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PROGESTERONE INCREASES OLIGODENDROGLIAL CELL PROLIFERATION IN RAT CEREBELLAR SLICE CULTURES

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Abstract—We have previously demonstrated that progesterone significantly increases the rate of myelination in organotypic slice cultures of 7-day-old rat and mouse cerebellum. Here, we show that progesterone (20 μM) stimulates the proliferation of oligodendrocyte precursors in cultured cerebellar slices of 7-day-old rats. The steroid increased the number of pre-oligodendrocytes ($NG2^+$, $O4^+$) and to some extent of oligodendrocyte precursors, corresponding to an earlier developmental stage (nestin$^+$, PDGFαR$^+$, $NG2^+$, $O4^-$). Progesterone stimulated the proliferation of both $NG2^+$ and $O4^+$ cells as shown by increased double-immunolabeling with the cell proliferation marker Ki67. The mitogenic effect of progesterone was inhibited by the progesterone receptor antagonist mifepristone (10 μM) and could not be mimicked by its GABA-active metabolite 3α,5α-tetrahydroprogesterone (allopregnanolone), even at the high concentration of 50 μM. Results indicate that progesterone first strongly and transiently stimulates the proliferation of oligodendrocyte precursors, and that it may thereafter accelerate their maturation into myelinating oligodendrocytes. Although oligodendrocyte precursors may be a direct target for the actions of progesterone, their number may also be indirectly influenced by the effects of the steroid on neurons and microglial cells, since treatment of the cerebellar slices with progesterone enhanced staining of the neuronal cytoskeleton marker microtubule-associated protein-2 and increased the number of $OX\text{-}42^+$ microglia. A small percentage (about 0.1%) of the $NG2^+$ cells transiently became $OX\text{-}42^+$ in response to progesterone. These results point to novel mechanisms by which progesterone may promote myelination in the CNS, specifically by stimulating the proliferation and maturation of oligodendrocyte precursors into myelinating oligodendrocytes.

Key words: progesterone, oligodendrocytes, microglial cells, cerebellum.

There is a growing recognition that the physiological functions of gonadal steroid hormones need to be extended beyond their role in reproduction. Thus, progesterone (PROG) has been shown to play an important role in the viability of neurons and in the formation of myelin sheaths (Schumacher et al., 2002, 2004). Moreover, PROG present in nervous tissues originates either from the steroidogenic endocrine glands as this steroid easily crosses the blood–brain barrier, or from local synthesis. Steroids which can be synthesized within the nervous system qualify as neurosteroids (Baulieu, 1997).

An important role for PROG in myelination has first been demonstrated in the regenerating mouse sciatic nerve and in co-cultures of sensory neurons and Schwann cells (Koenig et al., 1995). More recently, it has been shown that the administration of PROG allows to reduce aging-associated morphological abnormalities of myelin in the rat sciatic nerve and that the progesterone receptor (PR) of myelin-forming Schwann cells is a pharmacological target for the treatment of peripheral neuropathies (Azcoitia et al., 2003; Sereda et al., 2003). Recent work has shown that the increase in peripheral myelin protein synthesis by progestins involves the activation of both intracellular PR and membrane $GABA_A$ receptors located on Schwann cells (Magnaghi et al., 2001). The PROG metabolite 3α,5α-THP (3α,5α-tetrahydroprogesterone, allopregnanolone), is a potent positive modulator of $GABA_A$ receptors (Lambert et al., 2003).

There is now increasing evidence that PROG also promotes myelination by oligodendrocytes in the CNS. Already several years ago, PROG has been shown to increase the number of myelin basic protein (MBP)-positive oligodendrocytes in cultures of glial cells prepared from neonatal rat brain (Jung-Testas et al., 1989). More recently, the prolonged administration of PROG was found to improve the slow and inefficient remyelination that occurs in the brain of old male rats (Ibanez et al., 2003, 2004). In organotypic slice cultures of 7-day-old (P7) rat and mouse cerebellum, PROG significantly increased the rate of myelination, evaluated by immunofluorescence analysis of MBP (Ghoumari et al., 2003). As in peripheral nerves, the promyelinating effects of PROG in cerebellar slices involved both the intracellular PR and $GABA_A$ receptors, although activation of the former was required. In fact, PROG had no effect on myelination in PR knockout mice (Ghoumari et al., 2003).

PROG may accelerate CNS myelination by stimulating the proliferation or maturation of oligodendrocyte precursors. Such a mechanism was suggested by the observation that PROG also increased the immunostaining of two

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Abbreviations: DIV, days *in vitro*; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; NeuN, neuronal nuclei; OPC, oligodendrocyte progenitor cell; PBS, phosphate-buffered saline; PBSGTA, phosphate-buffered saline 0.12 M (pH 7.4) containing 0.25% Triton X-100, 0.2% gelatin, 0.1% sodium azide; PDGFαR, platelet-derived growth factor α receptor; PR, progesterone receptor; PROG, progesterone; P7, 7-day-old, postnatal day 7; RU486, mifepristone; 3α,5α-THP, 3α,5α-tetrahydroprogesterone, allopregnanolone; 5α-DHP, 5α-dihydroprogesterone.

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other markers of the oligodendroglial lineage in cerebellar slices, namely, galactocerebroside (GalC) and O4 antigen (Ghoumari et al., 2003). Thus, PROG could be part of the growth factors involved in the regulation of oligodendrocyte precursor proliferation and maturation (Miller, 2002).

As summarized in Fig. 1, the differentiation of oligodendrocytes progresses through a series of stages with distinct functional, morphological and antigenic characteristics (Hardy and Renolds, 1991; Levine et al., 2001; Gago et al., 2003). The oligodendrocyte precursor cells express platelet-derived growth factor α receptors (PDGFαR) (Pringle and Richardson, 1993; Grinspan et al., 1995), the intermediate filament protein nestin and the chondroitin-sulfate proteoglycan NG2 (Gallo and Armstrong, 1995; Nishiyama et al., 1996a; Dawson et al., 2000) (Fig. 1). Both PDGFαR and NG2 are highly co-localized on oligodendrocyte precursor cells, and it has been proposed that their interaction may be necessary for the cells to effectively respond to the mitogenic stimulus of PDGF (Nishiyama et al., 1996b). The oligodendrocyte precursor cells arise in germinal zones and then migrate throughout the CNS (Hardy and Reynolds, 1991; Pringle and Richardson, 1993; Lee et al., 2000). In the cerebellum, $NG2^+$ cells already appear during the last embryonic days and then proliferate over the first 10 days of postnatal life (Levine et al., 1993).

Co-expression of NG2 and O4 antigen defines a distinct stage of oligodendroglial maturation, referred to as pre-oligodendrocytes (Reynolds and Hardy, 1997). As $O4^+$ cells continue to differentiate, they no longer express NG2 and begin to produce GalC, an early marker of differentiated oligodendroglia (Compston et al., 1997). Fully mature myelinating oligodendrocytes continue to be labeled by the O4 antibody, as it also recognizes components of the myelin sheaths such as sulfatides (Bansal et al., 1992; Reynolds and Hardy, 1997) (Fig. 1).

