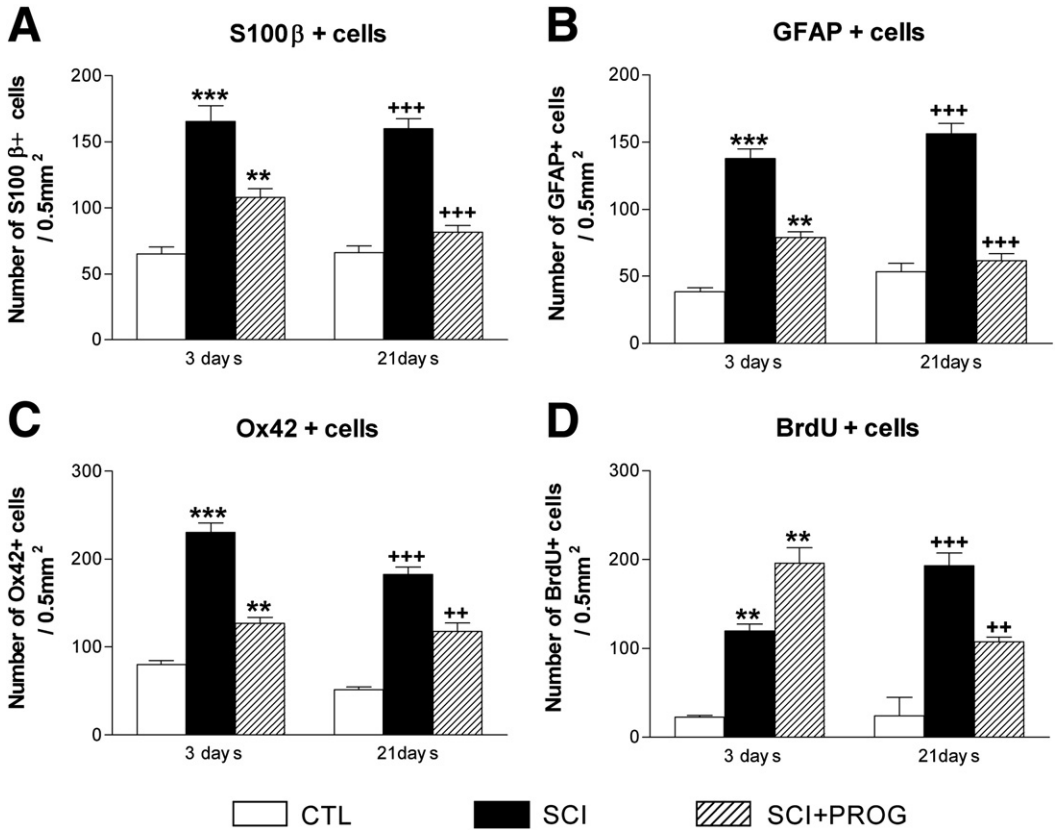


**Fig. 1.** Effects of progesterone on the number of S100β (A), GFAP (B), OX-42 (C) and BrdU (D) positive cells in control spinal cords (white columns), injured spinal cords (SCI, dark columns) and steroid-treated lesioned rats (SCI + PROG, cross-hatched columns) after 3 or 21 days of injury. Results represent the number of inmunopositive cells per 0.5 $mm^2$ (mean ± S.E.M.; 5 rats per group). Statistical comparison between groups was performed by one-way ANOVA followed by Tukey post-hoc test. The density of S100β, GFAP, OX42 and BrdU positive cells increased 3 days after injury (***$p<0.001$ vs CTL, **$p<0.01$ vs CTL) and also 21 days after injury (+++$p<0.001$ vs CTL). Progesterone treatment for 3 days significantly decreased S100β, GFAP and Ox-42 positive cell number (A–C: **$p<0.01$ vs SCI) and stimulated the number of BrdU positive cells above levels of rats with SCI (D: **$p<0.01$ vs SCI). Progesterone treatment for 21 days significantly decreased S100β, GFAP, Ox-42 and BrdU positive cell number (+++$p<0.001$, ++$p<0.01$ vs SCI).

characteristics indicated that SCI markedly increased the number of S100β+ (B), GFAP+ (E) and OX-42+ cells (H) and moderately increased BrdU+ profile number (K). Furthermore, in response to injury, astrocytes as well as microglial cells presented a very reactive phenotype and enlarged bodies. Given during 3 days to rats with SCI, progesterone down-regulated the number of S100β+ (C), GFAP+ (F) and OX-42+ cells (I) but also attenuated the reactive appearance of these cell types. However, more BrdU-labeled nuclei were observed in progesterone-treated rats with SCI (L), a finding explained by the stimulation of NG2+ cell density previously described in rats with SCI receiving a 3 day-course of progesterone treatment (Labombarda et al., 2009). In agreement with the last report (Labombarda et al., 2009), the present work also confirmed that CC1+ oligodendrocytes were reduced 3 days after injury (sham-operation: 525 ± 43,1/0.5 $mm^2$; SCI :220.6 ± 34.4/0.5 $mm^2$ ) whereas progesterone showed no effect in the injured group (189 ± 23.5).

Typical photomicrographs from spinal cord sections of sham-operated controls, rats with SCI and rats with SCI given progesterone for 21 days are shown in Fig. 3. SCI markedly increased the number of S100β+ (B), GFAP+ (E) and OX-42+ cells (H) and moderately increased BrdU+ profile number (K). A more reactive phenotype and enlarged body of astrocytes and microglia were also present in sections from rats with chronic SCI (Figs. 3B; E and H). Twenty one days of progesterone treatment decreased the immunostaining and number of S100β+ (C), GFAP+ (F) and OX-42+ cells (I), normalized their morphological reactivity, and decreased BrdU-labeled nuclei from rats with SCI (L). Progesterone treatment for 21 days normalized the CC1+ oligodendrocyte number which was reduced in steroid-naïve injured rats (sham-operation: 509, 7 ± 40.7/0.5 $mm^2$; SCI :266 ± 39.2 ; SCI plus progesterone : 472, 6 ± 25.7).

