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Neurofilament-Tubulin Binding Site Peptide NFL-TBS.40–63 Increases the Differentiation of Oligodendrocytes In Vitro and Partially Prevents Them From Lysophosphatidyl Choline Toxiciy

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During multiple sclerosis (MS), the main axon cytoskeleton proteins, neurofilaments (NF), are altered, and their release into the cerebrospinal fluid correlates with disease severity. The role of NF in the extraaxonal location is unknown. Therefore, we tested whether synthetic peptides corresponding to the tubulin-binding site (TBS) sequence identified on light NF chain (NFL-TBS.40–63) and keratin (KER-TBS.1–24), which could be released during MS, modulate remyelination in vitro. Biotinylated NFL-TBS.40–63, NFL-Scramble2, and KER-TBS.1–54 (1–100 μM, 24 hr) were added to rat oligodendrocyte (OL) and astrocyte (AS) cultures, grown in chemically defined medium. Proliferation and differentiation were characterized by using specific antibodies (A2B5, CNP, MBP, GFAP) and compared with untreated cultures. Lysophosphatidyl choline (LPC; 2×10^{-5} M) was used to induce OL death and to test the effects of TBS peptides under these conditions. NFL-TBS.40–63 significantly increased OL differentiation and maturation, with more CNP^+ and MBP^+ cells characterized by numerous ramified processes, along with myelin balls. When OL were challenged with LPC, concomitant treatment with NFL-TBS.40–63 rescued more than 50% of OL compared with cultures treated with LPC only. Proliferation of OL progenitors was not affected, nor were AS proliferation and differentiation. NFL-TBS.40–63 peptide induces specific effects in vitro, increasing OL differentiation and maturation without altering AS fate. In addition, it partially protects OL from demyelinating injury. Thus release of NFL-TBS.40–63 caused by axonal damage in vivo could improve repair through increased OL differentiation, which is a prerequisite for remyelination.

Key words: astrocyte; axon–glia interactions; demyelination; microtubule; neurofilament

Multiple sclerosis (MS) is the most common chronic inflammatory and demyelinating disease of the central nervous system in adults. Its etiology is unknown, and there is no curative treatment (see, e.g., Castro-Borrero et al., 2012). Lesions are characterized by incomplete remyelination by oligodendrocytes (OL) and axon degeneration (Trapp et al., 1998; Bitsch et al., 2000; Luchinetti et al., 2000; Schirmer et al., 2011). Axonal lesions include expression of abnormal nonphosphorylated forms of the main axon cytoskeleton proteins, neurofilaments (NF; Hoffman and Cleveland, 1988), and decreased NF and β-tubulin (TUB; Trapp et al., 1998; Fressinaud et al., 2005). It has been proposed that these alterations might, in turn, render axons improper for remyelination (Chang et al., 2002). Axon loss is also encountered outside the lesions, in the normal-appearing white matter (Evangelou et al., 2000; Siffrin et al., 2010), and axon damage is a major concern because it results in disability (Rudick et al., 1999; Wegner et al., 2006; Siffrin et al., 2010; Schirmer et al., 2011). Neuronal and axonal damage could result from demyelination (Raine and Cross, 1989) or be a distinct consequence of inflammation itself (DeLuca et al., 2006). Importantly, restoring remyelination capacity increases axon survival experimentally (Irvine and Blakemore, 2008). During this pathological process, NF are released into the cerebrospinal fluid (CSF) of MS patients (Lycke et et al., 1998; Teunissen et al., 2005), where their concentration correlates with disease severity (relapse rate and disability; Gresle et al., 2011; Kuhle et al., 2011).

The mechanisms of axon alterations in MS, as well as the reasons for the failure of remyelination are largely unknown. Experimentally, NF are susceptible to proteolysis (Saatman et al., 2003; Hall and Lee, 2005), and NF light chain (NFL)-positive macrophages have been

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identified in MS lesions (Huizinga et al., 2012). However, the appearance of NF or their proteolytic fragments during the evolution of MS is poorly documented, as are their functions when they are present outside of the axonal compartment. On the other hand, proteomic analysis of myelin purified from healthy subjects constantly reveals the presence of NF (Taylor et al., 2004; Werner et al., 2007; Ishii et al., 2009). Previously, we have observed in vitro that purified NF fractions and some of their associated axon cytoskeleton proteins (tubulin, MAPs, tau, synapsin 2, spectrin β2) specifically increase OL progenitor (OLP) proliferation and/or differentiation (Fressinaud et al., 2012). Moreover, NF and tubulin protect OL from injury when they are challenged with lysophosphatidyl choline (LPC), a well-known demyelinating compound, in vitro (Fressinaud and Eyer, 2013). This suggests that NF released during MS lesion might regulate OL fate and remyelination in vivo, although this phenomenon could be hampered by the release of altered isoforms of NF (see above) and modifications in proteolysis as described for experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Jain et al., 2009). Thus, to determine more precisely the putative roles of extra-axonal NF and their peptides, we have taken advantage of synthetic peptides corresponding to the sequence that binds tubulin (TBS) on NFL described from the laboratory (Bocquet et al., 2009), and we have tested their effects on pure OL cultures. One of these peptides was also demonstrated to penetrate into glioblastoma cells and inhibits their proliferation in vitro as well as in vivo by disrupting the microtubule network (Berges et al., 2012). In addition, we used a simple paradigm of OL toxicity and demyelination with LPC (Fressinaud, 2005) to test whether TBS peptides could improve recovery in this model.

MATERIALS AND METHODS

Synthetic Peptides

Biotinylated peptides (greater than 95% purity) were synthesized according to the TBS sequence identified in the NFL chain (NFL-TBS.40–63: YSSYSAPVSSSLSVRRSYSSSSGS) and keratin (Ker-TBS.1–24: MSIRVTQKSYKMSTSGPRAFSSRS; Millegen, Toulouse, France). Scrambled peptides with the same amino acids as NFL-TBS.40–63, although in random sequence (NFL-SCR2: SLGSPSSSVRASYSSSRSYVYSSS), were also used as controls (Bocquet et al., 2009). Peptides were dissolved in water and used at 10 μM final concentration unless otherwise specified.