Organotypic slice cultures provide a powerful tool for studying maturational processes such as myelination (Berger and Frotscher, 1994; Gähwiler et al., 1997). In the present study, we used organotypic slice cultures of P7 rat cerebellum to examine the effects of PROG on oligodendrocyte precursor proliferation and differentiation. At this developmental stage, a large number of Purkinje cells survive and axons start to be myelinated (Dusart et al., 1997; Ghoumari et al., 2000; Notterpek et al., 1993).

Our results show that PROG induces a strong and transient increase in the proliferation of $NG2^+$ and $O4^+$ pre-oligodendrocytes within the cerebellar slices. The mitogenic effect of PROG on oligodendrocyte precursors was mediated by the intracellular PR and could not be mimicked by the GABA-active metabolite 3α,5α-THP. PROG also significantly increased the proliferation of oligodendroglial cells at an earlier $NG2^+$ developmental stage, when cells still express nestin. In addition, PROG may favor the differentiation of pre-oligodendrocytes into premyelinating $O4^+$ and $GalC^+$ oligodendrocytes, in agreement with our previous observation that PROG increases O4- and GalC-immunoreactivities in cerebellar slices. Concerning other cell types, PROG did not influence the number of neurons, but increased the density of the neuronal cytoskeleton,

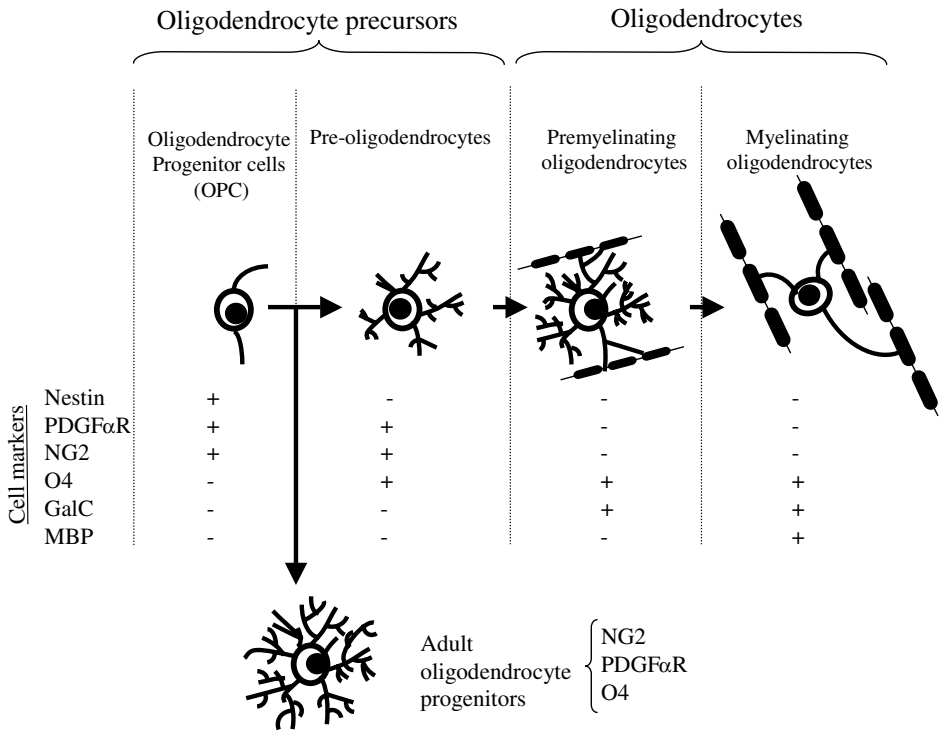

Fig. 1. Schema illustrating the different stages of development of the oligodendrocyte lineage. Different cell markers were used to identify the developmental status of the glial cells (NG2, a membrane chondroitin sulfate proteoglycan; O4 antigen) (adapted from Levine et al., 2001; Gago et al., 2003).

stained with an antibody against microtubule-associated protein-2 (MAP-2). Treatment with PROG also increased the number of OX-42$^+$ microglial cells and resulted in the transient co-expression of OX-42 and NG2 in a small percentage of cells.

EXPERIMENTAL PROCEDURES

Slice cultures

Cerebellar slices were prepared from P7 Sprague–Dawley rats (Janvier, Le Genest St Isle, France). For each experiment, at least three animals and 18 slices were used. All procedures concerning animal care and use were carried out in accordance with the European Community Council Directive (86/609/EEC). All animal procedures were approved by the animal care and use committee at the institute. After decapitation, brains were dissected out into cold Gey's balanced salt solution containing 5 mg/ml glucose (GBSS-Glu) and meninges were removed. Cerebellar parasagittal slices (350 μm thick) were cut on a MacIlwain tissue chopper and transferred onto membranes of 30 mm Millipore culture inserts with 0.4 μm pore size (Millicell, Millipore, Bedford, MA, USA). Slices were maintained in culture in six-well plates containing 1 ml of medium at 35 °C in an atmosphere of humidified 5% CO_2. The medium was composed of 50% basal medium with Earle's salts (Invitrogen, Gaithersburg, MD, USA), 2.5% Hanks' balanced salts solution (Life Technologies), 25% horse serum (Life Technologies), L-glutamine (1 mM) and 5 mg/ml glucose.

Steroid treatments

PROG was applied to the medium of P7 rat cerebellar slice cultures in ethanol. The final concentration of ethanol was 0.1%. Control media contained the same amount of vehicle. Based on our previous study (Ghoumari et al., 2003), the concentration of PROG used in the present study was 20 μM. The 5α-reduced metabolites of PROG, 5α-DHP and 3α,5α-THP, were added to the culture medium at 50 μM, and the PR antagonist mifepristone (RU486) at 10 μM. Cerebellar slices taken from P7 animals were cultured for 1, 3, 7 and 15 days *in vitro* (DIV). Medium, with newly applied steroids, was replaced once after 2 or 3 days. Steroids were purchased from Steraloids and RU486 has been previously provided by Roussel-Ucclaf.

Antibodies and staining procedures

The following primary antibodies were used: rabbit polyclonal antibody against Calbindin D-28K (1/10,000 dilution; Swant, Bellinzona, Switzerland) to visualize Purkinje cells, monoclonal antibodies against MBP (1/1000 dilution; Chemicon International, Temecula, CA, USA), mouse anti-oligodendrocyte marker O4 monoclonal antibody (1/50 dilution, Chemicon International), mouse anti-NG2 chondroitin sulfate proteoglycan monoclonal antibody (1/1000 dilution, Chemicon International), rabbit anti-NG2 chondroitin sulfate proteoglycan polyclonal antibody (1/1000 dilution, Chemicon International), mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1/1000 dilution Chemicon International), mouse monoclonal anti-MAP-2 (clone HM-2, 1/1000 dilution, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal antibody RTU-Ki67-MM1 and rabbit polyclonal antibody NCL-Ki67p (1/500 dilution, Novocastra Laboratories, Newcastle, UK), mouse anti-nestin monoclonal antibody (1/1000 dilution, Chemicon International), rabbit polyclonal anti-PDGFαR antibody (clone AB900, 1/20,000 dilution, kindly provided by Dr. Akiko Nishiyama from University of Connecticut, Storrs, CT, USA), monoclonal anti-microglia/macrophages (OX-42$^+$) (1/500 dilution, Serotec, UK), monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (clone G-A-5, 1/1000 dilution, Sigma), polyclonal rabbit anti-S100 antibody (code No. Z-0311, 1/100 dilution, Dakocytomation, Glostrup, Denmark) to label glial cells.