*Double immunofluorescence to discern the phenotype of BrdU+ cells*

The phenotype of dividing cells (i.e., cells showing nuclear BrdU incorporation), was investigated by double immunofluorescence staining of spinal cord sections of steroid-naïve and progesterone-treated SCI rats. Using this approach, we determined the number of NG2+/BrdU+, S100β+/BrdU+, GFAP+/BrdU+, Ox-42+/BrdU+ and CC1+/BrdU+ double-labeled cells at 3 days (Fig. 4) and 21 days (Fig. 5). As performed for single labeling, data were combined for white and gray matter regions, and expressed as % double labeled cells with respect to cells found in sham-operated rats. In the 3 day experiment, BrdU+ cells showed an uneven colocalization with the different cell markers. Thus, NG2+/BrdU+ cells, which increased three-fold after SCI, were further increased following progesterone treatment (Fig. 4A). SCI potently stimulated the BrdU+ proliferating S100β+ astrocytes; however, in contrast to the increased density of NG2+/BrdU+ cells, progesterone treatment for 3 days normalized S100β/BrdU double-labeled cells. Interestingly, BrdU incorporation was absent from GFAP+ cells pertaining to the control, SCI or the SCI plus progesterone-treated groups, suggesting that 3 days after SCI, the increase of GFAP+ cells (Fig. 1B) was mostly due to astrocyte hypertrophy rather than hyperplasia.

Fig. 4A also shows that SCI increased and progesterone treatment attenuated OX42 cell proliferation (i.e. OX-42+/BrdU+ cells). In summary, the double-labeling experiment carried out 3 days after

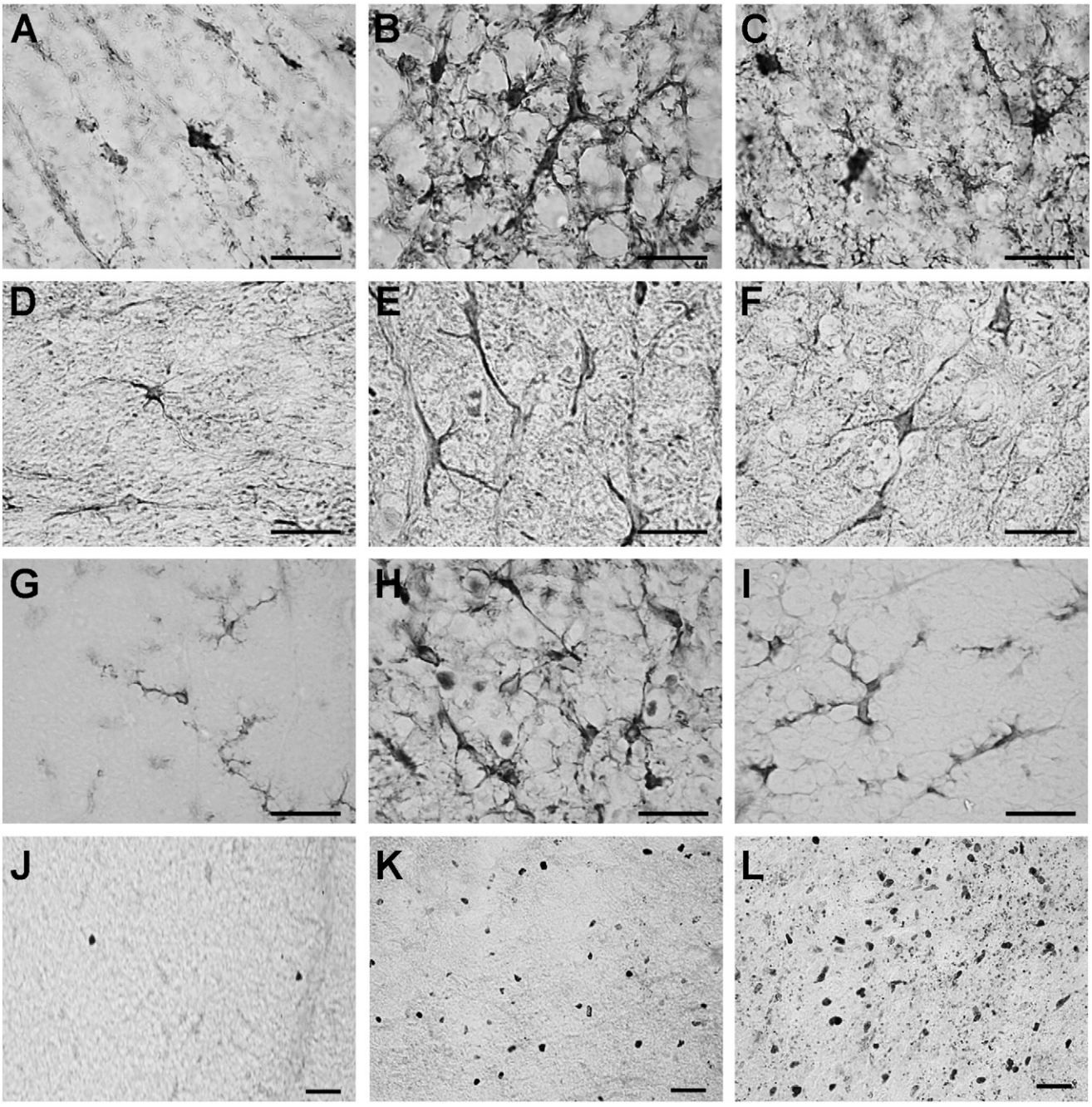


**Fig. 2.** Representative photomicrographs taken from the white matter lateral funiculus at 3 days after SCI, from sham-operated controls (A, D, G, J), rats with SCI (B, E, H, K) and rats with SCI given progesterone (C, F, I ,L).The images represent from top to bottom, S100β+, GFAP+, Ox-42+, and BrdU+ cells. SCI markedly increased the number of S100β+ (B), GFAP+ (E) and OX-42+ cells (H) and moderately increased BrdU+ labeled nuclei (K). Progesterone treatment of rats with SCI down-regulated the number of S100β+ (C), GFAP+ (F) and OX-42+ cells (I) but increased the density of BrdU+ proliferating cells (L). Scale bars for A–L: 25 μm.

