Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule

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Many factors have been shown to promote myelination, but few have been shown to be inhibitory. Here, we show that polysialylated-neural cell adhesion molecule (PSA-NCAM) can negatively regulate myelin formation. During development, PSA-NCAM is first expressed on all growing fibers; then, axonal expression is down-regulated and myelin deposition occurs only on PSA-NCAM-negative axons. Similarly, in cocultures of oligodendrocytes and neurons, PSA-NCAM expression on axons is initially high, but decreases as myelination proceeds. Importantly, if expression of PSA-NCAM is prematurely decreased in cultures, by either antibody-mediated internalization or enzymatic removal of the PSA moieties with endoneuraminidase N (endo-N), myelination increases 4- to 5-fold. In the optic nerve, premature cleavage of PSA moieties by intravitreous injection of endo-N also induces a transient increase in the number of myelinated internodes, but does not interfere with the onset of myelination. Previously, we showed that axonal electrical activity strongly induced myelination, which could be prevented by tetrodotoxin (TTX), an action potential blocker. Interestingly, removal of PSA moieties does not reverse the inhibition of myelination by TTX. Together, this suggests that myelination is tightly controlled by both positive (electrical activity) and negative (PSA-NCAM expression) regulatory signals.

Several observations demonstrate the influence of axons on the final stage of oligodendrocyte maturation, myelin formation. The report that, *in vitro*, oligodendrocytes will myelinate only axons, and not dendrites, nor the processes of oligodendrocytes and astrocytes, strongly suggests the existence of an axonal recognition signal specific for oligodendrocyte (1). The timing of oligodendrocyte differentiation has been shown to be controlled by the down-regulation of Jagged1 along axon (2). Similarly, although highly purified mature oligodendrocytes maintain a basal level of expression of the major myelin proteins, the addition of neurons to these cultures results in a significant increase in the transcription rate of these myelin-specific genes (3). Axons are also needed for the compaction of the myelin sheath. In neuron-free cultures, the pseudomyelin structures present at the tip of oligodendroglial processes (flat, rafts of membrane), or the accumulation of these processes around carbon fibers (4), never display the characteristic, tight compaction of normal myelin wrapped around an axon. Recently, by using different sodium channel-specific neurotoxins, either blocking (tetrodotoxin, TTX), or stimulating (α-scorpion toxin) neuronal electrical activity, we showed, both *in vitro*, in neuron/oligodendrocyte cocultures, and *in vivo*, in the optic nerve, that electrical activity plays a key role in the induction of myelination (5).

At the axon:oligodendrocyte interface, adhesion molecules are candidate molecules that could convey the axonal signal to initiate myelination. Adhesion molecules have the potential to not only bring the axon and glial cell into close apposition, but also to transduce the signals between these cells (6). The neural cell adhesion molecule (NCAM), a member of the Ig superfamily, has been well-studied (7) and is likely to play an active role in these processes. There are several isoforms of this molecule, which result from differential splicing and posttranslational modifications. Expression of the different NCAM isoforms are developmentally regulated, and all are capable of bearing long homopolymers of α2,8-linked sialic acid, transiently, attached to the fifth Ig domain. Polysialic acid (PSA) moieties on NCAM not only prevent homophilic NCAM–NCAM adhesion, but also serve more generally as negative regulators of cell–cell interactions (8–10). PSA-NCAM is abundantly expressed on all growing fiber tracts in the developing central nervous system and persists in certain areas of adult brain known to exhibit plasticity (11–13). Expression of the highly sialylated form of NCAM is developmentally down-regulated, and, in the optic nerve of the mouse, PSA-NCAM progressively disappears from retinal ganglion cell axons during the first two postnatal weeks, with a time course that parallels myelination (14). How expression of PSA-NCAM at the cell surface is regulated is poorly understood (9, 10). Several reports, however, have shown that PSA-NCAM expression at the axonal surface may be modulated by electrical activity along axons (15, 16).

Here, we have investigated the role of PSA-NCAM on neuron/oligodendrocyte interactions both in an *in vitro* system of myelination and *in vivo* in the optic nerve, and provide evidence that the process of myelination depends on the down-regulation of PSA moieties on the axonal surface. But removal of PSA is not sufficient to promote myelination, as it did not alleviate the TTX-induced inhibition of myelin formation. These results suggest that the onset of myelination depends on axonal regulatory signals, both negative, like the down-regulation of PSA-NCAM, and positive, mediated by the neuronal electrical activity.