The organotypic cultures were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 1 h at room temperature. After washing in phosphate-buffered saline (PBS), slices were taken off the Millicell membranes and processed for immunocytochemistry. In all cases, slices were incubated for 1 h in phosphate-buffered saline 0.12 M (pH 7.4) containing 0.25% Triton X-100, 0.2% gelatin, 0.1% sodium azide (PBSGTA) and lysine (0.1 M), before the overnight incubation with the primary antibodies (diluted in PBSGTA). The following secondary antibodies were used: goat anti-rabbit CY3 (1/200 dilution; Jackson ImmunoResearch Laboratories, Inc., West Baltimore Pike, USA), and goat anti-mouse Alexa Fluor488 (1/1000 dilution, Molecular Probes, Leiden, Netherlands). After 2 h incubation, slices were washed several fold in PBS, mounted with mowiol (Calbiochem) and analyzed using a fluorescent microscope (Axiovert 135 M Zeiss, Carl Zeiss Inc., Germany).

Quantification of oligodendrocyte staining in cerebellar slice cultures

Oligodendrocyte staining in organotypic slice cultures of rat cerebella was measured using a confocal Zeiss LSM 410 (Carl Zeiss Inc., Germany) image analyzing system. Images were acquired with confocal configuration (488 nm and 543 nm excitations). For each measurement, a region of interest was located around the deep nuclei zone and the apical ends of each lobule, according to our observation that in addition to myelination, glial cells are more dense around the deep nuclei zone than in other regions in the cerebellum. The immunolabeling density was measured in these areas, using NIH Image software. Staining density was quantified on a continuous scale of 0–255 (darkest). In order to minimize differences among the respective measurement, we set as control an arbitrary level of staining at 100. The staining density was evaluated as: % (light pixels/light+dark pixels).

The density of MAP-2 staining, studied in the present report, was also quantified using this equation. However, OX-42$^+$ microglial cells were numbered on semi-thin sections (1 μm) using a fluorescent microscope (Axiovert 135 M Zeiss, Carl Zeiss Inc.). Microglial cells were quantified at least in three fields (300 μm^2) within the same slice and more than three slices per experiment were used.

Three different experiments were performed for the quantification of NG2$^+$ cells, O4$^+$ and the double-labeled NG2$^+$/O4$^+$ cells. In each experiment, two rat cerebellum ($N=6$) were used and at least three slices from each cerebellum were analyzed ($n=18$). NG2$^+$, O4$^+$ and NG2$^+$/O4$^+$ cells were counted on thin-section confocal images (1 μm) within an area of 0.1 mm^2 around the deep nuclei region of cerebellar slices.

Statistical analysis

Data were expressed as mean±S.E.M. of at least 18 cerebellar slices ($n=18$) from three independent animals ($n=3$) and in three independent experiments. Newman-Keuls tests after one-way ANOVA were used for multiple group comparisons and Student's *t*-test for two group comparisons. The level of significance was set at $P \leq 0.05$.

RESULTS

We have previously shown that PROG accelerates myelination in cerebellar slices from P7-rats, as evaluated by immunofluorescence analysis of MBP (Ghoumari et al., 2003). Whether PROG promotes myelination by stimulating the proliferation of oligodendrocyte precursor cells is the main report of the present study. This possibility was

suggested by the observation that PROG also increased the density of other oligodendroglial markers, namely $O4^+$ and $GalC^+$ (Ghoumari et al., 2003), which are expressed by immature and myelinating oligodendrocytes (Reynolds and Hardy, 1997).

PROG increases $NG2^+$ and $O4^+$ staining in cerebellar slices

To examine whether PROG increases the staining of markers of oligodendrocyte precursors in P7-cerebellar slices, we quantified NG2- and O4-immunostaining by using the NIH Image software (Fig. 2). The proteoglycan NG2 is already expressed by oligodendrocyte progenitor cells (OPC), which still do not express the O4 antigen ($NG2^+/O4^-$) (Gallo and Armstrong, 1995; Dawsson et al., 2000). Its co-expression with the O4 antigen defines a later and distinct stage of the oligodendroglial lineage, referred to as pre-oligodendrocytes ($NG2^+/O4^+$). Pre-myelinating and fully mature oligodendrocytes no longer express NG2, but continue to be labeled with the O4 antibody ($NG2^-/O4^+$) (Bansal et al., 1992; Reynolds and Hardy, 1997) (Fig. 1).

Cerebellar slices were cultured for 1, 3 or 7 days in the absence (controls) or presence of 20 μM PROG. The culture medium containing PROG was replaced once at 3 DIV. At 1, 3 or 7 DIV slices were fixed and immunostained for NG2 and the O4 antigen. Treatment of the cerebellar slices with 20 μM PROG for 3 days increased the density of NG2 stain (1.5-fold) and O4 stain (two-fold) when compared with controls (Fig. 2). However, at 7 DIV, the density of NG2 stain in PROG-treated slices decreased to levels comparable to those observed in control slices. In contrast, the density of O4 stain continued to increase and reached levels three-fold above the controls in the PROG-treated slices. These changes in staining density in response to PROG were mainly observed and quantified around the deep nuclei area of the cerebellum.

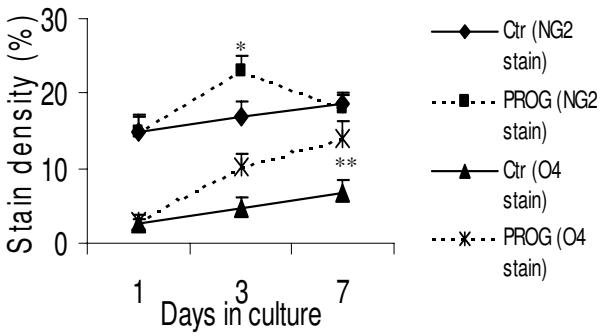

Fig. 2. PROG increased the staining densities of oligodendrocyte precursor cells labeled with antibodies against NG2 or the O4 antigen. P7 cerebellar slices were treated with 20 μM PROG or with vehicle (0.1% ethanol) for 1, 3 or 7 DIV. NG2 stain and O4 stain were quantified by using the NIH Image software and were expressed as % of light pixels (means±S.E.M.). The measurements were performed on multiple images, acquired with a confocal Zeiss LSM 410. These multiple images (1 μm thin) formed a mosaic of 4×4 images in order to integrate maximal NG2- or O4-staining on slices. NG2- and O4-immunostaining markedly increased in PROG-treated slices when compared with controls (Ctr) at 3 DIV. At 7 DIV, the density of O4 stain continued to increase in PROG-treated slices, whereas the density of NG2 stain decreased to control levels (* $P \leq 0.05$, ** $P \leq 0.01$ when compared with control by Newman-Keuls tests after one-way ANOVA).