SCI, suggested that progesterone enhanced the proliferation of NG2+ cells but inhibited the proliferation of S100β+ astrocytes and microglial cells. To further investigate if the above-mentioned effects of SCI and progesterone treatment on the diverse glial populations originated from changes of proliferation and/or activation of resident cells, we also determined the number of Ng2+/BrdU−, S100β+/BrdU−, GFAP+/BrdU− and Ox-42+/BrdU− cells (Fig. 4B). Stereological analysis showed that SCI increased all cell populations lacking BrdU incorporation but decreased CC1+ cells (Fig. 4B), effects that may involve cellular activation, increased antigen expression, cell hypertrophy and demyelination (Grossman et al., 2001; Ling et al., 1990; Wang and Bordey, 2008). In sharp contrast to the effects of SCI alone, progesterone decreased the non-proliferating fraction of astrocytes and microglial cells (Fig. 4B), but it substantially increased the fraction of non-proliferating NG2+ (i.e. BrdU negative) progenitors. To conclude, a 3 day exposure of the lesioned animals to progesterone depressed proliferation and activation of astrocytes and microglial cells. The reverse action was obtained for NG2+ progenitors, which proliferate and activate under the short-term treatment with progesterone.

We also performed double labeling colocalization to elucidate the phenotype of cells differentiating after 21 days of steroid exposure (Fig. 5). In this experiment, double labeled NG2+/BrdU+ cells were

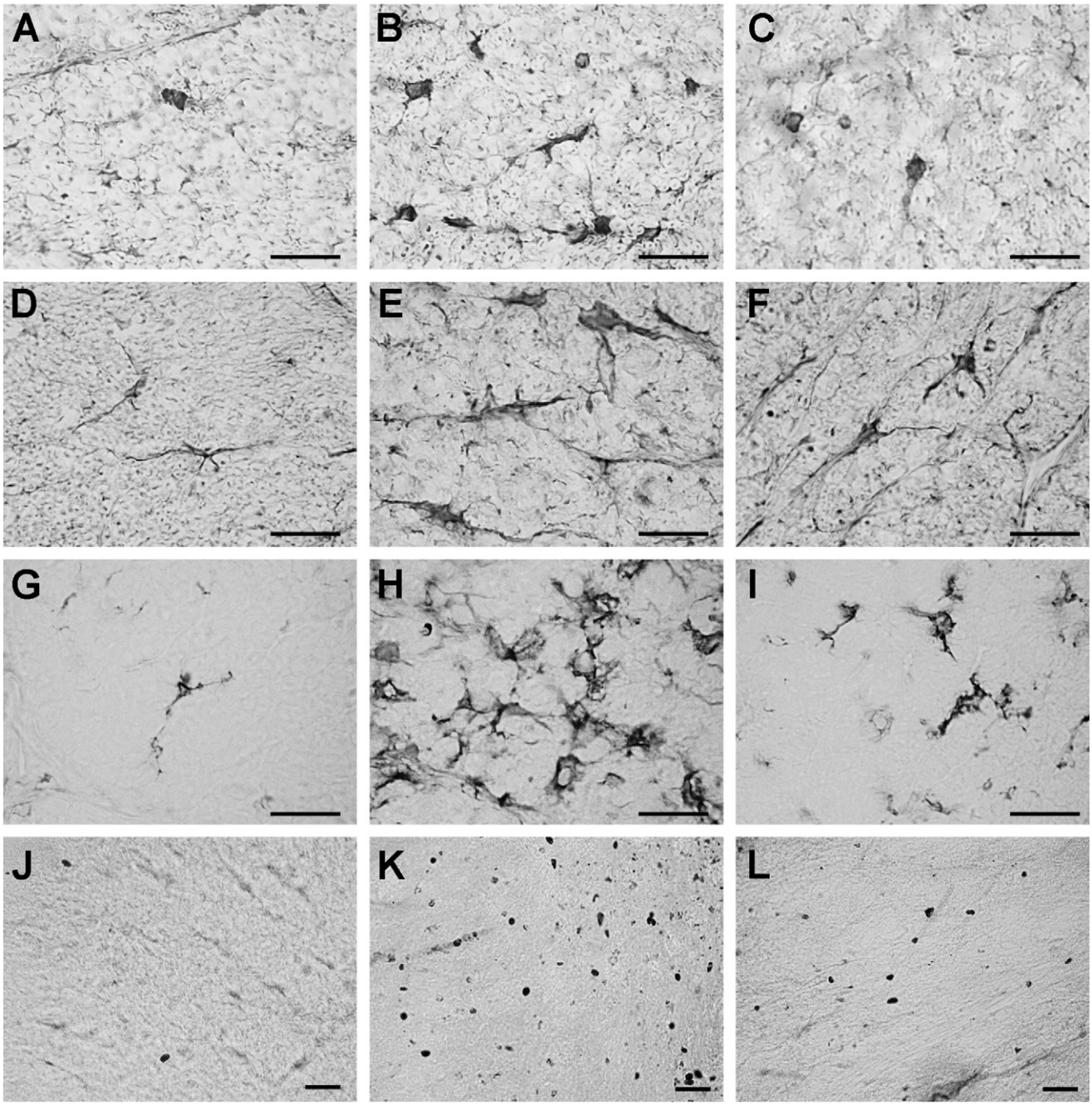


**Fig. 3.** Representative photomicrographs taken from the white matter lateral funiculus at 21 days after SCI, from sham-operated controls (A, D, G, J), rats with SCI (B, E, H, K) and rats with SCI given progesterone (C, F, I ,L).The images represent from top to bottom, S100β+, GFAP+, Ox-42+, and BrdU+ cells. SCI markedly increased the number of S100β+ (B), GFAP+ (E) and OX-42+ cells (H) and moderately increased BrdU labeled nuclei (K). Progesterone treatment to rats with SCI, down-regulated the number of S100β+ (C), GFAP+ (F), OX-42+ cells (I) and BrdU+ cells. Scale bars for A–L: 25 μm.

abundant in rats with SCI but barely present in the spinal cord of the sham-operated or the SCI plus progesterone group (Fig. 5A). Single labeled NG2+/BrdU− cells were also higher in SCI rats compared to sham-controls and to progesterone-treated rats (Fig. 5B). In the last case, the decrease probably reflected differentiation of NG2+ cells into a more mature population that lost this marker (Levine et al., 2001) In the 21 day experiment, stereological analysis of the number of S100β+ and GFAP+ cells retaining a BrdU immunoreactive nucleus produced similar data for both markers (Fig. 5A). Double-labeled GFAP+/BrdU+ appeared now in spinal cord-lesioned animals, suggesting that astrocytes of the S100β+/BrdU+ type undergoing proliferation at 3 days of SCI, were time converted into GFAP+ astrocytes. In the progesterone-treated SCI rats, double labeled GFAP+/BrdU+ astrocytes significantly decreased with respect to SCI alone, because this cell type derived from an already diminished S100β+/BrdU+ population. In addition, progesterone could decrease GFAP expression in astrocytes.