Materials and Methods

Cell Cultures. Forebrains were removed from 15-day-old OF1 mouse fetuses (Iffa Credo), dissociated mechanically, then enzymatically by digestion with trypsin (0.025%) (Biological Industries, Beit Haemek, Israel) for 15 min at 37°C. After washing, the pellet was passed gently through a nylon mesh (63-μm pores), then resuspended in DMEM (Seromed, Noisy le Grand, France)

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Abbreviations: Endo-N, endoneuraminidase N; DIV, days *in vitro*; MBP, myelin basic protein; PSA-NCAM, polysialylated-neural cell adhesion molecule; TTX, tetrodotoxin; PFA, paraformaldehyde.

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containing 10% FCS (Eurobio, Les Ulis, France). Cells (5×10^4) were plated on poly-L-lysine-coated (Sigma) 14-mm diameter glass coverslips (OSI, Maurepas, France) in 24-well plates (Costar). Cells were seeded in 20 μl of DMEM containing 10% FCS to facilitate attachment for 30 min, then 500 μl of culture medium was added to each well. Standard culture medium consisted of B-S medium (17) supplemented with 0.5% FCS, 1% penicillin-streptomycin (Biological Industries), and recombinant platelet-derived growth factor AA 10 ng/ml (PDGF-AA; Upstate Biotechnology, Lake Placid, NY).

Antibodies. Mouse monoclonal anti-myelin basic protein (MBP) antibody (IgG1; Euromedex, Mundolsheim, France) was used diluted 1/500. Mouse monoclonal anti-MAP-2 (1/100, IgG1) and TuJ1 antibodies (1/1000, IgG2a) were gifts from A. Frankfurter (University of Virginia, Charlottesville). Mouse IgM monoclonal anti PSA-NCAM antibody, under the form of ascitic fluid containing approximately 6 mg antibody/ml, was used diluted 1/100 to 1/500. For control experiments, two mouse monoclonal IgM were used: the nonrelevant K5-2 antibody (1/500), and A2B5 antibody (1/5; American Type Culture Collection). Fluorochrome-conjugated goat antibodies against mouse IgG1, IgG2a, and IgM were from Southern Biotechnology Associates and used diluted 1/100.

Immunolabeling. In cocultures, coverslips were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 10 min and then saturated with DMEM containing 10% FCS and 50% sheep serum for 20 min. For immunostaining on optic nerve sections, mice (OF1) were anaesthetized with Imalgene 500 (Rhône-Merieux, France) and perfused intracardially with 4% PFA. Brain and optic nerves were removed, postfixed with 4% PFA for 2 h at room temperature, then incubated overnight in sucrose (15% in PBS) at 4°C. Tissues were embedded in 7.5% gelatin/15% sucrose in 0.12 M phosphate buffer and frozen in melting isopentane. Cryostat sections (25 μm) were postfixed in 4% PFA, then in 70% ethanol (10 min each), then incubated for 1 h in PBS containing 50% sheep serum and 10% FCS, before overnight incubation with anti MBP and anti PSA-NCAM mAb diluted in PBS containing 0.2% gelatin and 0.2% Triton X-100. Sections were then incubated in secondary antibodies for 1 h, before being mounted in Fluoromount-G (Southern Biotechnology Associates) and examined either with a Leica (Deerfield, IL) DRMB fluorescent photomicroscope or by confocal microscopy (Omnichrome ion laser power supply on a Leica DRMB microscope).

Masking and Removal of PSA Sites. Anti-PSA-NCAM mAb were added to the culture medium between 10 and 18 days *in vitro* (DIV). Fresh antibody solution was added twice a week, at the same time as the culture medium was changed. In control experiments, anti-PSA-NCAM mAb were replaced by either K5-2 or A2B5 mAbs. The anti-PSA activity was titrated out by preincubating the antibody with increasing concentrations (between 0.1 and 100 mM) of colominic acid (Sigma) before its addition to the culture medium. A concentration of 1 mM of colominic acid was sufficient to block the anti-PSA-NCAM immunoreactivity. In cocultures, endoneuraminidase N (endo-N) was used at 0.8 units/ml, a concentration sufficient to suppress all sialylated sites after 1 h of incubation. It was added to culture medium from 10 to 18 DIV and changed twice a week. For *in vivo* experiments, deeply anesthetized P4 mice were injected with 1 μl of endo-N (3.5 units/μl) in the right intravitreous space using a 10-μl Hamilton syringe through a 34-gauge needle. Control animals received 1 μl of PBS.

TTX Treatment. To determine whether blockade of electrical activity could influence PSA expression on neurons as a function of time *in vitro*, the percentage of TuJ1-positive neurons expressing PSA-NCAM was evaluated in cultures treated for 2 days (between 11 and 13 DIV) with TTX (10^{-6} M). Cultures were examined at 2, 4, 6, and 8 days after TTX removal. In some experiments, myelination was quantified in cultures treated simultaneously, between 10 and 14 DIV, by TTX (10^{-6}M) and endo-N (0.8 units/ml), and compared with cultures treated by TTX alone.