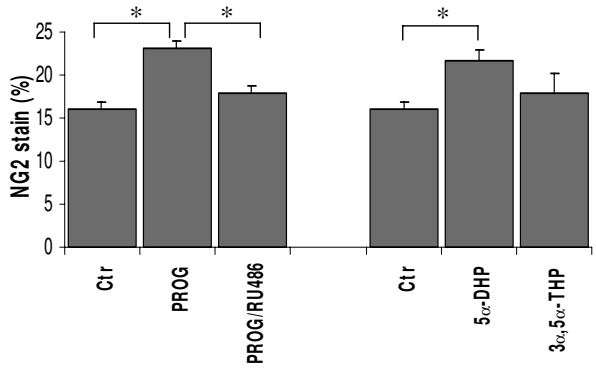

Fig. 3. The effect of PROG on NG2 stain involves intracellular PRs. Cerebellar slices from P7-rats were treated for 3 days with PROG (20 μM), PROG (20 μM)+RU486 (10 μM), 5α-DHP (50 μM), 3α,5α-THP (50 μM) or 0.1% ethanol vehicle (controls, Ctr). The increase in the NG2 stain by PROG was inhibited by RU486. 5α-DHP, but not 3α,5α-THP, also significantly increased the NG2 stain (mean±S.E.M., * $P \leq 0.05$ when compared with the control group or to the PROG+RU486 group by Newman-Keuls tests after one-way ANOVA).

The increase in NG2 and O4 staining by PROG involves intracellular PR

The effect of PROG on $NG2^+$ staining in the cerebellar slices was significantly reduced by the concomitant treatment with 10 μM RU486, a PR antagonist. In addition, the PROG metabolite 5α-dihyroprogesterone (5α-DHP, 50 μM), which binds to the PR, also stimulated $NG2^+$ staining. In contrast, 3α,5α-THP (50 μM), which has no affinity for the PR, had no effect (Fig. 3). Together, these results show that the effect of progestins on OPC markers is mediated by the intracellular PR.

PROG increases the proliferation of $NG2^+$ and $O4^+$ oligodendrocyte precursors

The marked increase in $NG2^+$ and $O4^+$ staining at 3 DIV in response to PROG strongly suggested an increase in the number of oligodendrocyte precursor cells. To test whether PROG stimulated their proliferation, cerebellar slices were cultured in the presence or absence of 20 μM PROG for 3 days and were then double-immunolabeled with antibodies against the cell proliferation marker Ki67 and against one of the phenotypic markers NG2 or O4 antigen.

PROG stimulated the proliferation of both $NG2^+$ and $O4^+$ cells, as shown by the increase in their Ki67 immunolabeling (Fig. 4). In PROG-treated slices, the total number of $Ki67^+/NG2^+$ cells and $Ki67^+/O4^+$ cells was significantly higher at 3 DIV in comparison to controls (Fig. 4C). However, at 7 DIV, fewer cells were labeled with the Ki67 antibody (results not shown). Thus, as revealed by Ki67 immunolabeling, PROG stimulated the proliferation of oligodendrocyte precursors mainly at 3 DIV.

PROG increases the density of NG2$^+$/O4$^+$ pre-oligodendrocytes

Double-immunolabeling revealed a sharp, about four-fold increase in the number of cells co-expressing NG2 and the O4 antigens (NG2$^+$/O4$^+$) in response to PROG at 3 DIV (Fig. 5). Thus, it is most likely that PROG is mitogenic for pre-oligodendrocytes, characterized by the expression of these two markers (Fig. 1). However, at 7 DIV, the number of NG2$^+$/O4$^+$ cells dropped again to control levels. These observations suggest a short but strong proliferating response of pre-oligodendrocytes to PROG treatment. In contrast, the number of NG2$^+$/O4$^+$ cells did not significantly change over the 7 day culture period in non-treated control slices. PROG treatment caused a modest but significant increase in NG2$^+$ cells between 1 DIV and 3 DIV, suggesting a small proliferating response also of the OPC.

A very marked increase in the number of O4$^+$ cells (Fig. 5) and of GalC$^+$ cells (not shown) was observed between 3 and 7 DIV, precisely at the time the number of NG2$^+$/O4$^+$ cells dropped, suggesting an accelerated maturation of pre-oligodendrocytes into premyelinating oligodendrocytes.

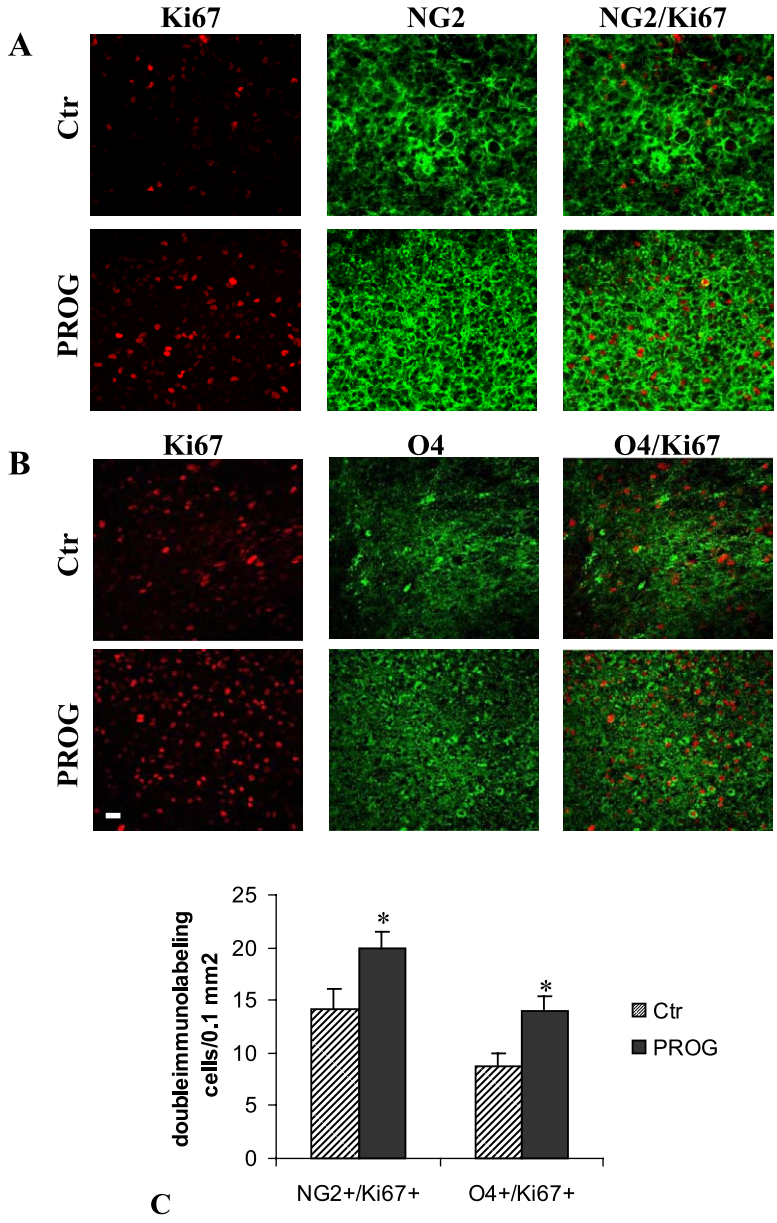

Fig. 4. PROG stimulated the proliferation of NG2$^+$ and of O4$^+$ oligodendrocyte precursor cells. P7 cerebellar slices were treated with PROG (20 μM) or with vehicle (controls, Ctr). At 3 DIV, slices were double-immunolabeled with antibodies against the cell proliferation marker Ki67 (red) and against one of two oligodendrocyte cell markers (green), either NG2 (A) or O4 antigen (B). The number of Ki67-marked cells was markedly increased after PROG treatment when compared with controls. Likewise, both Ki67/NG2 and Ki67/O4 double-immunolabeling were increased by PROG. Thin sections (1 μm) were used for quantification and images were acquired with a confocal Zeiss LSM 410 (C) (mean±S.E.M., * $P \leq 0.05$ when compared with control by Student's *t*-test) (scale bar=20 μm).