Cell Cultures

Glial cell cultures from newborn Wistar rat (breeding by the Faculty of Medicine, University of Angers) brains were performed as described by Fressinaud et al. (2012). The studies were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care and use of laboratory animals. After mechanical dissociation, brain hemispheres, freed from their meninges, were grown in primary glial cell cultures, enriched in OL, in Waymouth's medium supplemented with 10% calf serum (Gibco, Invitrogen, Cergy Pontoise, France). After 3 weeks, OL were separated from the astrocytic underlayer by flushing some medium over the cultures and were grown in secondary cultures. Before the subculture, contaminating microglial cells were removed by preplating on plastic Petri dishes. Then, OL recovered with the medium were seeded on poly-L-lysine-precoated glass coverslips in multiwell dishes (24 wells) and grown in chemically defined medium (CDM) composed of Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 4 g/liter glucose, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 mg/ml bovine serum albumin, and 30 nM selenium. These cultures are at least 95% pure, and the OL synthesize myelin-like membranes (Fressinaud et al., 1993, 1996).

Primary rat astrocyte (AS) cultures were prepared following a modification of this protocol as described elsewhere (Fressinaud et al., 1991, 2012). Cultures prepared on poly-L-lysine-precoated glass coverslips in multiwell dishes were grown in DMEM supplemented with 10% fetal calf serum (Gibco), and after 20 days the medium was replaced by CDM (see above).

Secondary OL cultures, and AS cultures, were treated for 1–3 days as follows (Fig. 1): 1) control (CONT): CDM alone; or 2) synthetic peptides: NFL-TBS.40–63, NFL-SCR2, or KER-TBS.1–24 (Bocquet et al., 2009) added once to the CDM (diluted in water, final concentrations: 0.25, 0.5, 1. 5, 10, 50, and 100 μM were tested). Proliferation was assessed by bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) incorporation (10 μg/ml for 16 hr) prior to fixation (day 2). Differentiation and maturation were analyzed 3 days after treatment with peptides (day 4).

To test the effect of peptides in demyelinating conditions, OL grown in CDM were allowed to adhere and grow processes on the coverslips for 24 hr after seeding and before lysophosphatidyl choline (LPC; 2×10^{-5} M final concentration, L-α-lysophosphatidylcholine from egg yolk, >99% pure; Sigma) was added for 24 hr on day 2 (Fressinaud, 2005; Fressinaud and Eyer, 2013; Fig. 1). At the same time, peptides (10 μM; see above) were added to sister-cell LPC-treated cultures. On day 3, LPC and peptides were removed, and the dishes were rinsed once. Then, they were grown further in CDM alone for 2 days and analyzed on day 5 for differentiation and maturation experiments. Control cultures grown in CDM only were performed at the same time.

Immunocytochemistry of Cultures

OL progenitors were labeled with anti-A2B5 monoclonal antibody (Ab; 1:100 dilution; Chemicon, Temecula, CA), differentiated (CNP^+) OL with anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (monoclonal Ab; 1:100 dilution; Sigma), and mature OL with anti-myelin basic protein (MBP) polyclonal Ab (1:100 dilution; Dako, Trappes, France), after fixation with 3% paraformaldehyde as previously described (Fressinaud et al., 1993, 1996). Astrocytes were labeled with polyclonal anti-GFAP Ab (1:100 dilution; Dako; Fressinaud et al., 1991). Cell proliferation of OLP and AS was assessed by anti-BrdU monoclonal Ab (1:400 dilution; Sigma), and double immunolabeling with anti-A2B5 (OLP) or -GFAP (AS) Ab. Labeling was revealed by goat anti-rabbit Ig G-Alexa Fluor 488 (MBP,

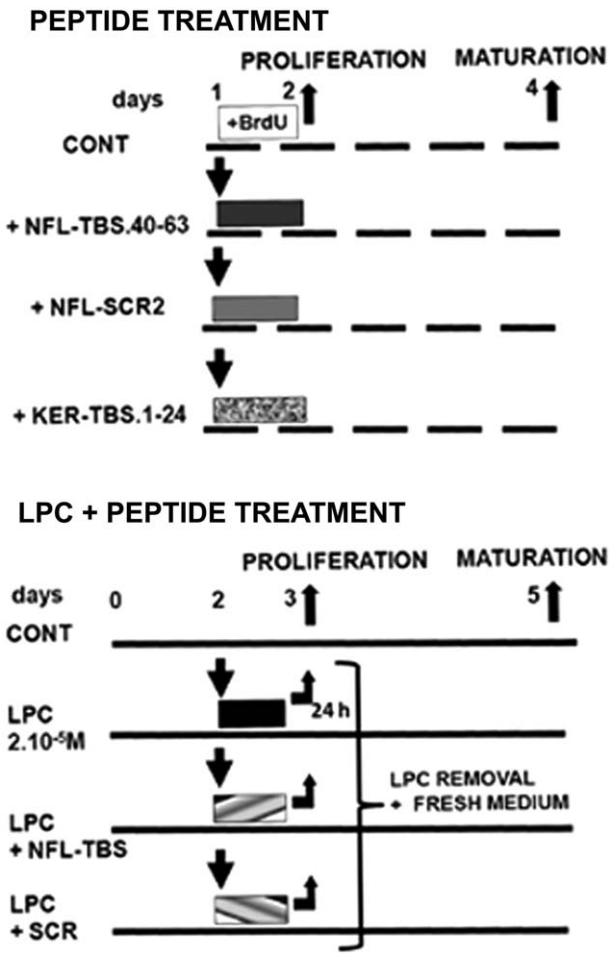

Fig. 1. Graph of the experimental time schedules used under basal conditions (upper panel) or with LPC treatment (lower panel). All cells were grown in chemically defined medium. Untreated controls (CONT) and cells grown with the different peptides and/or LPC were analyzed after 24 hr of exposure for proliferation experiments or 3 days later for differentiation and maturation.

GFAP; Invitrogen, Cergy Pontoise, France), or anti-mouse Ig M-Alexa Fluor 488 (A2B5; Invitrogen), or anti-mouse Ig G-Alexa 568 (CNP; BrdU)-conjugated secondary antibodies (1:200 dilution; Invitrogen; Fressinaud et al., 2012).

To specify further the mechanisms of action of TBS peptides, their effects on OL cytoskeleton were also analyzed by double immunocytochemistry with anti-CNP, anti-MBP, anti-βTUB (1:2,000 dilution; Sigma), anti-MAP, and anti-Tau antibodies as previously described (Fressinaud et al., 2012).