Quantification of Myelination. In cocultures, myelination was assessed at 22–24 DIV by counting the total number, per coverslip, of myelinated segments, identified as bright MBP-positive double lines, interrupted at nodes of Ranvier. The total number of mature MBP-positive oligodendrocytes was counted in the same experiments. To evaluate the number of neurons under different culture conditions, we used an anti-MAP-2 mAb, which stains robustly the neuronal cell body and thus allows a reliable quantification. In the optic nerve, myelination was assessed between P2 and P10 by counting the number of MBP$^+$ internodes in five successive longitudinal sections. For each nerve, the same length was counted starting from the lamina cribosa (retinal extremity). Results were expressed as the mean ± SEM of at least three independent experiments, with five cultures or three optic nerves per condition. Statistical analysis was performed by using the Student's t test.

Electron Microscopy. After washing with PBS, cultures grown on uncoated plastic wells were fixed in 2.5% glutaraldehyde for 30 min at room temperature, then postfixed in OsO4 for 30 min. After dehydration in a graded series of ethanol solutions, cultures were embedded in Resin Epoxy (Polysciences). The block of epoxy was then dipped in liquid nitrogen. Ultrathin sections (70–90 nm) were cut parallel to the surface of the cultures. After counterstaining with 5% uranyl acetate and lead citrate, sections were examined with a JEOL 1200 EX electron microscope operated at 70 kV.

Results

***In Vitro*, Expression of PSA-NCAM Is Down-Regulated at the Time of Myelination.** Dissociated cultures were prepared from E15 embryonic cerebral hemispheres, as previously described (1). This preparation allows myelination to occur and has proved to be well adapted for the study of oligodendrocyte/neuron interactions during myelin formation. Mature nonmyelinating oligodendrocytes, identified as MBP-positive cells with a highly branched morphology, were first detected at 11–12 DIV. At 16–18 DIV, some of the MBP$^+$ oligodendrocytes underwent a dramatic reduction in the number of their processes. The remaining processes wrap around nearby axons to form myelinated internodes, easily detected by their double-outline appearance after immunostaining with anti-MBP (Fig. 1*C*). The peak of myelination, estimated as the number of myelinated internodes per coverslip, was observed around 22–24 DIV, followed a few days later by the appearance of lipid droplets, suggestive of myelin breakdown. The number of myelinated segments per coverslip varied from one experiment to another, but was very similar within an experiment. The oligodendrocyte/axon ratio ranged from 1:1 to 1:20, with a mean ratio of 1:5.

Expression of PSA-NCAM on neurons was analyzed as a function of time *in vitro* (Fig. 1*A*). Until 12 DIV, more than 95% of the TuJ1-positive neurons expressed PSA-NCAM, both on the cell body and the neurites. Very few neurons (<5%), usually characterized by the very short length of their neurites, did not express detectable levels of PSA-NCAM. At 16 DIV, 86% of neurons were still expressing PSA-NCAM on their neurites, but the cell bodies were no longer stained (Fig. 1*B*). The percentage of PSA-NCAM-positive neurons dropped abruptly down to 16%