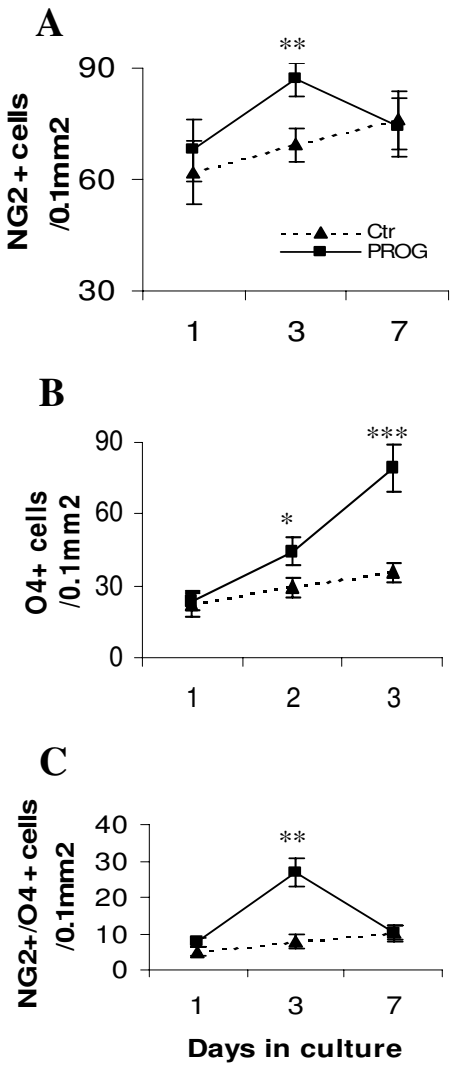

Fig. 5. PROG increased the density of $NG2^+/O4^+$ pre-oligodendrocytes and stimulates their maturation into $NG2^-/O4^+$ myelinating oligodendrocytes. P7 cerebellar slices were treated with 20 μM PROG or with vehicle for 1, 3 or 7 DIV and double-immunolabeled with antibodies against NG2 and O4 antigen. $NG2^+$ (A), $O4^+$ (B) and $NG2^+/O4^+$ cells (C) were counted on thin-section confocal images (1 μm) within an area of 0.1 mm^2 around the deep nuclei region of cerebellar slices. Quantification of oligodendrocyte positive cells was performed at 1 DIV, 3 DIV and 7 DIV. (C) Note that at 3DIV, PROG significantly increased the number of $NG2^+/O4^+$ cells. However, at 7 DIV, no difference in the density of $NG2^+/O4^+$ cells was observed between the PROG-treated and the control slices (mean±S.E.M., *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ when compared with the control groups by Newman-Keuls tests after one-way ANOVA).

Together, these results suggest that PROG strongly and rapidly stimulates the proliferation of $NG2^+/O4^+$ pre-oligodendrocytes and to a smaller extent of $NG2^+$ cells belonging to an earlier stage of maturation. The proliferating response of the oligodendrocyte precursors is short-lasting (approximately 3 days). Thereafter, there is an accelerated maturation toward $O4^+$ cells and $GalC^+$ pre-myelinating oligodendrocytes.

PROG increases the density of nestin$^+$, PDGFαR$^+$ and NG2$^+$ oligodendrocyte precursors

In addition to the strong stimulation of $NG2^+/O4^+$ pre-oligodendrocyte proliferation, treatment with PROG also significantly increased the number of $NG2^+$ cells, and it is thus conceivable that PROG may also stimulate progenitor proliferation at an earlier maturational stage. Indeed, NG2 starts to be expressed by oligodendrocyte precursors prior to O4 antigen (Fig. 1).

To investigate this possibility, we have studied the effects of PROG on the density of stain of nestin, PDGFαR and NG2 cell markers in P7 cerebellar slices at 3 DIV. The $O4^-$ OPC express high levels of the intermediate filament protein nestin, which is strongly down-regulated during the $O4^+$ developmental stages (Gallo and Armstrong, 1995). Similar to NG2, receptors for PDGF are present on both OPC and pre-oligodendrocytes, and PDGF is a potent mitogen for these cells (Noble et al., 1988; Calver et al., 1998; Levine et al., 2001) (Fig. 1).

Cerebellar slices were treated with 20 μM PROG or ethanol vehicle for 3 days and were then immunolabeled with specific antibodies against nestin, PDGFαR or NG2. The staining density of cells immunopositive for these markers was determined by NIH Image software analysis. Results confirmed that treatment with PROG significantly increased the NG2 stain (1.5-times). The density of nestin stain (1.5-times) and of PDGFαR stain (1.25-times) was also increased by PROG (Fig. 6). As reported above for NG2 and O4 stain, PROG also increased the nestin and PDGFαR stain, double-labeled with Ki67 (results not shown). These findings suggest that PROG may already be mitogenic for oligodendrocyte precursors prior to the expression of O4.

Does PROG increase the density of cells not belonging to the oligodendrocyte lineage?

To address the question of the cell-specificity of PROG actions, we have first examined its effects on neurons. Indeed, in the P7 cerebellar slices Purkinje cells still die by apoptosis (Dusart et al., 1997; Ghoumari et al., 2000), and PROG may thus have indirectly increased the number of oligodendroglial cells by promoting neuronal survival. This was not the case, as PROG treatment did not affect the number of calbindin-positive Purkinje cells (Fig. 7A, A′). Moreover, PROG did not influence the number of neurons stained with an anti-NeuN antibody (Fig. 7B, B′). However, in response to PROG, we have observed an increase in the density of the neuronal cytoskeleton, stained with an antibody against MAP-2 (Fig. 7C, C′). This cytoskeletal protein plays a prominent role in the growth and maintenance of neurites during neuronal differentiation and in neuronal plasticity (Tucker, 1990; Johnson and Jope, 1992). P7 cerebellar slices were treated with 20 μM PROG for 7 days and maintained in culture for up to 15 days to allow the observation of neurite differentiation during an extended period. Expression of MAP-2 showed an about two-fold increase in PROG-treated slices when compared with non-treated controls.