Analyses of OL and AS Cultures

The effects of peptides on cultures were analyzed on day 2 of the subculture for proliferation and on day 4 for maturation, following the main criteria listed below (Fressinaud, 2005): 1) number of total cells (number of cells per optic field observed by phase-contrast microscopy); 2) proliferation: after BrdU incorporation and double immunolabeling with anti-BrdU, anti-A2B5, or anti-GFAP antibodies (see below); 3) differentiation and maturation features: immunocytochemistry using anti-A2B5 (OL progenitors), anti-CNP (differentiated OL), anti-MBP (mature OL), and anti-GFAP (astrocytes) antibodies; 4) morphology: numbers of processes, membranes, and putative myelin balls were evaluated after immunolabeling of OL with anti-CNP or -MBP antibodies.

More precisely, morphological changes were quantified after CNP immunostaining by counting the number of processes per CNP^+ OL and scoring the presence of membranous extensions. Cells with a morphology suggestive of a high level of differentiation, i.e., more than five ramified processes, membranous extensions, or myelin balls (Sperber and McMorris, 2001; Bauer et al., 2009), were quantified and designated as CNP^+ mb. Their relative percentage was compared with the total number of CNP^+ OL (Fressinaud et al., 2012). The effects of LPC and peptides were determined by using the same protocol on day 5. The proliferation (BrdU incorporation) and differentiation (GFAP immunolabeling) of AS were analyzed similarly after 1 and 3 days of peptide treatment, respectively, by counting labeled cells compared with untreated control cultures.

Mechanisms of NFL-TBS.40–63 Peptide Effects on OL Cultures

Biotinylated peptides were localized using streptavidin coupled to Alexa Fluor A488 (1:200 dilution; Invitrogen) in double-labeling experiments with anti-CNP Ab revealed by anti-mouse IgG coupled to Alexa Fluor 568. Observations were made also by confocal microscopy to specify further the intracellular location of the peptides (Olympus FV300; Service Commun d'Imagerie et d'Analyse Microscopique, Faculty of Medicine, University of Angers).

To detect the putative activation of several signalling pathways by NFL-TBS.40–63, double immunocytochemistry of OL cultures with anti-A2B5 or anti-CNP Ab was performed as follows. Activation of Fyn tyrosine kinase was assessed by using polyclonal antiphospho-Src family Ab and monoclonal nonphospho-Src Ab (1:100 dilution; Cell Signalling, Danvers, MA). Similarly MEK (rabbit polyclonal Ab antiphospho-MEK1; 1:40 dilution; R&D Systems, Minneapolis, MN), Akt (pan Akt monoclonal Ab; 1:100 dilution; R&D Systems), and Rho (rabbit polyclonal Ab anti-Rho A/B/C; 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) modifications were tested. Modifications of PTEN and phospho-GSK pathways were analyzed using double immunolabeling with PTEN rabbit monoclonal Ab directed against total PTEN (1:100 dilution; Cell Signalling), or phospho-GSK-3αβ (Ser 21/9) rabbit polyclonal Ab (1:50 dilution; Cell Signalling), and anti-CNP mouse monoclonal Ab (see above).

Sampling and Statistical Analysis

Each experiment was run five times, with different batches of peptides preparations, in three samples for each of the treatments (control, and different dilutions of the TBS peptides). Similarly, experiments with LPC were performed five times in triplicate. Counting of labeled cells was performed on 20 optical fields distributed randomly on each coverslip. Results were averaged and compared with appropriate tests (Mann and Whitney test, Student's *t*-test adapted to small samples, variance analysis [ANOVA, F test]).

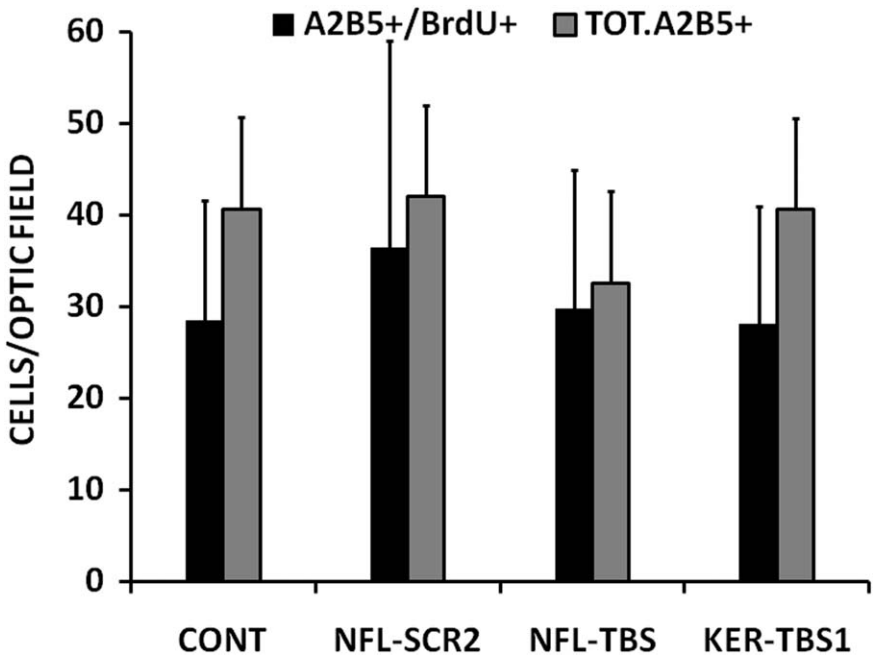

Fig. 2. Effects of the NFL-TBS.40-63 peptide (NFL2; 10 μM final concentration) on OL progenitor cell (total $A2B5^+$ cells, gray bars) proliferation ($A2B5^+/BrdU^+$ cells, black bars) compared with untreated control cultures (CONT). Results are mean of labeled cells per optic field. Cultures were analyzed after 1 day in chemically defined medium and compared with control peptides (NFL-scramble: NF178T- and Ker-TBS.1–24). Differences from controls are not significant.

