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NEUROFILAMENTS AND NFL-TBS.40–63 PEPTIDE PENETRATE OLIGODENDROCYTES THROUGH CLATHRIN-DEPENDENT ENDOCYTOSIS TO PROMOTE THEIR GROWTH AND SURVIVAL *IN VITRO*

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Abstract—Neurofilaments (NF) are released into the cerebrospinal fluid (CSF) during multiple sclerosis (MS), but their role outside the axon is still unknown. *In vitro* NF fractions, as well as tubulin (TUB), increase oligodendrocyte (OL) progenitor proliferation and/or their differentiation depending on the stage of their purification (Fressinaud et al., 2012). However the mechanism by which NF enter these cells, as well as that of synthetic peptides displaying NFL-tubulin-binding site (NFL-TBS.40–63) (Fressinaud and Eyer, 2014), remains elusive. Using rat OL secondary cultures we localized NF, TUB, and NFL-TBS.40–63 by double immunocytochemistry and confocal microscopy. After treating OL cultures with NF P2 (2nd pellet of the purification), or TRITC-TUB, these proteins were localized in the cytoplasmic processes of myelin basic protein (MBP+) expressing OL. Similarly biotinylated NFL-TBS.40–63 synthetic peptides and KER-TBS.1–24 were detected in OL progenitors, differentiated (CNP+) and MBP+ OL. In addition, NFL-TBS.40–63 colocalized with cholera toxin, a known marker of endocytosis, within the cells. Pretreatment of OL by methyl β cyclodextrin abolishes both cholera toxin and NFL-TBS.40–63 uptake, indicating endocytosis. Clathrin-dependent endocytosis was further confirmed by treatment with dynasore, a dynamin inhibitor, which inhibited the uptake of peptides, as well as NFP2 fractions, by 50%. This study demonstrates that axon cytoskeletal proteins and peptides can be internalized by OL through endocytosis. This process could be involved during demyelination, and the release of axon proteins might promote remyelination.

Key words: axon–glia interactions, demyelination, microtubule, multiple sclerosis.

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Abbreviations: CDM, chemically defined medium; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CSF, cerebrospinal fluid; CTXB, cholera toxin subunit B; DAPI, 4′,6′-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; KER, keratin; LPC, lysophosphatidyl choline; MBP, myelin basic protein; MES, 4-morpholineethanoesulfonic acid sodium salt; MS, multiple sclerosis; NF, neurofilaments; OL, oligodendrocyte; OLP, oligodendrocyte progenitor; TBS, tubulin-binding site; TUB, tubulin.

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INTRODUCTION

The expression of neurofilaments (NF) and other axon cytoskeleton proteins is dramatically decreased in demyelinated lesions during multiple sclerosis (MS) (e.g. Trapp et al., 1998; Fressinaud et al., 2005; Schirmer et al., 2011), even in the early stages (Bitsch et al., 2000). Moreover NF subunits are released in the cerebrospinal fluid (CSF) of MS patients (Lycke et al., 1998; Teunissen et al., 2005), and their concentration correlates with disease severity (Gresle et al., 2011; Kuhle et al., 2011). However the role of NF in this extra-axonal location is unknown. In order to address this crucial question, we have demonstrated *in vitro* that purified NF fractions increase the proliferation, differentiation and maturation of oligodendrocytes (OL) (Fressinaud et al., 2012). In addition, they protect OL from lysophosphatidyl choline ((LPC) a demyelinating agent) toxicity (Fressinaud and Eyer, 2013). These properties of NF are shared by other cytoskeleton proteins such as tubulin (TUB), microtubule-associated proteins, tau, synapsin and spectrin β2 which also increase the proliferation and differentiation of OL *in vitro* (Fressinaud et al., 2012). In the CSF of MS patients TUB is also increased (Madeddu et al., 2013). The role of NF was further confirmed by the observation that peptides corresponding to the tubulin-binding sequence (TBS) of NF light subunits (NFL) (Bocquet et al., 2009; Berges et al., 2012) also increase the differentiation of OL, as well as their survival when challenged with LPC *in vitro* (Fressinaud and Eyer, 2014).