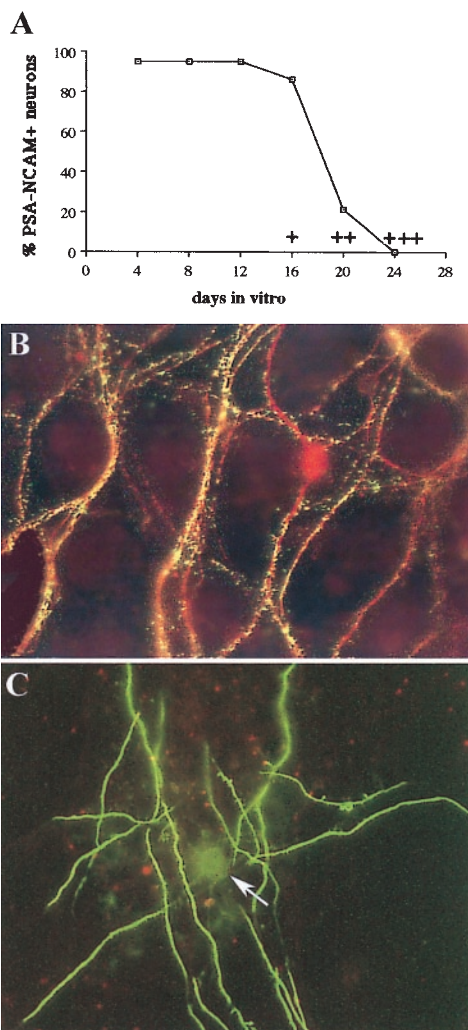

Fig. 1. Down-regulation of neuronal PSA-NCAM is concomitant with the onset of myelination. Cultures derived from embryonic (E15) mouse hemispheres were maintained for various periods of time before being double-labeled with anti-PSA-NCAM and either TuJ1 to label neurons (*B*) or anti-MBP mAb to label myelinated internodes and oligodendrocytes (*C*). (*A*) The percentage of PSA-NCAM-expressing neurons (TuJ1-positive cells) is plotted as a function of time in culture. The intensity of myelination in sister cultures was evaluated by counting the number of myelinated MBP positive internodes and is indicated by + (onset), ++ (moderate), and +++ (maximum). (*B*) Immunostaining of TuJ1-positive neurons (red) with anti-PSA-NCAM mAb (green) at 16 DIV. Most of the neurites appear in yellow because of the superposition of the two labeling. (*C*) A field of myelinated internodes linked to a single oligodendrocyte at 24 DIV. Only rare PSA-NCAM dots (red) are visible, contrasting with the intense labeling of MBP-positive myelinated internodes (green). Arrow points to the myelinating oligodendrocyte cell body, which is not in the same plane of focus and is moderately stained with the anti-MBP mAb, as after the onset of myelination, most of the MBP migrates out the oligodendrocyte cell body. (*B* and *C*, magnification ×320.)

at 20 DIV, and, at 24 DIV, no more neurons were PSA-NCAM-positive.

When this kinetics of expression was compared with the appearance of myelinated segments, a clear inverse relation was observed, the reduction of PSA-NCAM on neurons being coincident with the detection of the first myelinated segments, and the complete disappearance of neuronal PSA-NCAM at 24 DIV corresponding to the peak of myelination (Fig. 1 *A* and *C*).

Masking and Removal of PSA-NCAM Increases Myelination. This temporal relation suggests that the disappearance of PSA-NCAM could be a prerequisite for myelination to occur. To test this hypothesis, two series of experiments were designed. One was aimed at blocking the PSA site by antibodies, the other at removing the PSA moieties from the axon surface by the use of the specific enzyme endo-N. Antibodies directed against the PSA moiety of NCAM were added to the culture medium between 10 and 18 DIV, and myelination quantified at 22–24 DIV. Masking of PSA sites induced a 5.2-fold increase ($P < 0.001$) in the number of myelinated internodes compared with control untreated cultures (Fig. 2*A*). This effect was not associated with a modification in the number of either MBP-expressing oligodendrocytes or MAP-2 (microtubule-associated protein 2)-positive neurons (not shown). It was concentration-dependent, and no more effect was observed when the working dilution was increased four times, with intermediate values assayed with a 2-fold dilution. No increase in the number of myelinated internodes was observed in cultures treated with anti-PSA-NCAM mAb that had previously been adsorbed with 1 mM colominic acid (a polymer of α2,8-linked sialic acid), showing that the promyelinating effect of anti-PSA-NCAM mAb was mediated by its binding to the PSA antigenic determinant. Addition in the culture medium of a nonrelevant mAb (K5-2), belonging to the same subclass (IgM) of immunoglobulins as anti-PSA-NCAM mAb, had no effect on myelination (Fig. 2*A*). We then tested the effect of the addition of A2B5 mAb (mouse IgM), which under our culture conditions bind to the surface of neurites, but not to PSA-NCAM. Substitution of anti-PSA-NCAM by A2B5 mAb had no effect on myelination, which is an additional argument in favor of the specific influence of the PSA moiety on myelination (Fig. 2*A*).

These data could indicate that the antibody-mediated masking of the PSA groups on the cell surface by use of antibodies prevents their interaction by blocking a signaling effect to oligodendrocytes. Alternatively, the crosslinking of PSA groups at the cell surface by IgM antibodies (which are pentameric) may induce an internalization of the PSA-NCAM molecule. To differentiate between these two hypothesis, we added the secondary antibody alone (anti-IgM) to anti-PSA-NCAM mAb-treated cultures, either live or fixed and permeabilized with 4% PFA. Binding of the secondary antibody was clearly visible on cultures that had been permeabilized (Fig. 2*C*), but no staining was observed on unfixed cultures (Fig. 2*D*). These data are very suggestive of an internalization of PSA-anti-PSA-NCAM complexes.