RESULTS

NFL-TBS.40–63 Peptide Increases OL Differentiation and Maturation In Vitro

Analyses of OL cultures grown in CDM revealed that the NFL-TBS.40–63 peptide (10 μM) regulated OL development. Its effects were selective, in that it significantly increased OL differentiation and maturation, whereas OLP proliferation was unchanged (Figs. (2 and 3)) compared with untreated control cultures. After 24 hr of treatment with NFL-TBS.40–63, neither the number of $A2B5^+$ OLP nor that of proliferating ($BrdU^+$) cells was increased compared with untreated controls (Fig. 2). The other peptides (NFL-scramble 2 and KER-TBS.1–24) also were without effect on OLP proliferation (Fig. 2). On the contrary, 3 days after treatment with NFL-TBS.40–63, differentiation appeared to be stimulated, because the percentage of differentiated (CNP^+) cells had increased by more than 90% ($P < 0.01$) compared with untreated controls (Fig. 3B,C). Moreover, differentiated cells (expressing CNP) and extending membranes or numerous (>5 ramified processes; Fig. 3B) thin, branched processes (CNP^+ mb) had increased by 120% (Fig. 3C). These cells correspond to highly differentiated OL (e.g., Sperber and McMorris, 2001).

Finally, cell maturation was also enhanced by the NFL-TBS.40–63 peptide after 3 days, because MBP^+ cells had increased above controls by more than 170% ($P < 0.01$; Fig. 3C). Similarly to what was observed for CNP^+ OL, MBP^+ cells displayed a more well-developed shape in peptide-treated cultures than their untreated counterparts (Fig. 3G). Dose–response experiments (1–100 μM) did not reveal significant modifications of the NFL-TBS.40–63 effects, and a maximal response was obtained for 10 μM concentration. The differentiation was slightly increased at higher concentrations (not shown).

Effects of Other Peptides

The scrambled NFL peptide (NFL-SCR2), composed of the same amino acids as NFL-TBS.40–63 but distributed randomly, did not alter OLP proliferation (Fig. 2). It slightly enhanced OL maturation, with an increase in MBP^+ cells of 65% above control values, which did not reach significance (Fig. 3C).

The KER-TBS.1–24 peptide (corresponding to the tubulin binding site of keratin) had effects similar to those of NFL-TBS.40–63, though to a lower extent. It did not upregulate OLP proliferation (Fig. 2). Nevertheless, it induced a slight increase in OL with a very differentiated morphology (CNP^+ mb; +55% above control values) as well as an increase in the number of mature cells (MBP^+) by 125% above controls (Fig. 3C; $P < 0.01$).

Specificity of NFL-TBS.40–63 Peptide Effects on OL

To determine whether the effects of the TBS peptides were specific to OL or could also affect other cell types, the same peptides (NFL-TBS.40–63, NFL-SCR2, and KER-TBS.1–24) were assayed in pure AS cultures

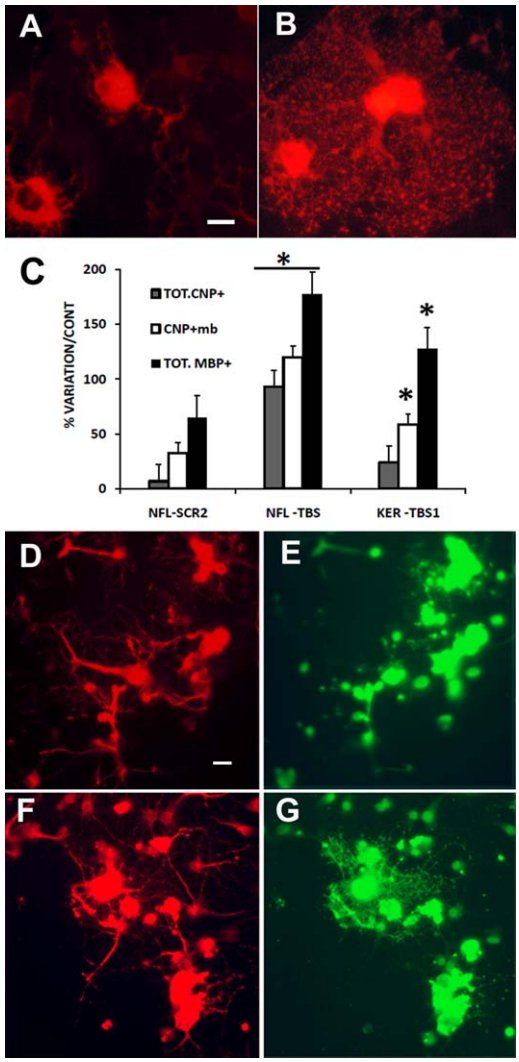

Fig. 3. **A,B:** Immunocytochemistry with anti-CNP antibody showing the effects of the NFL-TBS.40-63 peptide (B; 10 μM final concentration for 3 days) on OL differentiation compared with untreated control cultures (A). Note numerous and complex processes in NFL-TBS.40-63-treated cells, characteristic of highly differentiated OL, referred to as CNP^+ mb (>5 ramified processes and/or membranous extensions). **C:** Effects of the NFL-TBS.40-63 peptide (10 μM final concentration) on OL differentiation (CNP^+ cells) and maturation (MBP^+ cells) after 3 days in chemically defined medium. The NFL-TBS.40-63 increases significantly ($^\star P < 0.01$) all the criteria analyzed: total number of CNP^+ cells ($TOT.CNP^+$), number of CNP^+ mb OL (which depicts high morphological differentiation), and total number of mature OL (TOT. MBP^+ cells). Results are expressed as percentage of variation vs. untreated control (CONT) cultures and compared with control peptides (NFL-SCR2, and Ker-TBS.1–24). Differences with CONT are also significant for CNP^+ mb and MBP^+ OL only with KER-TBS1 ($^\star P < 0.01$), whereas the scramble NFL-SCR2 peptide was statistically inefficient. **D–G:** Double immunolabeling of mature OL cytoskeleton with anti-β-tubulin (D,F) and anti-MBP (E,G) antibodies. Note the increased length and complexity of the cytoskeleton with β-$tubulin^+$ and MBP^+ processes in cultures treated with NFL-TBS.40-63 (F,G) compared with untreated control (D,E). Scale bars = 10 μm in A (applies to A,B); 10 μm in D (applies to D–G). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

under the same conditions (1–3 days of treatment in CDM). In accordance with previous results (Berges et al., 2012), none of the peptides significantly modified the proliferation of AS, because the number of $BrdU^+/GFAP^+$ cells remained unchanged. Similarly, AS differentiation was not affected in the range of concentrations tested (1–100 μM), and GFAP immunolabeling did not show differentiation or morphological changes (not shown).