As the level of NF in the CSF of MS patients increases with disease progression, it can be hypothesized that either (i) NF concentration is not optimal for promoting OL growth (maximal effect was obtained *in vitro* for 200 ng/mL (Fressinaud and Eyer, 2013)), or that (ii) as time progresses, disease activity overwhelms this compensatory mechanism. Indeed, we have demonstrated that repeated injuries are deleterious for OL in which case growth factors (such as PDGF) are less potent in rescuing them compared to a single injury – (Fressinaud, 2005). In addition, OL ability to remyelinate lesions is decreased in MS (Fressinaud, 2007). This points out a decreased plasticity and regenerating capability of OL during the disease. So it is possible that the NF compensatory mechanism may function during the early stages of the disease only.

Thus the release of NF, and of TUB, during demyelination *in vivo* could regulate OL outcomes and remyelination. Nevertheless, a signaling pathway mediating the NF effects has not yet been identified. Previous results suggest that NFL-TBS.40–63 was localized intracellularly after incubation with OL (Fressinaud and Eyer, 2014). Similarly, a TBS-peptide derived from keratin (KER-TBS.1–24) appeared to localize in OL although it had no significant effect on their growth (Fressinaud and Eyer, 2014). This needed to be confirmed, as well as the mechanism by which NF or peptides penetrate the cells since these proteins do not display a sequence signal corresponding to typical cell-penetrating proteins (Dupont et al., 2007; Prochiantz, 2008).

EXPERIMENTAL PROCEDURES

NF purification procedures

Three different batches of NF were purified from rat brain as previously described (Fasani et al., 2004; Fressinaud et al., 2012; Fressinaud and Eyer, 2013). These NF are predominantly phosphorylated, although there is a small proportion of poorly phosphorylated NF (Perrot et al., 2008). Briefly, after CO_2 inhalation the brains from 15 adult Sprague–Dawley rats were homogenized in MES buffer, and centrifuged at 100,000*g* for 1 h at +4 °C. The first pellet, containing mostly membranes and myelin, was removed. The supernatant was made 4 M glycerol and incubated for 2 h at +4 °C to prevent microtubule assembly, and then centrifuged as above. The resulting pellet (P2) was homogenized in MES (4-morpholineethanoesulfonic acid sodium salt) buffer, and this procedure was repeated up to the 5th pellet to obtain P5 NF enriched fractions (Fressinaud et al., 2012). The amount of proteins in each sample was determined. Proteins from each purification step were separated on a 7.5% acrylamide SDS–PAGE according to Laemmli (1970), and then transferred onto nitrocellulose membranes for immunoblotting analysis (Towbin et al., 1979). Primary antibodies (monoclonal mouse anti-NFH, -NFM, -NFL, -α and -β TUB, 1:2000 dilution (Sigma, St Louis, MO, USA)) were used to determine using Western blots the purity of NF proteins recovered. In OL cultures NFP2 and NFP5 were used at 50 μg/mL (final concentration) to allow their visualization by immunocytochemistry (maximal biological activity: 200 ng/mL (Fressinaud et al., 2012)). Both NF fractions are enriched in NF, although some TUB is also present, mainly in NFP2 (Fressinaud et al., 2012).

NF subunits were also tested. NF are heteropolymers composed of three subunits (NFH, NFM, NFL) corresponding respectively to high (200 kDa), medium (160 kDa) and light (68 kDa) apparent molecular weight isoforms. To determine if NF fractions' effects could be ascribed to a specific subunit, purified NFL, NFM and NFH (bovine spinal cord, >98% purity, Progen, Heidelberg, Germany) were added, alone or in combination, to OL cultures (see below). The concentrations used were calculated based on subunit distribution within native NF (NFL 5/NFM 3/NFH 1) (Perrot et al., 2008), and on the concentration of NF fractions previously demonstrated to have maximal activity (200 ng/mL) (Fressinaud et al., 2012). Thus cultures were treated with either NFL 66 ng/mL final concentration, and/or NFM 94 ng/mL, or NFH 39 ng/mL. Similarly, concentrations 10 times higher were tested on proliferation after 20 h of treatment, and on differentiation and maturation after 72 h of treatment.