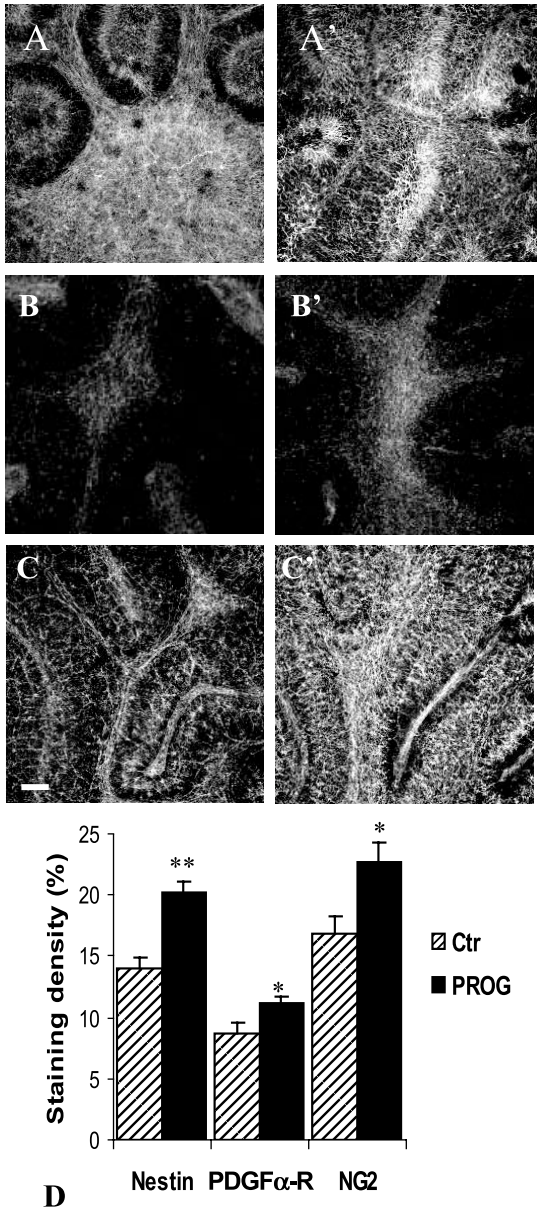

Fig. 6. PROG increased the staining of nestin, PDGFαR and NG2. Cerebellar slices of P7 rats were cultured for 3 days in the absence or presence of 20 μM PROG. To visualize high glial cell staining, a region of interest was selected around the deep nuclei zone and the apical ends of each lobule. Oligodendrocyte precursor cells were immunolabeled with antibodies against nestin (A, A′), PDGFαR (B, B′) or the chondroitin sulfate proteoglycan NG2 (C, C′). Slices treated with PROG (A′, C′) showed higher staining density of nestin and NG2 when compared with controls (A, C). A small but significant difference in PDGFαR staining was also observed between PROG-treated slices (B′) and vehicle-treated control slices (B). The densities of Nestin stain and of NG2 stain were increased about 1.5-fold whereas the one of PDGFαR about 1.25-fold by PROG (D) (mean±S.E.M., * $P \leq 0.05$ and ** $P \leq 0.01$ when compared with controls by Student's *t*-test) (scale bar=20 μm).

Different glial cell markers such as GFAP and S-100 were used to study PROG influences on their expression. Fig. 8 shows no significant difference in GFAP and S-100 immunolabeling between PROG-treated and non-treated slices. However, the density of OX-42$^+$ microglia/macrophages was increased by PROG. This was shown by treating P7 cerebellar slices for 3 days with PROG (20 μM) (Fig. 8C, C′). PROG induces proliferation of OX-42$^+$ microglia/macrophages as shown by the increase in their Ki67 immunolabeling (Fig. 8D). The number of OX-42$^+$ microglial/macrophage cells, counted in different fields within the slices, was higher in PROG- treated slices than in controls (control: 41±8; +PROG: 57±10 cells/300 μm^2) (Fig. 8D). However, the morphology of the OX-42$^+$ cells was not changed by PROG. A small part of the cells was characterized by

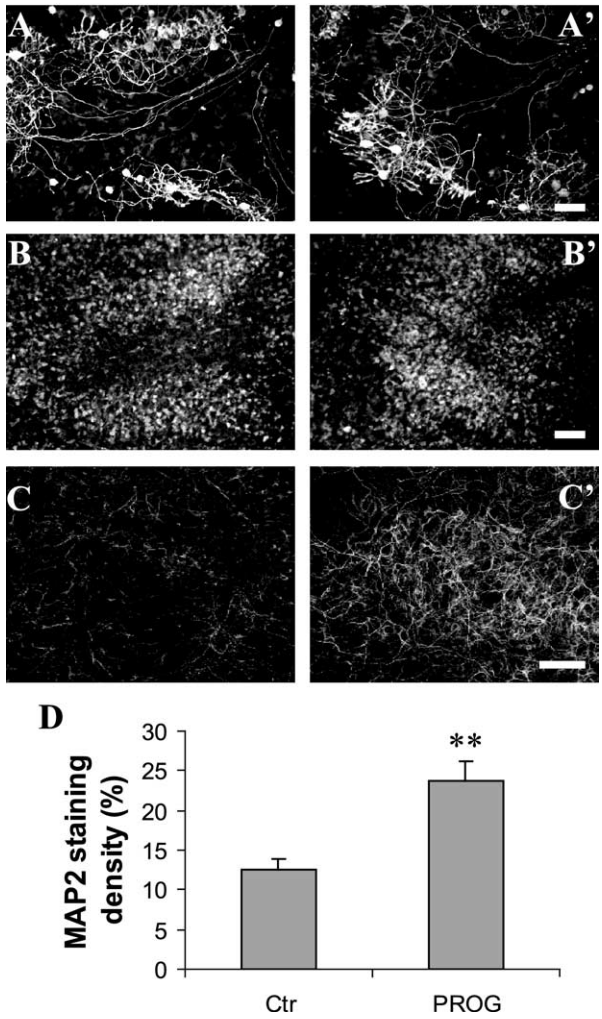

Fig. 7. Effect of PROG treatment on neurons. Cerebellar slices of P7 rats were cultured for 3 or 15 days in the absence or presence of 20 μM PROG. Sections were labeled at 3 DIV with antibodies against the Purkinje cell marker Calbindin D-28K (A, A′) and NeuN (which labels neurons except Purkinje cells) (B, B′). Counting Purkinje cells and quantifying NeuN stain did not reveal any difference between PROG-treated and control slices (data not shown). At DIV 15, staining of the neuronal cytoskeleton by antibodies against the MAP-2 was more intense in PROG-treated slices (C′) than in controls (C). (D) MAP-2 staining density was quantified in these slices by using the NIH Image software and was expressed as % of light pixels (mean±S.E.M., ** $P \leq 0.01$ when compared with control by Student's *t*-test) (scale bars=50 μm).