Mechanisms of Action and Signalling Pathways

When OL cultures were treated with NFL-TBS.40–63 peptide, subtle morphological changes of the OL cytoskeleton were observed by double immunolabeling of MBP^+ OL for βTUB (Fig. 3F). In comparison with controls (Fig. 3D), there was an increase in the number, length, and complexity of OL processes that expressed both MBP and βTUB (Fig. 3F,G). Nevertheless, there was no other modification of the OL cytoskeleton. In particular βTUB and MBP displayed a colocalization pattern similar to that observed in controls (Fig. 3D,E), and fluorescence had the same homogeneous aspect. Also, double immunolabeling of MBP^+ OL with MAPs and Tau revealed their colocalization with MBP in the processes that did not differ from controls (not shown).

Immunocytological localization of biotinylated peptides revealed by streptavidin allowed the evaluation of their distribution in the cell body and proximal parts of main processes in CNP^+ OL (Fig. 4D,F,G). Quantification confirmed that most of the cells were labeled (80% for NFL-TBS.40–63 and 95–100% for NFL-SCR2 and KER-TBS.1–24). This was confirmed by confocal microscopy (Fig. 4F,G).

Several putative signaling pathways were explored to characterize the mechanisms of action of the NFL-TBS.40–63 peptide. The following pathways known to be involved in OL differentiation and myelination were analyzed: 1) the Src family to which Fyn belongs (Krämer et al., 1999; Biffiger et al., 2000; Klein et al., 2002; White et al., 2008); 2) Akt, which has been involved in OL survival and myelination (Flores et al., 2008; Narayanan et al., 2009); 3) Rho (Kippert et al., 2007) and phospho-MEK, which participate in OL cytoskeleton regulation (e.g., Boggs et al., 2008); and 4) PTEN and phospho-GSK, which were reported to alter axon regeneration and OL maturation (Harrington et al., 2010; Azim and Butt, 2011).

We did not observe modifications in OL culture immunostaining for Src family proteins after NFL-TBS.40–63 treatment. The intensity, the cellular localization of the staining, and the number of labeled OL were similar to those of untreated controls (not shown). Also, pan-Akt, PTEN, phospho-GSK, phospho-MEK (Fig. 5D–F), and Rho (Fig. 5J–L) immunostaining was not affected under similar conditions. MEK immunolabeling of $A2B5^+$ OLP was observed in 78.9% ± 6.8% of untreated controls (Fig. 5A–C) vs. 84% ± 4% of NFL-TBS.40–63-treated cultures (Fig. 5D–F). Rho immunostaining was observed in about 40% ± 10% of $A2B5^+$ OLP in control (Fig. 5G–I) and in treated cultures (Fig.

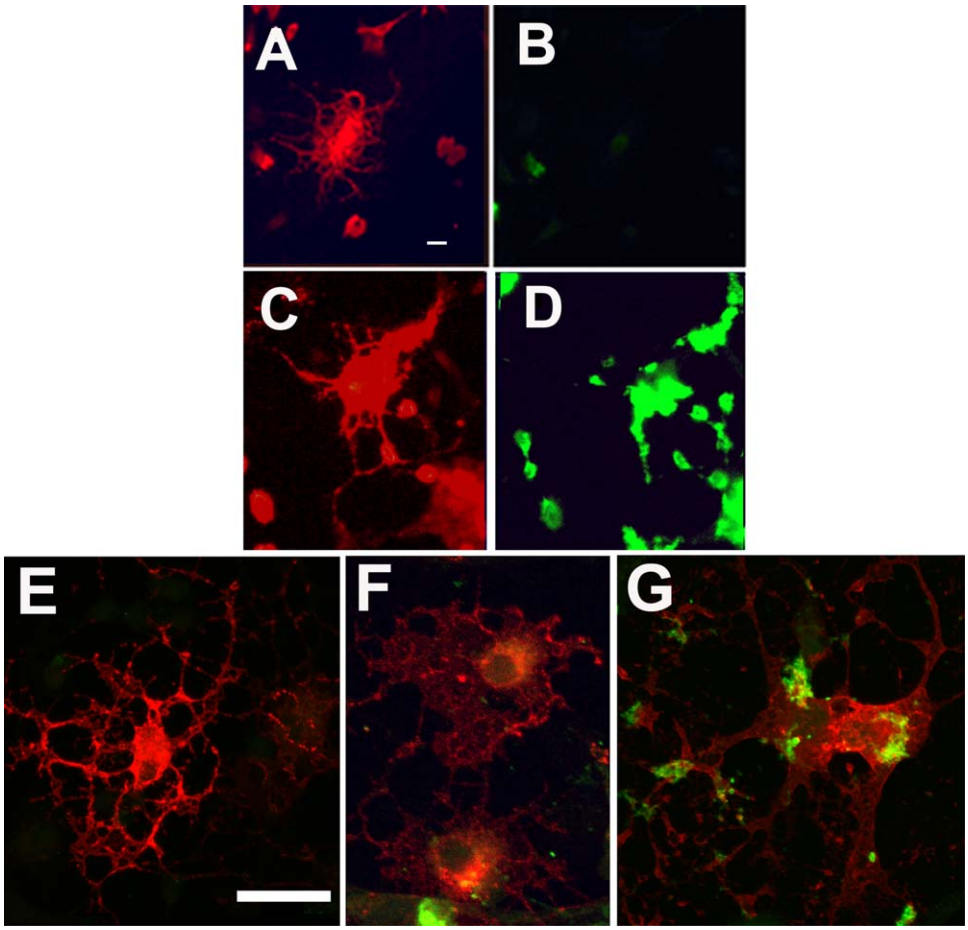

Fig. 4. Localization of NFL-TBS.40-63 (C,D,F) in OL by optic (**A–D**) and confocal microscopy (**E–G**) using double immunocytochemistry. Differentiated OL are labeled by anti-CNP (A,C,E–G; revealed with Alexa-fluor 568, red). Biotinylated NFL-TBS.40-63 entry into CNP^+ OL (C,F) is revealed by streptavidin coupled to Alexa Fluor 488 (D,F, green), A,B and C,D correspond to the same optic fields, (F) to the merge of CNP and NFL-TBS confocal microphotographs. Untreated control cultures labeled with CNP (A,E) show less well-differentiated cells than NFL-TBS.40-63-treated cultures, and there is no labeling by Alexa Fluor 488 (B,E). E–G: Confocal microscopy (merge of fields) confirms the localization of biotinylated NFL-TBS.40-63 (F) and KER-TBS.1–24 (G) into CNP^+ OL in OL cultures treated with these peptides (F,G), whereas there is no staining with streptavidin coupled to Alexa Fluor 488 in untreated control cultures (E). Scale bars = 10 μm in A (applies to A–D); 20 μm in E (applies to E–G). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

5J–L). In controls, about 8% of CNP^+ OL expressed Rho as well as 5.5% of treated cells (not shown).