To further demonstrate that the promyelinating effect of the anti-PSA-NCAM mAb was linked to the removal of the PSA moiety from the cell surface, cultures were treated between 10 and 18 DIV with endo-N, which specifically cleaves the PSA groups. In preliminary experiments, we verified that endo-N, used at a concentration of 0.8 units/ml, was sufficient to eliminate all of the immunodetectable PSA groups after 1 h of incubation. Endo-N was then added at 10 DIV in culture medium, renewed at 14 DIV, and finally removed by washing at 18 DIV. Endo-N treatment induced a 4.8-fold increase in the number of myelinated internodes, similar to the increase observed in cultures treated with the anti-PSA mAb (Fig. 2*A*).

The effect of PSA removal on myelination was associated to an increase in the percentage of myelinating oligodendrocytes, but was not related to a modification of the axon/oligodendrocyte ratio, which varied from 1:1 to 1:20, similar to the control cultures (see above). Electron microscopic analysis showed that myelinated axons were more frequent in anti-PSA-NCAM mAb-treated cultures compared with control cultures. Well compacted myelin, with 5–25 wraps, were observed in both treated and control cultures (Fig. 2*B*).

Although removal of PSA moiety increases the number of myelinated internodes, it did not induce a precocious onset of myelination. The first myelinated segments were detected at the