Therefore, while injury promoted astrocyte maturation, progesterone did not modify this process. Regarding microglia/macrophages, distribution of the OX-42 marker with or without BrdU, showed a similar pattern at 3 and 21 days, since at both times SCI increased double and single labeled OX-42 cells and progesterone down-regulated these populations. Important data also derived from the analysis of CC1+/BrdU+ and CC1+/BrdU− cells (Figs. 5A and B).The absence of BrdU labeling of CC1+ oligodendrocytes in progesterone-

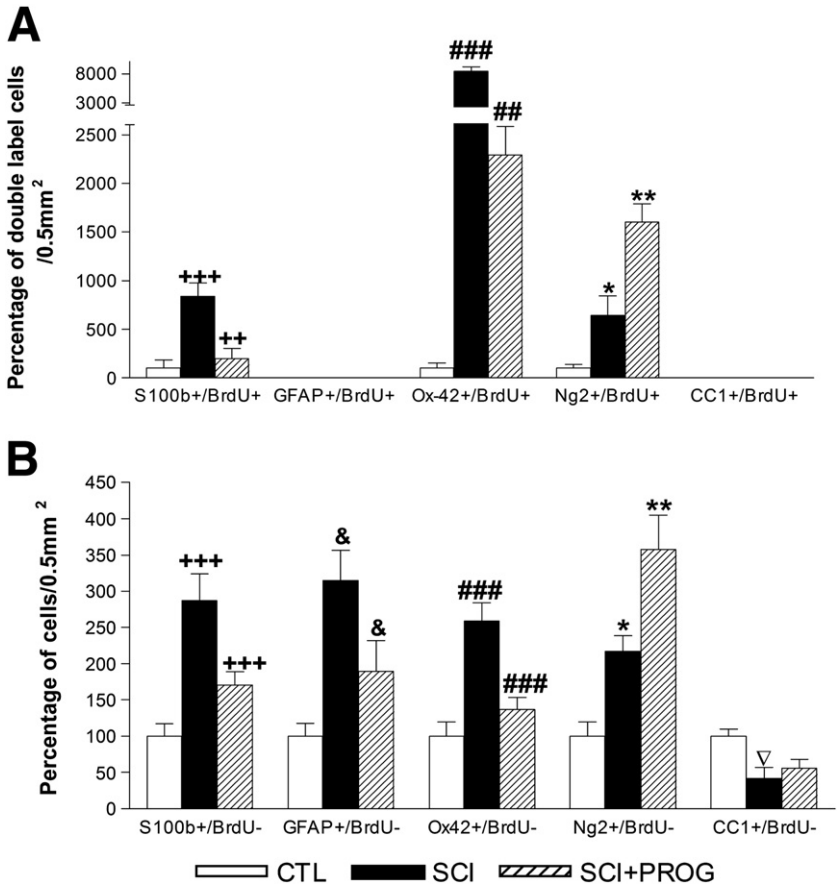


**Fig. 4.** Effects of SCI and progesterone treatment for 3 days on the proliferation of NG2, S100β, GFAP, Ox42 and CC1 positive cells. BrdU was injected to lesioned animals 3 days after SCI; rat perfusion and spinal cord fixation took place 2 h later. (A). Effects of SCI and progesterone treatment on the density of NG2+/BrdU+; S100β +/BrdU+; GFAP+/Brd+; Ox-42+/BrdU+ and CC1+/BrdU+ cells (B). Effects of SCI and progesterone treatment on single labeled cells bearing cell specific markers but BrdU−. The number of double-or single-labeled cells counted for each marker in white and gray matter regions was combined and expressed as % inmunopositive cells respect of sham-operated rats per 0.5 $mm^2$ (mean ± S.E.M.; n = 5 rats per group). Statistical comparisons were performed by one-way ANOVA followed by post-hoc Tukey post-hoc test. A and B, SCI increased % inmunopositive cells for NG2 (*$p<0.05$), S100β (+++$p<0.001$) and Ox-42 (###$p<0.001$, SCI vs their respective CTL). Progesterone treatment further increased the number of NG2 double labeled and NG2+/BrdU− cells (**$p<.001$ vs SCI) and inhibited proliferation of S100β+ astrocytes (++$p<0.01$; +++ $p<0.001$) and OX-42+ microglial cells (##$p<0.01$; ###$p<0.001$ vs SCI). In rats with SCI (plus or minus progesterone treatment) CC1+/BrdU− oligodendrocytes were reduced (∇$p<0.05$ vs sham-operated rats).

naive rats with SCI, implied a block of OPC differentiation into mature cells (Fig. 5A). In addition, this group showed a decrease of pre-existing oligodendrocytes (CC1+/BrdU− phenotype), possible due to an apoptotic mechanism (Fig. 5B). However, 21 days of progesterone treatment increased double labeled CC1+/BrdU+ cells, supporting that progesterone induced the differentiation of a proportion of early proliferating progenitors into mature CC1+ cells. At this time period, CC1+/BrdU− cell density was enhanced compared with the same group at 3 days, suggesting that some NG2+/BrdU− or highly dividing NG2+/BrdU + cells ultimately differentiated into CC1+ cells.

Fig. 6 shows photomicrophotographs representing the double immunofluorescence colocalization and confocal stack with orthogonal views taken from the white matter lateral funiculus. This procedure exemplified our criteria to consider a particular cell as double-labeled. These representative photographs show differentiated oligodendrocytes from a 21 day progesterone-treated rat with SCI (A, A′ and A″), proliferating OPC following 3 days of progesterone treatment of a SCI rat (D, D′, D″), the double colocalization of BrdU with GFAP in mature astrocytes after 21 days of SCI (B, B′, B) and BrdU coinciding with OX-42 in microglial cells (C, C′, C″).