NFL-TBS.40–63 Peptide Protects OL From LPC Toxicity In Vitro

Since NF could be released by axon lesion during demyelination in MS, we explored whether NFL-TBS.40–63 peptides could prevent OL injury when cells are exposed to lysolecithin (LPC), a known demyelinating agent that we had characterized in these cultures (e.g., Fressinaud et al., 1996; Fressinaud, 2005). As previously described, 2×10^{-5} M LPC for 24 hr (Fig. 6B) induced the death of half of the cells compared with untreated controls (Fig. 6A), and there was no spontaneous recovery 2 days later. Also, after LPC treatment, surviving cells displayed an altered morphology with short, truncated ramifications, or lack of processes (Figs. 6B, 7C,D).

On the contrary, 2 days after LPC removal, in cultures treated simultaneously with LPC and NFL-TBS.40–63 (Fig. 6C), OL were more numerous, and some of the cells had the characteristic multiprocess morphology of differentiated OL as in untreated control cultures. These effects were obvious by simple observation of living cells via phase-contrast microscopy of cultures (Fig. 5C), but they were only partial; LPC- + NFL-TBS.40–63-treated cells could still be distinguished from untreated controls (Fig. 7). This was not the case with NFL-SCR2 peptide, which did not decrease cell death compared with cultures treated with LPC only and was also unable to improve morphological alterations (Fig. 6D).

These results were confirmed by immunocytochemistry. Compared with cultures treated with LPC alone (Fig.

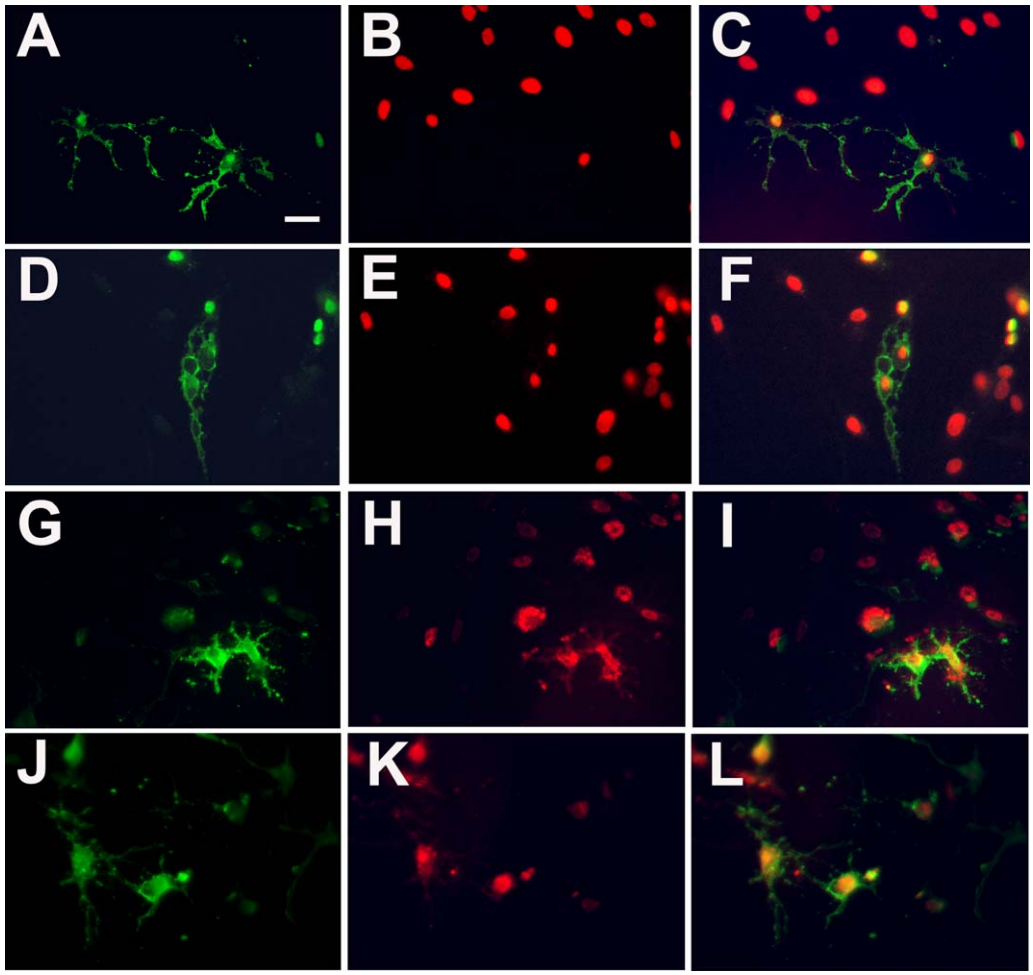

Fig. 5. Microphotographs of untreated control (**A–C,G–I**) and cultures treated with NFL-TBS.40-63 for 24 hr (**D–F,J–L**). Double immunolabeling for A2B5 (A,D,G,J), phospho-MEK (B,E), or Rho (H,K). Merged images of the same fields (C,F,I,L). Note $A2B5^+$/MEK^+ and $A2B5^+$/Rho^+ OL progenitors in control as well as is treated cultures. The number of cells labeled for phospho-MEK and for Rho in NFL-TBS.40-63-treated cultures is similar to control. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

7C,D), NFL-TBS.40–63 addition resulted in increased numbers of differentiated and mature cells evidenced by a 110% increase of the total number of CNP^+ and CNP^+ mb OL (Fig. 7E–G), whereas MBP^+ cells increased by 180% ($P < 0.01$). The peptide did not induce OL proliferation, so this result was suggestive of an enhanced survival, the peptide preventing LPC toxicity in part of the OL. A slight effect was observed with concomitant treatment of cells with LPC and KER-TBS.1–24, which increased only the percentage of CNP^+ mb and of MBP^+ cells, whereas NFL-SCR2 was inefficient (Fig. 7G).