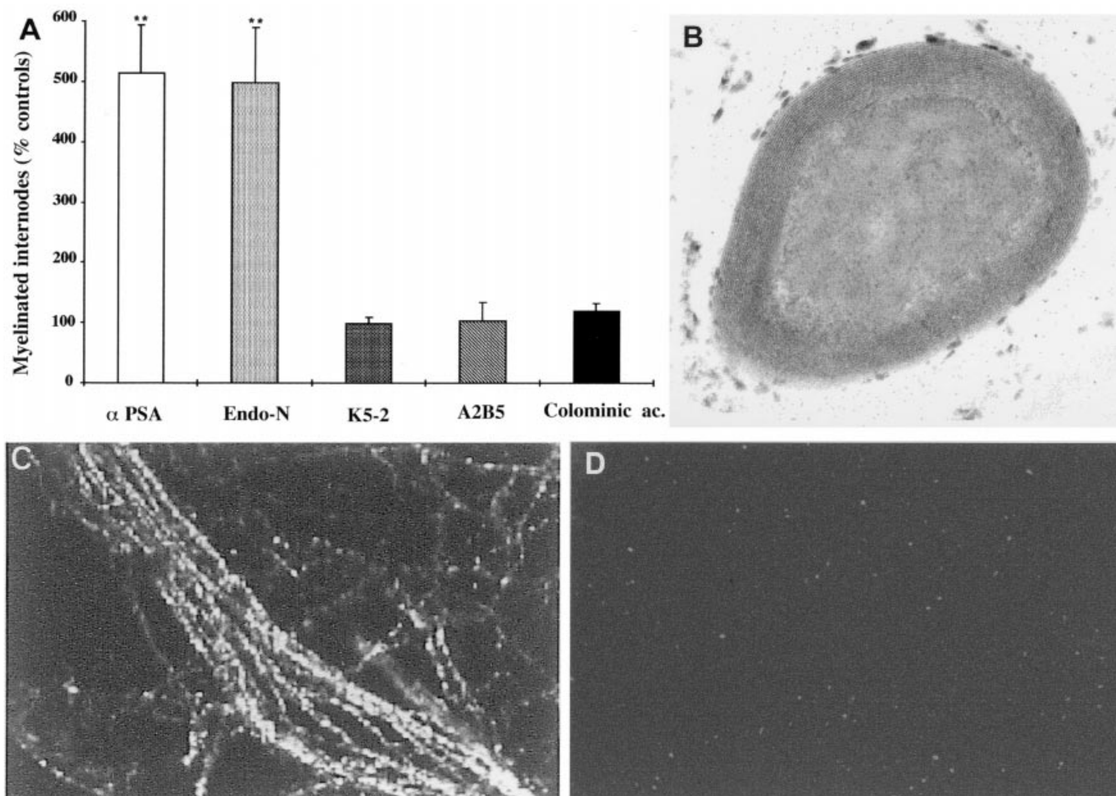

Fig. 2. Removal of PSA moieties increases myelination. (*A*) Anti-PSA-NCAM mAb (α-PSA) or endo-N were added to the culture medium between 10 and 18 DIV, and myelination was quantified at 22–24 DIV by counting the number of MBP-positive myelinated internodes per coverslip. Controls of specificity of anti-PSA mAb were cultures treated with either K5-2 mAb, an irrelevant mouse IgM used at the same concentration as anti-PSA, or A2B5 mAb, an IgM that binds to axons, but not to PSA-NCAM, or anti-PSA mAb adsorbed with 1 mM colominic acid, a polymer of α2,8-linked sialic acid. Anti-PSA mAb and endo-N-treated cultures showed a 5.2- and 4.8-fold increase in myelination, respectively. Results are expressed as the mean ± SEM of three experiments in quintuplicate. (**, $P < 0.001$; Student's *t* test). (*B*) At the ultrastructural level, the myelin formed in anti-PSA-treated cultures appears normal and well compacted. (*C* and *D*) Anti-PSA-NCAM mAb treatment induced an internalization of the PSA-anti-PSA-NCAM complex. Anti-PSA-NCAM mAb were added to culture medium between 10 and 18 DIV. Cultures were then either fixed (*C*) in 4% PFA or maintained alive (*D*) before being incubated with the fluorescein-conjugated secondary antibody alone. Staining was only visible on fixed cultures (*C*), demonstrating internalization of anti-PSA-NCAM mAb. (Magnification: *B*, ×35,000; and *C* and *D*, ×350.)

same time whether in the presence or absence of anti-PSA-NCAM mAb (not shown), suggesting that induction of myelination requires, in addition, the expression of developmentally regulated permissive signals.

TTX-Induced Inhibition of Myelination Is Not Associated with Modification of PSA Expression. Blocking the neuronal electrical activity by addition of TTX to the culture medium has been reported to inhibit myelination, without affecting the viability of either oligodendrocytes or neurons (5). Indeed, when TTX (10^{-6} M) was applied for 4 days between 10 and 14 DIV, the number of myelinated internodes, counted at 24 DIV, i.e., at the estimated peak of myelination, was decreased by 74.3% (Fig. 3). In avian, it has been shown that *in ovo* injection of curare, which blocks both the neuromuscular junction and the spontaneous electrical activation of the motoneurons, resulted in an increase in spinal motoneurons PSA expression (16). To investigate whether the TTX-induced inhibition of myelination could be related to an up-regulation of PSA expression at the axonal surface, we tested the ability of endo-N treatment to counteract the TTX-induced inhibition of myelination. Simultaneous addition of endo-N and TTX to the cultures did not revert the TTX-induced inhibition of myelination (Fig. 3). There was even a slight, although not statistically significant, synergistic effect of TTX inhibition by endo-N treatment. In a separate set of experiments, we investigated the effect of a 4-day TTX treatment on the expression of neuronal PSA-NCAM. No significant difference was observed in the percentage of neurons expressing PSA-NCAM, or in the intensity of the immunolabeling, between treated and control cultures (not shown). Moreover, under our culture conditions,

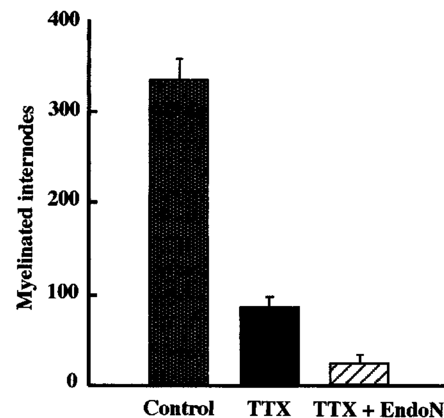

Fig. 3. TTX-induced inhibition of myelination is not reversed by PSA removal. TTX (10^{-6} M) was added for 4 days (10–14 DIV) to the culture medium, and myelination was quantified at 22 DIV. Myelination was decreased by 74.3% in TTX-treated culture. This TTX-induced inhibition of myelination was not prevented by removal of PSA sites by endo-N (0.8 units/ml) added simultaneously in the culture medium between 10 and 18 DIV.

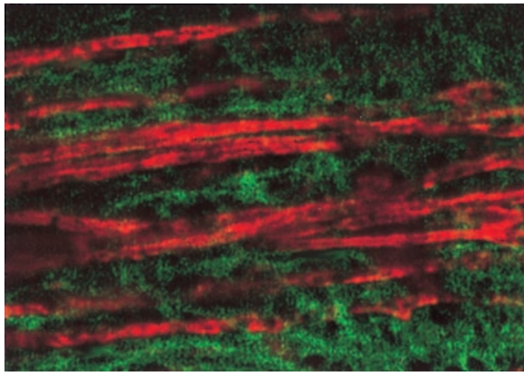

Fig. 4. *In vivo*, myelin is deposited along PSA-NCAM-negative axons. Confocal photographs of cryostat sections of a P7 mouse optic nerve immunostained with anti-MBP (red) and anti-PSA-NCAM (green) mAb illustrating a complete exclusion of the two labeling. MBP-positive myelin sheaths are deposited only around PSA-negative fibers. (Magnification, ×900.)

TTX treatment did not delay the normal down-regulation of neuronal PSA-NCAM.