Synthetic peptides

Biotinylated peptides (more than 95% purity) were synthesized according to the TBS sequence identified in NFL chain (NFL-TBS.40–63: YSSYSAPVSSSLSVRRSY SSSSGS) and keratin (KER-TBS.1–24: MSIRVTQKSY KMSTSGPRAFSSRS) (Millegen, Toulouse, France, or Genecust, Dudelange, Luxembourg). NFL-TBS is the active site, and has 100% sequence homology with the binding site of TUB on NFL. KER-TBS.1–24 binds TUB on keratin but is inactive on OL; it was used to identify the role of the TBS sequence in the entry of peptides into OL. The scrambled peptide, with the same amino-acids as NFL-TBS.40–63 but in a random sequence (NFL-SCR: SLGSPSSSVRASYSSSRSYVYSSS), was also used as a control (Bocquet et al., 2009; Berges et al., 2012). NFL-SCR has no biological effect on OL (Fressinaud and Eyer, 2014). Peptides were dissolved in water and used at 50 μM final concentration – determined to allow convenient detection – except if otherwise specified (maximal biological effects are observable at 10 μM (Fressinaud and Eyer, 2014)).

Labeled TUB

Labeled TUB ((TUB*), X-rhodamine TUB, bovine brain, Cytoskeleton, Denver, CO, USA) was added to OL cultures at 2 μg/mL, for 20 h (Fressinaud et al., 2012). Incubation was followed by immunolabeling with anti-A2B5 or -MBP antibody (see below) to localize and identify which cells of the OL lineage could have bound TUB*. As with experiments performed with NF and NFL-TBS.40–63 peptides, the inhibition of TUB* uptake by dynasore, an inhibitor of clathrin-dependent endocytosis, was assayed (see below).

Cell cultures

Preparation of secondary OL cultures. Glial cell cultures from newborn Wistar rat (Breeding from the Faculty of Medicine, University of Angers (SCAHU)) brains were performed as described (e.g. Fressinaud et al., 2012). 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 Corp., Cergy Pontoise, France). After 3 weeks, OL were separated from the astrocytic underlayer by flushing some medium over the cultures, and grown in secondary cultures. Contaminating microglial cells were removed by preplating on plastic Petri dishes before the subculture. 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, Saint Aubin, France), supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 4 g/L 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 OL synthesize myelin-like membranes (Fressinaud et al., 1993, 1996).

Culture treatments and demonstration of endocytosis. Secondary OL cultures were treated during a 20-h period as follows: (i) control (CONT): CDM alone; (ii) NFP2 or NFP5 fractions (50 μg/mL final concentration), (iii) NFL, NFM, and/or NFH (low and high concentration: see above), (iv) TUB* (2 μg/mL), (v) synthetic peptides: NFL-TBS.40–63, NFL-SCR, or KER-TBS.1–24 (Bocquet et al., 2009) were added once to the CDM (diluted in water, final concentrations: 0.25, 0.5, 1, 5, 10, and 50 μM were tested). Peptide concentration of 50 μM allowed a better detection by immunocytochemistry and was used thereafter.

The effects of NF subunits on OLP proliferation were determined after 20 h of bromodeoxyuridine (BrdU, 10 μg/mL) incorporation (20 h) and double immunocytochemistry for BrdU and A2B5 (see below). In addition, their effects on OL differentiation and maturation were analyzed after 72 h of treatment versus control (CDM alone) by immunocytochemistry to determine the morphology and the number of CNP+ or MBP+ OL per optic field (Fressinaud et al., 2012) (see below).

Alexa Fluor 555-labeled cholera toxin B subunit (10 μg/mL final concentration, Molecular Probes, Saint Aubin, france) was incubated with peptides (50 μM) for 4 h as a marker of endocytosis (e.g. Wernick et al., 2010). Methyl β cyclodextrin disrupts formation of clathrin-coated endocytic vesicles (Rodal et al., 1999; Duchardt et al., 2007), thus pre-treatment for 1 h with methyl β cyclodextrin (10 μg/mL final concentration, Sigma, St Louis, MO, USA) was used in sister cell cultures to test the inhibition of this process. Results were analyzed by confocal microscopy.