DISCUSSION

NFL-TBS.40–63 Peptide Upregulates OLP Differentiation

To our knowledge, regulation of OL development by axon cytoskeleton-derived peptides has not been reported previously. This investigation shows that the effects of NFL-TBS.40–63 were obvious and statistically significant even under demyelinating conditions. They were observed with several batches from different peptide preparations. Given the procedure of the synthesis, it is unlikely that NFL-TBS.40–63 peptides contain other molecules that could have explained the effects recorded. Moreover, the NFL-SCR2 peptide prepared similarly was inefficient. These effects were restricted and specific, because NFL-TBS.40–63 peptide induced differentiation and maturation of cells of the OL lineage, whereas it did not upregulate their proliferation. As previously reported, NFL-TBS.40–63 peptide had no effects on AS cultures, and it penetrates fewer than 10% of astrocytes in vitro (Berges et al., 2012). Thus, given the purity (≥95%) of the cultures (see, e.g., Fressinaud et al., 1993), it appears very unlikely that the peptide exerts its effects indirectly through putative minor contaminants (astroglia [≤3% of total cells] or microglia [≤2% of total cells]).

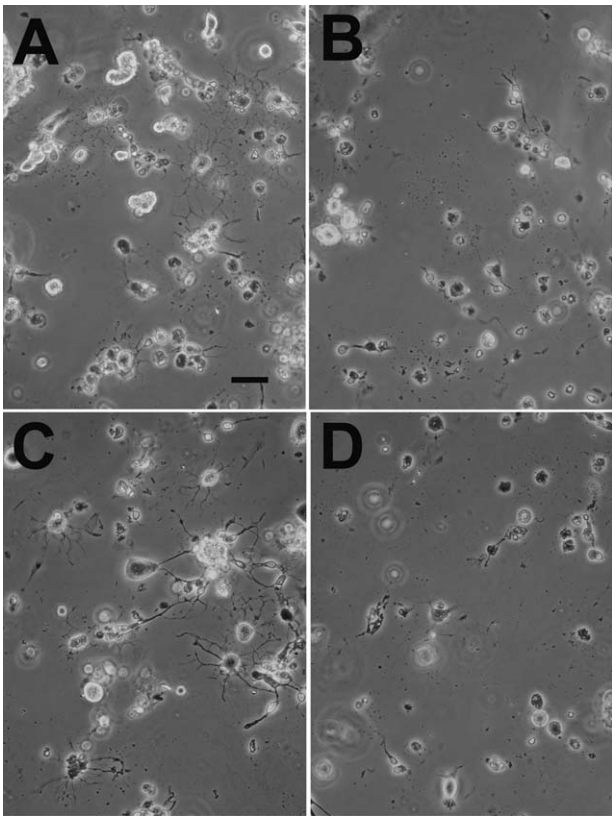

Fig. 6. Preventive effects of the NFL-TBS.40-63 peptide on LPC toxicity. In cultures treated for 24 hr with LPC and NFL-TBS.40-63 (**C**), the number of the cells is significantly increased compared with LPC alone (**B**) and differs only slightly from untreated control (**A**). Also, differentiated cell morphology with long and numerous ramified processes is preserved with NFL-TBS.40-63 peptide treatment. On the contrary, the NFL-SCR2 peptide has no effect when added with LPC (**D**). Phase-contrast microphotographs. Scale bar = 20 μm.

In cultures grown under standard conditions, NFL-TBS.40–63 increased the number of differentiated OL and mature cells. Proliferation was unchanged as demonstrated by BrdU incorporation and cell counts, so this could result only from a specific upregulation of terminal differentiation of some progenitors that remain at undifferentiated stage in these cultures (e.g., Fressinaud et al., 1993).

The demonstration of the effects of NFL-TBS.40–63 suggests that the TBS sequence of NFL could account, at least partially, for the activity of purified NF fractions previously observed (Fressinaud et al., 2012). Indeed, the second pellet recovered from NF purification (NFP2), as well as other cytoskeleton proteins (including TUB), induced OLP proliferation (Fressinaud et al., 2012), which is not the case for NFL-TBS.40–63 peptide. However the last fraction of NF purification, NFP5, had the same pattern of effects as the peptide, with only an increase in OL differentiation and maturation. In addition, purified NF fractions were ineffective in AS cultures (Fressinaud et al., 2012), similarly to NFL-TBS.40–63. Therefore, if peptides such as NFL-TBS.40–63 are released in vivo following axonal damage, they could potentially improve myelin repair through increased differentiation and maturation of OLP. However, the release of NF subunits, including NFL, in the CSF of MS patients has been identified (Lycke et et al., 1998; Teunissen et al., 2005), and NFL concentration correlates with disease severity (Gresle et al., 2011; Kuhle et al., 2011). In addition, OL that failed to differentiate are seen in contact with axons in MS lesions (Chang et al., 2002). Although these facts argue against a pro-remyelinating role of NFL-TBS.40–63, they have to be tempered by a set of other considerations. First, there has been no detailed molecular analysis of the NF released in the CSF during MS to our knowledge, whereas profound alterations of NF expression in MS lesions have been described (e.g., Trapp et al., 1998), so release of altered NF could suppress their effects. Second, altered proteolysis has been reported in the EAE model of MS (Jain et al., 2009); again, modifications in the peptides released could change their pro-remyelinating potential.

Mechanism of Action of the NFL-TBS.40–63 Peptide Is Equivocal

In glioblastoma cells, NFL-TBS.40–63 was demonstrated to bind TUB (Bocquet et al., 2009) and to disrupt the microtubule cytoskeleton (Berges et al., 2012). Similarly, the penetration of the peptide into CNP^+ OL was unequivocal. However, immunolabeling of the OL cytoskeleton for TUB, MAP, and Tau did not reveal specific changes in NFL-TBS.40–63-treated cultures, and in controls too the colocalization of MBP and cytoskeleton proteins was similar to that described in previous reports (Fischer et al., 1990; Müller et al., 1997; Gorath et al., 2001; Terada et al., 2005). This lack of disorganization of the cytoskeleton suggests that NFL-TBS.40–63 upregulates usual physiological pathways to increase OL differentiation and maturation. Interestingly, the same observation was made for treatment with purified NF fractions and other axon cytoskeleton proteins, which increased OL differentiation without altering cytoskeletal organization (Fressinaud et al., 2012). Physiologically, this pathway involves Erk1/2 MAPK and mTOR signaling sequentially (Guardiola-Diaz et al., 2012).