In the Optic Nerve, Premature Removal of PSA Increases Myelination *in Vivo*. The *in vivo* relevance of the above observations was then examined in the developing optic nerve. In a first series of experiments, we confirmed the inverse relation between axonal down-regulation of PSA-NCAM and the onset of myelination. Until P4, i.e., when the first MBP^+ oligodendrocytes were detected, all of the axons were strongly $PSA\text{-}NCAM^+$. At P5.5 and P6, some axons started to be PSA-NCAM-negative, which coincided with the timing of appearance of the first MBP^+ myelinated internodes. Deposition of myelin occurred only on PSA-NCAM-negative axons. Between P5 and P7, a total of 12 optic nerves were examined, and we never observed PSA-NCAM-expressing axons surrounded by an MBP^+ myelin sheath (Fig. 4). At P10, at a developmental stage when the vast majority of optic nerve axons were undergoing myelination, PSA-NCAM was no longer detectable. To investigate the effect of premature removal of PSA moieties, endo-N was injected intravitreously in the right eye at P4, and animal examined at P5, P5.5, and P6. Twenty-four hours after endo-N injection, PSA-NCAM was no longer detectable along the optic nerve. Interestingly, the disappearance of immunodetectable PSA-NCAM was also observed in the contralateral optic nerve, and, in most of the cases, extended in the adjacent ipsilateral forebrain. Despite complete removal of PSA moieties, there were no myelinated fibers at P5, and the first MBP^+-myelinated internodes were observed at P5.5, with the same timing as in normal or PBS-injected control animals. Thus, premature removal of PSA did not accelerate the onset of myelination. At P5.5, however, there was a 1.6-fold increase in the number of myelinated internodes in the endo-N-injected animals (38 ± 28, $n = 8$) compared with PBS-injected controls (24 ± 19, $n = 9$). This increase was no longer observed at P6.

Discussion

Our study shows that, both *in vitro* and *in vivo*, PSA-NCAM expression is down-regulated when myelination starts, and that early removal of PSA-NCAM on axons, either by targeting with antibodies, which induce its internalization, or by means of endo-N digestion of PSA moieties, results in an increase in the number of myelinated internodes. The amplitude of the promyelinating effect of endo-N-induced premature removal of PSA moieties was more important in cultures (4.8-fold increase) than *in vivo* (1.6-fold increase). A possible explanation for this difference is that, in the cocultures, most axons are not myelinated, allowing mature oligodendrocytes to respond to a promyelinating stimulus, whereas, in the optic nerve, all axons are committed to be myelinated, and there is a very narrow time window between the experimental removal of PSA (P4) and its normal developmentally regulated complete down-regulation (P10). In this respect, it will be of interest to examine myelination in double-transgenic animals in which both sialyltransferases (PST and STX, the two enzymes responsible for the synthesis of PSA moieties on NCAM) encoding genes have been invalidated. To our knowledge, however, this double mutant mouse is not yet available.

The effect of anti-PSA-NCAM mAb on myelination could be related to a steric effect of the antibody. However, this possibility is rather unlikely as an internalization of the PSA-anti-PSA-NCAM complex has been demonstrated, and a similar increase in myelination was reproduced by enzymatic removal of PSA moieties. The specificity of the biological effect observed with the anti-PSA-NCAM mAb is strengthened by the observation that the amplitude of the effect is antibody concentration-dependent and is no longer observed after adsorption of the antibody with colominic acid. Moreover, no effect on myelination was observed with an antibody (A2B5), which binds to axons, but not to PSA-NCAM. Our findings are in agreement with the recent report that in a nonmyelinating *in vitro* coculture system, endo-N-induced suppression of PSA residues favors the close alignment of oligodendroglial processes along axons (18). Taken together, these results clearly suggest that down-regulation of PSA-NCAM favors myelination. A negative regulation of myelination by PSA-NCAM could involve two different and hypothetical mechanisms: PSA-NCAM may act by either triggering a negative signal regulating oligodendrocytes maturation, or preventing by steric inhibition the establishment of a close contact between axons and oligodendrocytes processes. Our results do not allow to choose unambiguously between these two possibilities. Experimental arguments suggesting that PSA-NCAM is a recognition molecules have been mostly provided by the coculture model popularized by Doherty *et al.* (19), where PSA-NCAM on the neuron is in contact with a transfected monolayer that does not express the PSA form. In some respect, our coculture of neurons and oligodendrocytes is reminiscent of Doherty's model, and our data could thus be explained in terms of a PSA-NCAM-mediated negative signal regulating oligodendrocytes maturation. From a variety of studies, however, it has been proposed that PSA-mediated regulation of cell–cell interactions stems from its large hydrated volume or negative charges, or both (7, 20). These properties of PSA could impede *trans*-interactions between apposing cells, and it can, therefore, be suggested that PSA-NCAM is acting on the process of myelination by preventing the adhesion of oligodendrocyte processes to axons.