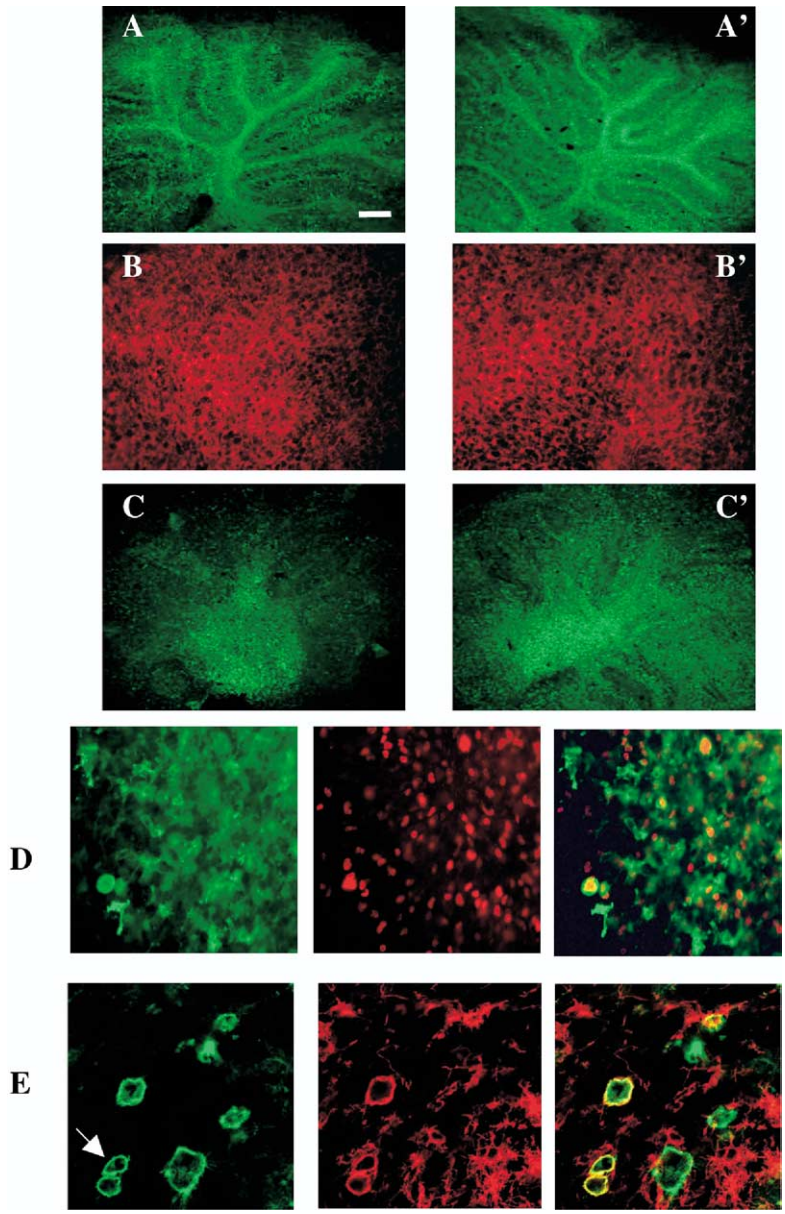

Fig. 8. PROG increased the density of OX-42$^+$ microglia/macrophages but not the one of GFAP$^+$ and S-100$^+$ glial cells. P7 cerebellar slices were treated with 20 μM PROG for 3 days. GFAP staining (A, A′) and S-100 staining (B, B′) were analyzed in PROG-treated and in non-treated slices. PROG did not influence the staining density of the GFAP and S-100 glial cell markers (compare A to A′ and B to B′). (C, C′) OX-42 staining was analyzed by confocal Zeiss LSM 410. The density of OX-42$^+$ cells was significantly increased in PROG-treated slices (C′) when compared with vehicle-treated controls (C). The proliferating effect of PROG on OX-42$^+$ microglial cells was confirmed using the cell proliferation marker Ki67. At 3 DIV, slices were double-immunolabeled with antibodies against Ki67 (red) and against OX-42$^+$ microglial cells (green) (D). OX-42$^+$ microglial cells were counted on semi-thin sections (1 μm) using a fluorescent microscope (Axiovert 135 M Zeiss, Carl Zeiss Inc.). Quantification revealed an about 1.4-fold increase in OX-42 staining in response to PROG (Control: 41±8; +PROG: 57±10 cells/300 μm^2). (E) PROG induces co-expression of NG2 and OX-42 antigens. Double immunolabeling and analysis of thin-section confocal images (0.7 μm) revealed that in the PROG-treated slices a small percentage (less than 0.1%) of NG2$^+$ cells (red) was also OX-42$^+$ (green). OX-42 staining was restricted to the cell surface. Note that only round and not ramified cells (arrow) co-expressed NG2 and OX-42 antigen (scale bars=400 μm in A, A′, 100 μm in B, B′, 400 μm in C, C′, 50 μm in D and 20 μm in E).

mobile ameboid phenotypes or by ramified processes, suggesting the presence of activated macrophages in the organotypic slice cultures. The labeling pattern observed with the OX-42 antibody was similar in all regions of the cerebellar slices, with more intense staining in the deep nuclear region (Fig. 8C, C′).

PROG induces co-expression of OX-42 and NG2 in precursor cells

It has been previously reported that at no stage of development, NG2$^+$ cells co-express OX-42 immunoreactivity, suggesting that they do not develop along a microglial

pathway (Nishiyama et al., 1997; Reynolds and Hardy, 1997; Dawson et al., 2000). However, recent studies have shown that NG2 can be transiently expressed by activated macrophages after brain or spinal cord injury (Bu et al., 2001; Jones et al., 2002). We thus decided to make sure that the observed increase in $NG2^+$ cells within our cerebellar slices in response to PROG did not reflect an increase in $OX\text{-}42^+$ cells.

Slices were double-labeled with NG2 and OX-42 antibodies. Results confirmed that most of the $NG2^+$ were in fact negative for OX-42. However, when slices were treated for 3 days with PROG (20 μM), a very small number of small and round $OX\text{-}42^+$ cells appeared to be NG2-reactive (Fig. 8E). As shown by confocal microscopy, the $NG2^+/OX\text{-}42^+$ cells represented no more than 0.1% of the total number of $NG2^+$ cells, and the above-described mitogenic effect of PROG on a large population of $NG2^+$ oligodendrocyte precursors cannot result from of the stimulation of microglial proliferation. Nevertheless, this is the first report of small $OX\text{-}42^+$ progenitor cells in the brain co-expressing the NG2 proteoglycan. It has previously been shown that activated microglia in the brain can encircle $NG2^+$ cells. However, in the small $NG2^+/OX\text{-}42^+$ cells present in our cerebellar slices, both NG2- and OX-42-immunoreactivity were present at the cell surface, indicating a real co-localization and that $NG2^+$ cells were not just phagocytosed by $OX\text{-}42^+$ microglia. At 7 DIV, co-localization of NG2 and OX-42 markers was not longer observed. We also confirmed that the $NG2^+$ cells are distinct from $GFAP^+$ astrocytes and from $NeuN^+$ and $MAP\text{-}2^+$ neurons within cerebellar slices cultured in the absence or presence of PROG.

DISCUSSION

Myelin formation is accelerated by PROG in organotypic slice cultures of P7 rat cerebellum (Ghoumari et al., 2003). In the present study, we demonstrate that the steroid stimulates the proliferation of oligodendrocyte precursors in the cerebellar slices after 3 DIV. We first showed that PROG increased NG2 and O4 stain. PROG stimulated the proliferation of cells expressing these markers, as shown by their staining with an antibody against the cell proliferation marker Ki67. Double-immunolabeling experiments revealed that at 3 DIV, PROG caused a marked increase in the number of $NG2^+$ and $O4^+$ cells, which was no longer observed at 7 DIV. These observations strongly suggest a transient mitogenic effect of PROG on pre-oligodendrocytes, which are characterized by the co-expression of NG2 and the O4 antigens (Fig. 1). The short duration of the increase in proliferation was not due to an exhaustion of PROG from the culture medium, because it was renewed at 3 DIV.