We did not identify a specific signalling pathway mediating the peptide effects. Several pathways involved in OL growth and myelination were analyzed. We did not detect the participation of Src family proteins, to which Fyn kinase belongs. This was tested because it is involved in OL process outgrowth (Klein et al., 2002) and in myelination (e.g., Sperber et al., 2001; White et al., 2008). Similarly, Akt, which regulates OL survival and myelination (Flores et al., 2008; Narayanan et al., 2009); MEK (Boggs et al., 2008); Rho (Liang et al., 2004); PTEN; and phospho-GSK (Harrington et al., 2010; Azim and Butt, 2011), were unchanged by the NFL-TBS.40–63 treatment of OL.

All the peptides assayed were proved to enter most OL. The mechanism of entry of these peptides into OL is unknown. In glioblastoma cell lines, it has been

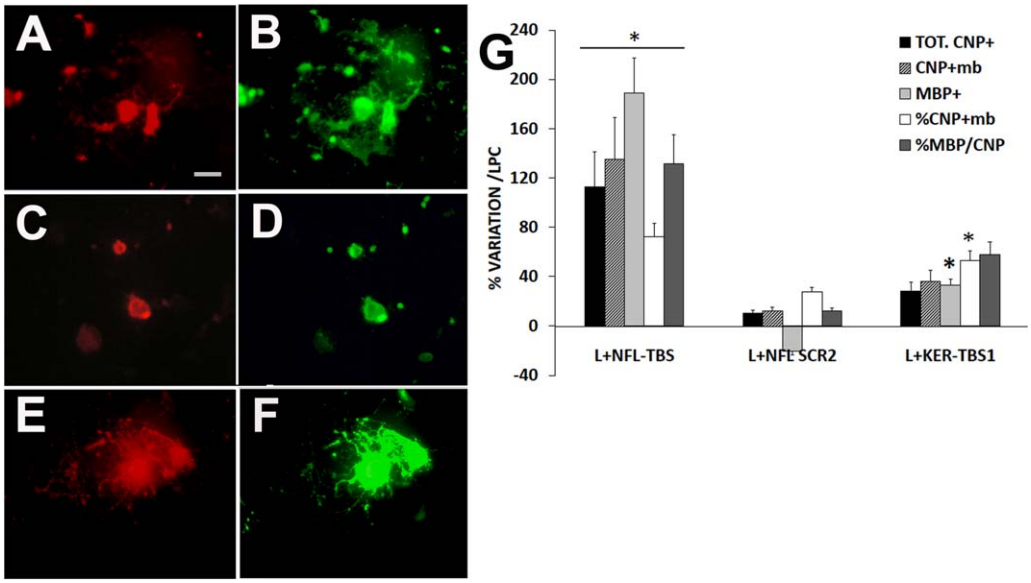

Fig. 7. Preventive effects of the NFL-TBS.40-63 peptide on LPC toxicity. In cultures treated for 24 hr with LPC and NFL-TBS.40-63 (**E,F**), differentiation and maturation of the cells are significantly increased compared with LPC (**C,D**) and are similar to untreated control cultures (**A,B**). Double immunolabeling for CNP (A,C,E) and MBP (B,D,F). **G:** In cultures treated for 24 hr with LPC and NFL-TBS.40-63, all the criteria of OL differentiation (total number of CNP^+ OL [$TOT.CNP^+$], number and percentage of cells with a very differentiated morphology [CNP^+ mb], and maturation [number of MBP^+ cells, ratio of MBP to CNP^+ OL]) are significantly increased ($^*P < 0.01$). Results are in percentage of variation compared with LPC alone. In cultures treated with LPC and KER-TBS.1–24, only the respective percentage of CNP^+ mb OL (cells with a very differentiated morphology) and MBP^+ OL are significantly upregulated ($^*P < 0.01$). Scale bar = 30 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

demonstrated that the NFL-TS.40–63 peptide as well as a synthetic peptide homolog of the TBS sequence of vimentin (Vim-TBS.58–81 peptide) enters the cells via endocytosis (Balzeau et al., 2012; Lépinoux-Chambaud and Eyer, 2013). Some molecules, lacking membrane receptor and known as "cell penetrating peptides" (CPP; Dupont et al., 2007; Prochiantz, 2008), contain a specific sequence accounting for this property. Nevertheless, TBS peptides do not share sequence homology with CPP.

Although the NFL-TBS.40–63 peptide enters glioblastoma cells via an energy-dependent endocytosis process, no membrane receptor for this peptide has yet been identified (Lépinoux-Chambaud and Eyer, 2013). This is also encountered with several other biologically active molecules. For example, myelin proteins alter OLP differentiation (Syed et al., 2008; Baer et al., 2009). In addition, soluble signalling from neuron to glia and involved in proteolipid (PLP) trafficking has been observed, without identification of a specific molecular pathway (Trajkovic et al., 2006). Alternately, structural proteins of the OL, such as MBP or MOG, could act as transducers for external signals (Dyer and Matthieu, 1994; Dyer et al., 1994; Marta et al., 2005).

In conclusion, the NFL-TBS.40–63 peptide specifically increases OL differentiation and maturation in vitro and partially protects them from demyelinating injury. It shares all these properties with the last fraction recovered from NF purification, NFP5, previously reported (Fressinaud et al., 2012; Fressinaud and Eyer, 2013). The homology of sequence with NFL and the similarity of effects might indicate that NFL-TBS.40–63 accounts for increased differentiation of OL observed with NFP5. On the contrary, TUB, which partially copurifies with NF by binding to TBS, increases OLP proliferation without delaying OLP differentiation or maturation in vitro. The presence of TBS on NF (Bocquet et al., 2009) might balance free and bound TUB. In vivo, this phenomenon may modulate the ratio of free axon cytoskeletal proteins available to regulate the OL response to demyelination (proliferation or differentiation).

Importantly, release of such a TBS peptide, or proteolytic fragments of NFL containing this sequence, during axon injury could improve remyelination in vivo. A single demyelinating event is often followed by remyelination, despite a transient decrease in NF and TUB expression (Jean et al., 2002). This could correspond to the release of untransformed NF. In MS lesions, on the contrary, altered NF expression (Trapp et al., 1998; Fressinaud et al., 2005) and abnormal NF proteolysis (Jain et al., 2009) might result in release of abnormal NF isoforms with a reduced capacity to stimulate OL. This might impair remyelination after several relapses in MS. This study brings new insights to explain the paucity of remyelination in MS and suggests that the profound alterations of axon cytoskeletal proteins that are observed in MS plaques might, in turn, inhibit remyelination.

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