PSA is heavily expressed on developing axons. It is also transiently expressed on immature glial cells. Recently, a role for PSA-NCAM in glial plasticity and migration of oligodendrocyte progenitors during development has been suggested (21, 22). In our study, the reported effect of PSA-NCAM removal on myelination is linked most probably to removal of axonal PSA and not of oligodendroglial PSA. Indeed, at 10 DIV, when either anti-PSA-NCAM mAb or endo-N were added in cultures, oligodendrocytes were no longer expressing PSA-NCAM (not shown). Moreover, no differences in the number of mature MBP-expressing oligodendrocytes were observed between treated and control cultures. Similarly, in the optic nerve, the short interval between endo-N injection and the increase in myelination makes it very unlikely that this effect is mediated by an acceleration of the maturation of oligodendroglial progenitors.

Our results do not suggest that modification of PSA-NCAM expression is the primary effect in the inhibition of myelination induced by blocking electrical activity in TTX-treated cultures. Suppression of PSA sites by endo-N did not prevent the drastic inhibition of myelination observed in TTX-treated cultures. Moreover, TTX treatment did not modify the percentage of PSA-expressing neurons during the first 2 wk of the culture, and did not interfere with the down-regulation of PSA expression after 15 DIV (not shown). This suggests that electrical activity and PSA-NCAM expression are acting on myelination by two independent mechanisms. This is in contrast with other reports suggesting that neuronal impulse activity is influencing PSA-NCAM expression. For example, during chick muscle formation, blockade of contractile activity results in a decrease of PSA-NCAM expression on muscle fibers, whereas an up-regulation is observed on axons (16). In contrast, in cultured cortical neurons treated with endo-N, restoration of PSA expression at the neuronal surface was completely prevented by TTX treatment, suggesting that electrical activity is required for PSA expression (15). In this respect, our observation that immunodetectable level of PSA-NCAM is not influenced by TTX is in agreement with the recent report that in the hypothalamo-neurohypophysial system, during lactation, overall levels of PSA-NCAM were not greatly altered by stimulation (23).

Our results suggest that expression of PSA-NCAM acts as a negative signal of myelination, probably by preventing adhesion of oligodendrocyte processes to axons. If PSA expression is considered as a negative regulator of myelination, one could hypothesize that persistence of axonal PSA expression in the adult brain would be restricted to unmyelinated fibers. This is indeed the case for mossy fibers of the dentate gyrus and axons from the supra optic and paraventricular nuclei, which remain unmyelinated throughout life, and have persistent PSA expression (11, 24).

However, the disappearance of these inhibitory signals from the axonal surface, although necessary, are not sufficient to allow myelination to proceed, suggesting the existence of positive regulatory axonal signals. We have previously shown that neuronal electrical activity induces myelination (5). Other observations support the role of electrical activity as an inducer of myelination. In the medial forebrain bundle, nigrostriatal fibers en route to the caudate nucleus and putamen are in close contact with myelinated fibers, and thus in the vicinity of mature myelin-forming oligodendrocytes. Nevertheless, although nigrostriatal fibers are PSA-NCAM-negative, they are not myelinated. It is of note, however, that nigrostriatal fibers are the axons from dopaminergic cells in the pars compacta, which are most of the time electrically silent (three to four action potentials per second). In myelinating cocultures and in the optic nerve, the premature removal of PSA moieties did not modify the timing of onset of myelination, suggesting that other parameters control the timing of wrapping of oligodendrocytes processes around axons. At the time when myelination starts, the electrical activity of retinal ganglion cells changes from a transient to a repetitive pattern of firing (25, 26). The molecular mechanism responsible for this electrical activity-mediated induction of the myelination process remains to be discovered. Myelination appears as a complex tightly controlled phenomenon: first, the down-regulation of negative signals allows axons to be permissive to the adhesion of oligodendrocyte processes; then, positively regulatory signals, mediated by the pattern of firing along the axons, trigger the ensheathment process.

In conclusion, our results could have important implications in the understanding of the pathophysiology of demyelinating diseases like multiple sclerosis. It has been demonstrated that in multiple sclerosis, there are attempts to remyelinate, but remyelination is, in most cases, insufficient after some years of evolution, despite oligodendrocyte sparing (27, 28). Our results showing that PSA-NCAM can act as a negative regulator of myelination could be extrapolated to central nervous system remyelination. In this respect, it could be speculated that reexpression of PSA at the surface of demyelinated axons could account for the partial failure of remyelination.

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