In summary, progesterone treatment exerted separate effects on NG2+, S100β+, GFAP+, OX-42 and CC1+ cells. Changes of GFAP+, S100β + and OX-42 cells indicate progesterone inhibition of astrocyte hyperplasia/hypertrophy and of proinflammatory cells. This glial cell scenario contrasted with progesterone stimulation of NG2 and CC1+ cells, a process related to remyelination of the lesioned tissue.

## Discussion

The present study revealed that progesterone attenuates the reaction of astrocytes and microglial/macrophages cells and enhances oligodendrocyte differentiation following spinal cord injury. The steroid, by inhibiting the proliferation and activation of astrocytes and microglial cells, created an anti-inflammatory environment that might favor remyelination, since the proliferating OPC became differentiated into mature oligodendrocytes after 21 days of progesterone treatment.

### *Changes in astrocytes following SCI and progesterone treatment*

Our study showed that 3 days post-surgery, SCI induced the proliferation of S100β cells, but did not change the number of GFAP+/BrdU+ reactive astrocytes. Since S100β is a marker of immature and mature astrocytes and is normally found in dividing astrocytes (Brozzi et al., 2009), our results suggest that injury may induce the proliferation of immature astrocytes. In agreement with others studies (Alonso, 2005) we did not find proliferation of reactive astrocytes in tissues analyzed distant from the glial scar formation (Buffo et al., 2008; Sofroniew, 2009). Thus, the increase in the number of GFAP+/BrdU− cells observed at this time point was likely due to cellular hypertrophy and activation of astrocytes. The use of S100β as a marker of astrocytes deserves a comment, because a subset of S100β positive cells could be Schwann cells invading the lesioned spinal cord (Totoiu and Keirstead,

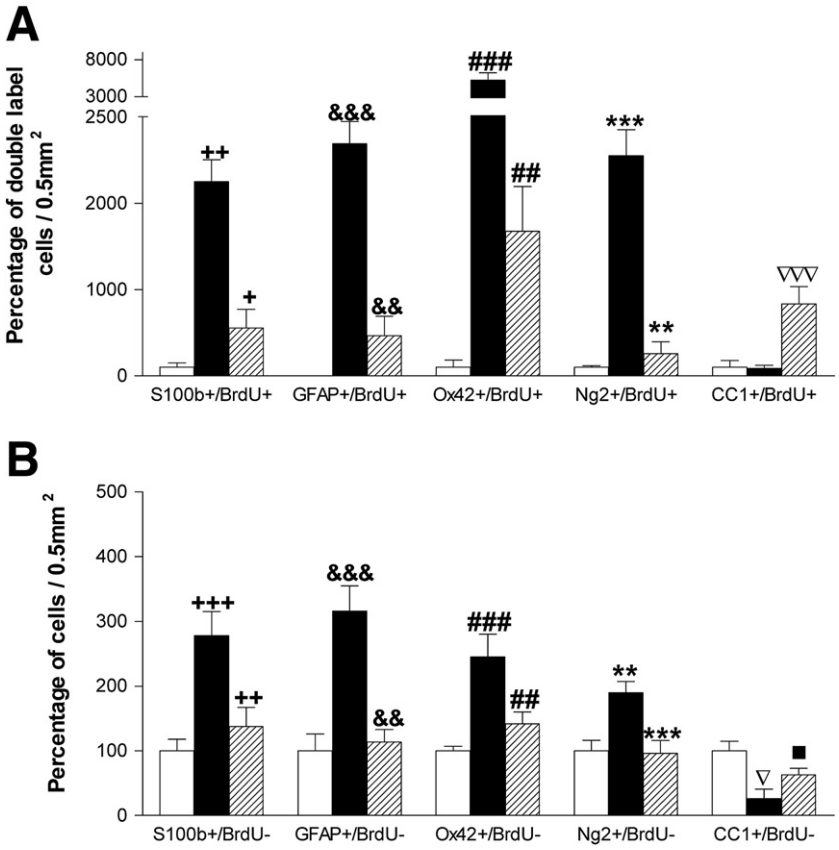


**Fig. 5.** Distribution pattern of individual phenotypes of BrdU+ cells 21 days after SCI with or without progesterone treatment. Animals were injected with BrdU 24–48 h post injury and sacrificed 18 days later (A) Quantitation of NG2+/Brd+−; S100β+/BrdU+; GFAP+/BrdU+; Ox-42+/BrdU+ and CC1+/BrdU+ cells (B) Quantitation of single labeled cells bearing cell specific markers but not BrdU. As done for data of Fig. 4, the number of double-or single label cells counted in white and gray matter regions was combined for each marker and results were expressed as % inmunopositive cells with respect to sham-operated rats per 0.5 $mm^2$ (mean ± S.E.M.; n = 5 rats per group. Statistical comparisons were performed by one-way ANOVA followed by Tukey post-hoc test). A and B: SCI increased all specific cell markers with the exception of CC1+ cells (dark columns, ++p<0.01; +++p<0.001; ###p<0.001; **p<0.01; ***p<0.001). SCI also induced maturation of astrocytes (GFAP+/BrdU+, &&&p<0.001 vs CTL). Progesterone treatment decreased all specific cell markers (+p<0.05; ++p<0.01; &&p<0.01; ##p<0.01). Progesterone treatment for 21 days decreased NG2+/BrdU+ cells (**p<0.01 vs SCI) and simultaneously stimulated the appearance of CC1+/BrdU+ double labeled cells (▽▽▽p<0.001 vs SCI) suggesting the differentiation of OPC into oligodendrocytes. B: SCI decreased mature oligodendrocytes (CC1+/BrdU−; ▽p<0.05 vs CTL), instead progesterone increased the number of CC1+/BrdU−: ■p<0.05 vs SCI.

2005). However, we discarded this possibility, because our studies were carried out away from the epicenter of the lesion, whereas Schwann cell invasion takes place.