PROG also caused a small, but nevertheless significant increase in the number of $NG2^+$ cells and stimulated the proliferation of cells expressing the early maturation marker nestin, suggesting that the steroid may already be mitogenic for cells at an earlier stage of maturation, such as OPC. The mitogenic effect of PROG on the $NG2^+$ precursor cells was not mimicked by its GABA-active metabolite 3α,5α-THP and involved the intracellular PR, as it could be blocked by the PR antagonist RU486. These results are in agreement with our previous findings that the promyelinating effects of PROG in cerebellar slices require PR activation, as they could also be blocked by RU486 and were not observed in slices from PR knockout mice (Ghoumari et al., 2003). They are also consistent with an earlier study reporting that both PR isoforms are highly expressed in the cerebellum during early postnatal life (Sakamoto et al., 2003). Interestingly, at this stage of development levels of PROG and expression of the enzymes involved in its synthesis, the cytochrome P450scc and the 3β-hydroxysteroid dehydrogenase, are temporarily elevated within the cerebellum (Ukena et al., 1999). It is thus likely that myelination may be developmentally regulated by locally synthesized PROG, pointing to an important role for neurosteroids during brain development (Robel et al., 1999; Mellon et al., 2001).

PROG may also promote the maturation of pre-oligodendrocytes into myelinating oligodendrocytes. Indeed, between 3 DIV and 7 DIV, when the number of $NG2^+/O4^+$ cells sharply decreased, the number of $O4^+$ and of $GalC^+$ myelinating oligodendrocytes markedly increased in response to PROG treatment. These results suggest that PROG first stimulates, in a rapid and transient manner, the proliferation of OPC, and subsequently promotes their differentiation into myelinating oligodendrocytes. However, the direct or indirect effect of PROG on oligodendrocyte maturation remains to be established. This effect could be in relation with the activation of some growth factor responses and thus may be context-dependent.

Although the number of neurons in the P7-cerebellar slices was not influenced by PROG, staining of the neuronal cytoskeleton marker MAP-2 was strongly increased. This finding is consistent with previous reports demonstrating that Purkinje cells are a target for PROG and that these neurons express the two PR isoforms as well as the enzymes involved in PROG synthesis (Ukena et al., 1999; Sakamoto et al., 2001, 2003). PROG may thus indirectly influence the proliferation and maturation of $NG2^+$ oligodendrocyte precursors via its actions on neurons. It has indeed been shown that axons stimulate OPC proliferation, and that co-culturing $NG2^+$ cells with neurons stimulates their proliferation (Levine, 1989; Barres and Raff, 1999). However, although it is very likely that the effects of PROG on neurons may have repercussions on oligodendrocyte precursor development, there is also evidence for a direct action of the steroid on these cells. First, several studies have shown that oligodendrocyte precursor cells continue to divide, even in the absence of neurons (Ueda et al., 1999; Greenwood and Butt, 2003). Second, we have observed that PROG also significantly increased the NG2 staining in cerebellar slices from P6-rats (control: 17 ± 1.7; +PROG: 23 ± 1.6 staining density, $P \leq 0.05$), at a developmental stage when Purkinje cells do not survive in explant cultures (Dusart et al., 1997; Ghoumari et al., 2000, 2002).

Astrogliosis is characterized by an increase in GFAP. In this study, no significant difference of $GFAP^+$ cells was observed between PROG-treated and non-treated slices.

This was confirmed by using another glial cell marker S-100. The density of cells displaying S-100 protein-like immunoreactivity was approximately identical in PROG-treated and non-treated slices.

During the first 2 postnatal weeks, microglial cells strongly proliferate in the rat and mouse CNS (Milligan et al., 1991; Alliot et al., 1999). Here, we show that PROG significantly increases the number of OX-42$^+$ microglia in postnatal cerebellar slices (1.4-fold control). Only very few of these cells had a large ameboid morphology characteristic of activated macrophages. This increase in the number of microglia by PROG may also have beneficial effects on oligodendrocyte precursors. Indeed, conditioned media from nonactivated microglial cells has been shown to promote the survival and maturation of OPC (Nicholas et al., 2001). In addition, PROG may protect the developing oligodendrocytes by inhibiting the activation of microglia and the production of cytotoxic cytokines (Benveniste et al., 1997; Drew and Chavis, 2000).

Despite the fact that PROG influenced the rapid maturation of NG2$^+$ cells along the oligodendroglial lineage, NG2$^+$ cells continued to be abundant and to proliferate at 7 DIV. There is increasing evidence that the numerous NG2$^+$ cells present in the developing brain may represent a heterogenous population of multipotent cells. Thus, two populations of NG2$^+$ cells have been distinguished throughout neonatal cortical development. Moreover, NG2$^+$ progenitor cells present in the postnatal mouse brain have recently been shown to generate not only oligodendrocytes, but also neurons and astrocytes *in vitro* (Belachew et al., 2003). They may also be at the origin of other types of glial cells, such as the recently identified synantocytes, which specifically contact neurons and axons at synapses (Butt et al., 2002).

Whether PROG, in addition to its effects on the oligodendroglial cells, also activates the development of other lineages arising from NG2$^+$ cells, remains to be investigated. NG2$^+$ cells present in the P7 cerebellar slices never expressed the neuronal markers NeuN, CaBP and MAP-2 or the astrocytic marker GFAP. However, we found that a small percentage (about 0.1%) of the NG2$^+$ cells transiently became OX-42$^+$ in response to PROG. The NG2$^+$/OX-42$^+$ cells in the cerebellar slices were small and round and were very distinct from recently described large ameboid NG2$^+$/OX-42$^+$ macrophages/microglial cells, which appear in the CNS after trauma (Bu et al., 2001; Jones et al., 2002). It is possible that the NG2$^+$/OX-42$^+$ cells belong to a lineage distinct from the one leading to oligodendrocytes, even though it is generally admitted that NG2$^+$ cells do not give rise to microglia (Nishiyama et al., 1997; Reynolds and Hardy, 1997; Levine, 1994; Dawson et al., 2000).

Different types of NG2$^+$ glia persists in the adult CNS (Horner et al., 2002; Nishiyama et al., 2002), a subpopulation of which corresponds to oligodendroglial progenitors, able to generate new oligodendrocytes and playing an important role in remyelination (Levine et al., 2001; Watanabe et al., 2002). Whether PROG continues to be mitogenic for adult oligodendrocyte progenitors remains to be demonstrated. However, this possibility is strongly suggested by the recent observation that treatment with PROG of adult male rats after spinal cord injury increases the number of NG2$^+$ cells as well as MBP-staining density (De Nicola et al., 2003). In that case, PROG could become particularly useful for stimulating remyelination in situations where the recruitment or differentiation of adult OPCs is impaired, such as during multiple sclerosis and in the aging brain (Chari et al., 2002; Sim et al., 2002). Indeed, PROG treatment has recently been demonstrated to have subtle beneficial effects on the slow and inefficient remyelination that occurs in old male rats (Ibanez et al., 2003, 2004).

CONCLUSION

Our results demonstrate that PROG is mitogenic for oligodendrocyte precursor cells during the postnatal development of the cerebellum. They also suggest that PROG may contribute indirectly by acting on other factors in promoting the maturation of NG2$^+$ progenitor cells along the oligodendroglial pathway. Whether PROG is also mitogenic for adult NG2$^+$ oligodendroglial progenitors, as suggested by several recent findings, remains to be established. This would confer a therapeutic potential for progestins to stimulate myelin repair.

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