The fact that 21 days after injury, double labeled GFAP+/Brdu+ astrocytes appeared, suggests maturation of the S100β +/BrdU+ cells that might have divided earlier, i.e., at 3 days. Furthermore the number of GFAP+ cells remained highly elevated 21 days post-injury, implying a chronic cellular hypertrophy and activation of these astrocytes. In contrast, 3 days of progesterone treatment inhibited the proliferation and the activation of astrocytes, suggesting progesterone attenuation of a harmful reaction to SCI. After 21 days of SCI, rats receiving progesterone still showed the inhibition of reactive astrogliosis, since the number of GFAP+ cells was lower than the cell density of injured rats. The scheme of Fig. 7 shows that only 9% of the total cells counted in the control spinal cord were astrocytes, meanwhile in rats with SCI, the total astrocyte number comprised 23% of the total cells evaluated.

Reactive astrogliosis is a common phenomenon in cases of nervous system injury, excitotoxic damage, aging, neurodegeneration, neuroinflammation, ischemia and metabolic diseases (Benarroch, 2005; Garay et al., 2007; Norenberg, 1994; Revsin et al., 2005; Sofroniew, 2009). Reactive astrocytes might have detrimental effects after injury, including exacerbation of the inflammatory cascade, release of neurotoxic levels of reactive oxygen species and glutamate and the compromise of the function of the blood–brain-barrier, increasing the formation of cytotoxic edema (Sofroniew, 2009). In many of these conditions, steroid hormones play an antigliotic role, by decreasing astrocyte proliferation and activation. The anti-gliotic effect has been already shown for many steroids, including estradiol, selective estrogen receptor modulators, progesterone, androgens and glucocorticoids (Crossin et al., 1997; De Nicola et al., 2006; 2009; Djebaili et al., 2005; Garcia-estrada et al., 1999). Since astrocytes express intracellular and membrane receptors for progesterone (Labombarda et al., 2000b, 2010b; Waters et al., 2008), chances exist for direct steroid effects. Examples for the effects of in vivo administered progesterone on astrocyte function include the inhibition of nitric oxide synthase, GFAP, the proinflammatory cytokines tumor necrosis factor alpha and interleukin 18 (Coughlan et al., 2005; Kipp et al., 2007; Labombarda et al., 2000a; Meyer et al., 2010; Melcangi et al., 1998). It is also known that a selective inactivation of astroglial NF-kB, a key regulator of inflammation and secondary injury cascade, leads to a dramatic improvement of functional recovery in models of SCI and multiple sclerosis (Brambilla et al., 2005, 2009). In this regard, inhibition of NF-kB by progesterone (Cutler et al., 2007; Pettus et al., 2005) points to this neuroactive steroid as a key regulator of astrocyte activation and secondary damage.

*The response of microglia/macrophages to SCI and progesterone treatment*

The steady proliferation and activation of microglial cells and infiltrating macrophages found after 3 and 21 days of SCI are a potential threat to the injured spinal cord. In rats studied after 21 days of SCI, microglial cells raised to 26% of the total number of glial cells,

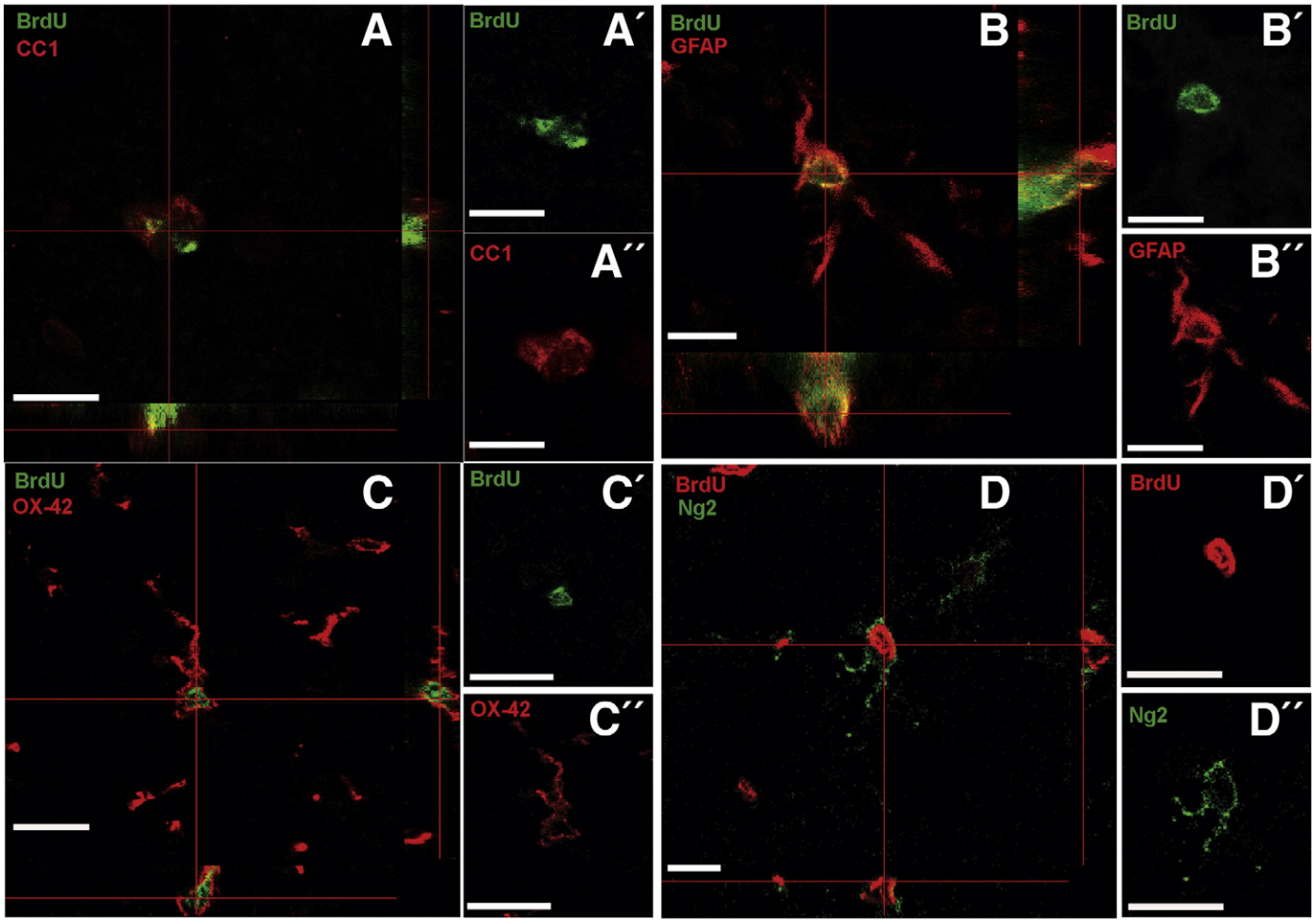


**Fig. 6.** Confocal images showing the phenotype of BrdU-double labeled cells. The images represent 12 μm thick confocal stack with orthogonal views sequentially acquired in a line-scanning mode through an optical section of 0.5 μm in the z-axis. A: BrdU+/CC1+; B: BrdU+/GFAP+; C: BrdU+/Ox-42+ and D: BrdU+/NG2+. Scale bar: A, B, C, D = 15 μm; A′, A″, B′, B″, C′, C″, D′, D″ = 10 μm.

representing an ~3 fold increase over control levels (Fig. 7). Following injury, microglial cells initiate a massive inflammatory response further contributed by astrocytes and blood-borne macrophages (Hanisch and Kettenmann, 2007). In rats with SCI, microglia/macrophages at the lesion site secrete the cytokine tumor necrosis factor alpha (TNFα), an inductor of oligodendrocyte apoptosis that interferes with NG2 cell proliferation (Wu et al., 2010). In spite of this, progesterone and in some instances its reduced derivatives antagonize microgliosis, LPS-induced nitric oxide release, LPS-inducible NOS and NO synthesis, and TNFα production by these cells (Drew and Chavis, 2000; Garcia-Segura and Melcangi, 2006; Muller and Kerschbaum, 2006). Stein has conclusively shown that progesterone reduces the inflammatory cascade that develops after traumatic brain injury (Stein, 2008). Our findings are in complete agreement with these reports, supporting that progesterone anti-inflammatory effects in rats with SCI reduce proliferation and activation of microglial/macrophage cells. In fact, OX-42+ cells were reduced from 26% in steroid-naïve rats to only 14% of the total glial population evaluated after prolonged progesterone treatment (Fig. 7). Microglial cells express the glucocorticoid, mineralocorticoid and estrogen receptor without evidence for a classical PR (Sierra et al., 2008). However, after SCI, microglial cells expressed the α isoform of the membrane PR (mPrα) (Labombarda et al., 2010b) that might be involved in the down-regulation of proliferation and activation of OX-42+ cells exerted by progesterone.

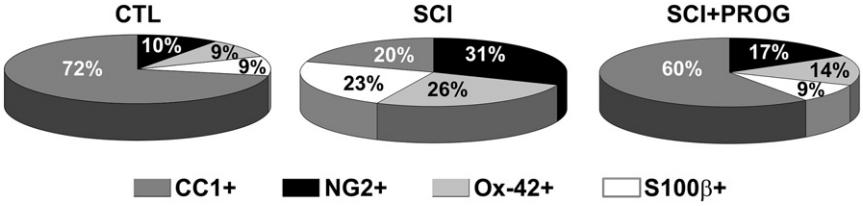


**Fig. 7.** Schematic distribution of glial cell populations after SCI and progesterone treatment for 21 days. Data are presented as % of each immunopositive cell group over the total number of cells counted in 0.5 $mm^2$ of the spinal cord. Group labeling as described in legend to Fig. 1. Microglial (Ox-42+) astrocytes (S100β+) and NG2 cells are the most abundant cell populations in the injured spinal cord. However progesterone treatment changed the distribution of glial cells, since abundance of astrocytes and microglia cells after SCI was replaced by CC1+ cells. Thus, the most abundant glial type in rats with SCI receiving progesterone was the mature oligodendrocyte (CC1+ cells).

It is possible that an early modulation by progesterone of the inflammatory response involving NF-kB inactivation in microglia/macrophages (Cutler et al., 2007; Pettus et al., 2005), leads to a secondary inhibition of the astrocyte reaction. Work is in progress in our laboratory to disclose the cytokines and pro-inflammatory factors that accompany the changes of microglia/macrophages in rats with SCI receiving vehicle or progesterone treatment.

*Progesterone effects on the oligodendrocyte lineage in rats with SCI*

Our experiments support that oligodendrocytes are acutely vulnerable in response to injury (Horky et al., 2006), since they were reduced by half 3 days following SCI. At this time period, however, progesterone did not stop the loss of CC1+ oligodendrocytes; instead, it enhanced the proliferation of BrdU+ immature oligodendrocytes (NG2+/BrdU+ cells). Thus, as early as 3 days following injury, progesterone was already emerging as a pro-myelinating factor, because successful remyelination requires a prior progenitor proliferation (Baumann and Pham-Dinh, 2001; McDonald and Belegu, 2006). In agreement with other reports, we found that SCI stimulated the OPC proliferation. However, successful remyelination may be aborted because progenitors cannot be converted into myelin-producing cells (Horky et al., 2006; Rabchevsky et al., 2007; Zai and Wrathall 2005). Regarding why NG2+ cells proliferate in response to progesterone, preliminary experiments employing real time PCR to determine mRNA levels of PDGFα receptor, a factor involved in NG2 proliferation (Zai et al., 2005), demonstrated that PDGFα receptor mRNA decreased after spinal cord injury (CTL: $0.89 \pm 0.09$ vs SCI: $0.44 \pm 0.05$, $p<0.05$) and was normalized by 3 days of progesterone treatment (SCI + progesterone $0.95 \pm 0.1$, $p<0.05$ vs. SCI, one way ANOVA and post-hoc test). Therefore, up-regulation of PDGFα receptor expression may be one factor explaining progesterone control of NG2 cell proliferation.

Twenty one days of continuous exposure to progesterone decreased the number of NG2+/BrdU+ cells in parallel with a significant increase of CC1+/BrdU+ cells, suggesting differentiation of NG2 cells into mature CC1+ oligodendrocytes. At this time period, there was a decrease of BrdU+ cells, in comparison with the increased number of this cell phenotype that occurred 3 days after SCI. The cause of the decreased number of BrdU+ cells in the 21-day progesterone-receiving group may have several explanations. First, the survival of some of these cells may be compromised, similar to the reported effects of estradiol in the control of cell proliferation and survival in the hippocampus (Barker and Galea, 2008). On the other hand, the decreased number of BrdU+ cells may be due to the dilution of labeling, caused by increased NG2 division after the last BrdU injection. In support of this hypothesis, we found in the group receiving progesterone for 21 days an increased number of CC1+/BrdU− cells, compared with the same group at 3 days, suggesting that surviving NG2+ / BrdU+ and /or NG2+/BrdU− cells differentiated into cells bearing the CC1+ /BrdU− phenotype.

In rats sacrificed 3 days after injury, our previous report has shown that progesterone increased the mRNA for myelin basic protein (MBP), one of the major central myelin proteins, and the mRNA for the oligodendrocyte transcription factors Olig2 and NKx2.2 which are related to oligondendrocyte differentiation (Labombarda et al., 2009). This change supports that an early modulation of the above mentioned transcription factors may be involved in progenitor differentiation into CC1+ mature oligodendrocytes. In the present work, we observed that 21 days of progesterone treatment, increased the percentage of CC1+ oligodendrocytes compared to progesterone-naive rats (Fig. 7). Although measurement of CC1 alone does not mean mature oligodendrocytes, previous work from our group has reported that under identical experimental conditions (i.e., SCI rats given 16 mg/kg progesterone for 21 days) there is an increase in proteolipid protein (PLP) immunostaining, using an antibody that recognizes PLP protein present in myelinating cells (Labombarda et al., 2009). Thus, changes of CC1+ cells observed under chronic progesterone treatment are accompanied by increases in PLP, an index of oligodendrocyte maturity.

Additionally, the dramatic changes in the endogenous synthesis and metabolism of progesterone observed during oligodendrogenesis in culture assigns a direct steroid role on the precursors (Gago et al., 2004). This possibility is supported by evidence for the classical intracellular PR and the mPRα in oligodendrocytes (Jung-Testas et al., 1996; Labombarda et al., 2010b; Mitterling et al., 2010). Progesterone's reduced metabolite allopregnanolone, which increases after SCI and progesterone treatment (Labombarda et al., 2006b), exerts promyelinating activity in the PNS and CNS (Azcoitia et al., 2003; Ciriza et al., 2004; Djebaili et al., 2001; Ghoumari et al., 2003) and enhances OPC proliferation in vitro (Gago et al., 2001, 2004). Thus, non-genomic effects on these cells cannot be discarded. Through these various mechanisms, progesterone may target NG2 and CC1+ cells favoring remyelination.

*Possible mechanisms of progesterone action on glial cells*

Although the molecular mechanisms employed by progesterone to modulate glial cell populations after SCI were beyond the scope of our current study, some possibilities are worth mentioning. As mentioned before, several forms of progesterone receptors have been described in glial cells of the spinal cord, including the classical PR, the membrane isoforms mPR α, mPRβ and 25DX ( Labombarda et al., 2000b; Labombarda et al., 2010b). One way to elucidate if effects on glial cells are genomic or membrane-mediated, would require the use of PR KO mice. Work is in progress in our laboratory to specify if effects of SCI and progesterone treatment obtained in male rats are reproduced in mice. Second; use of the antagonist RU486 would also implicate PR in progesterone actions. A problem may arise here because work performed in one of our intervening laboratories has shown that RU486 is itself neuroprotective (Ghoumari et al., 2006). The third point is the possibility that progesterone effects may not be direct but due to a local increase estradiol synthesis. This is an important issue because Lorenz et al.(2009)have shown that progesterone neuroprotection in damaged cortical neurons could be accounted by increased estradiol synthesis. This possibility has been explored in preliminary experiments carried out in the spinal cord of male rats treated with progesterone. Our results have shown that the levels of aromatase mRNA measured by qPCR did not differ from those found in control animals (CTL: $1.2 \pm 0.74$, SCI + PROG $0.67 \pm 0.2$ NS ANOVA, Labombarda et al., unpublished data) suggesting that progesterone effects could be direct. However, further experiments are needed to fully clarify the role of estradiol as a possible mediator of progesterone action.

## Conclusions

Our data supports that the responses of glial cells to SCI are controlled in a context-dependent manner by complex and combinatorial events involving different cells types. Microglia cells are a source of cytotoxic molecules leading to chronic inflammation and also promote astrogliosis, oligodendrocyte death, and inhibition of OPC proliferation and differentiation. Furthermore, reactive astrocytes can also stimulate microglial cells by releasing cytokines, which in turn amplified tissue damage. We demonstrated that SCI and progesterone greatly modified the distribution of glial cell populations in opposite directions (Fig. 7). After SCI, astrocytes and microglial cells increased from 9% to 23% and from 9% to 26%, respectively, compared with sham-operated rats. Simultaneously, mature oligodendrocytes decreased from 72% to 20% (Fig. 7). Progesterone, instead, created a pro-myelinating environment by changing the glial cell distribution, since 60% of the total cells counted were oligodendrocytes whereas astrocytes and microglia accounted for only 9% and 14%, respectively (Fig. 7). Decreased astrogliosis may be due to progesterone-induced

apoptosis of astrocytes (Arnold et al., 2008) and the inhibition of astrocyte proliferation mediated by a decay of the pro-inflammatory cytokines. Within this cellular and molecular context progesterone emerged as a cytoprotective molecule on oligodendrocytes and its progenitors, owing in part to the inhibition of the inflammatory and harmful interplay between astrocytes and microglia. On the other hand, the multiplicity of effects that progesterone and reduced derivatives exert on OPC proliferation/differentiation and on myelin genes makes us consider that progesterone targets the oligodendrocyte lineage. Whereas there is no current evidence that progesterone prevents apoptotic death of mature oligodendrocytes, it may increase survival of oligodendrocyte progenitors due to its anti-inflammatory properties. Thus, our observations further support the role of progesterone as a promising therapeutic agent for treatment of SCI.

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