Dynasore (diluted in dimethylsulfoxide (DMSO); 20 μM final concentration, Sigma, St Louis, MO, USA) was used as inhibitor of dynamin, a GTPase involved in clathrin-dependent endocytosis (Kirchhausen et al., 2008). Peptides, NFP2, or TUB* were incubated alone or in association with dynasore for 20 h in cultures grown in CDM. Quantitative results were obtained by counting CNP+ (or MBP+) OL that had incorporated peptides (or NFP2, or TUB*) in CDM plus DMSO (as controls), and in sister cultures treated concomitantly with dynasore and peptides, NFP2 or TUB* (see counting protocol below). Uptake of proteins and peptides was analyzed by confocal microscopy, performed in parallel to verify the endocytosis of the different molecules and its putative inhibition by dynasore.

Immunocytochemistry of cultures

Cells of the OL lineage were labeled with polyclonal Olig2 antibody (1:200, Abcam, Paris, France). OL progenitors were labeled with anti-A2B5 monoclonal antibody (Ab) (1:100 dilution, Chemicon, Billerica, MA, USA), differentiated (CNP+) OL with anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (monoclonal Ab, 1:100 dilution, Sigma Immunochemicals, St Louis, MO, USA), and mature OL with anti-myelin basic protein (MBP) polyclonal Ab (1:100 dilution, Dako, Trappes, France), after fixation with 3% paraformaldehyde and methanol, as previously described (e.g. Fressinaud et al., 1993). Labeling was revealed either by goat anti-rabbit Ig G – Alexa fluor 488 (Olig2, MBP) (Invitrogen, Cergy Pontoise, France), or anti-mouse Ig M – Alexa fluor 488 (A2B5) (Invitrogen), or anti-mouse Ig G – Alexa 568 (CNP)-conjugated secondary antibodies (1:200 dilution,

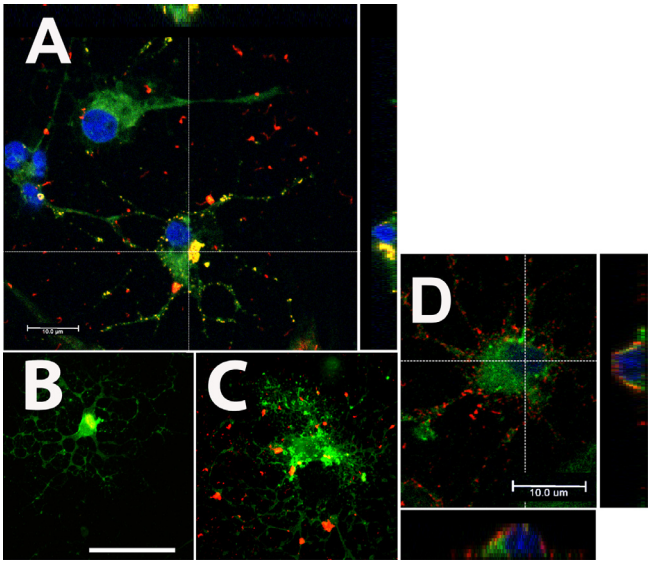

Fig. 1. Localization of neurofilament P2 fraction (red, A, C, D) in oligodendrocyte cultures labeled with anti-A2B5 (green, A), or anti-MBP antibodies (green, B–D). NF are visualized with anti-NFH antibody revealed by Alexa-fluor 568 (red). Panels represent merger of the same fields obtained by confocal microscopy for control (B) and NF-treated OL (A, C, D). The uptake of NFP2 by OL progenitors (A), and by mature OL (D) is demonstrated in orthogonal images (nuclei counterstain by DAPI). Scale bar = 10 μm in A and D, and 50 μm in B and C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Invitrogen) (Fressinaud et al., 2012). Anti-NFH mouse monoclonal Ab (1:200 dilution, Sigma, St Louis, MO, USA) was used followed by anti-mouse Ig G – Alexa 568 to reveal NF in MBP+ OL (Abs directed against NFL and NFM were also tested with similar results). Appropriate anti-NFH, -NFM and -NFL Abs were also used to localize NF subunits.

Proliferation of OLP in cultures treated by NF subunits was characterized by double immunocytochemistry with anti-BrdU Ig G monoclonal Ab (1:400 dilution, Sigma, St Louis, MO, USA) and anti-A2B5 Ab; the number of double-labeled OLP per optic field was compared to control cultures (CDM alone) (Fressinaud et al., 2012). Similarly the number of CNP+, and MBP+ OL per optic field was analyzed versus control.

Biotinylated peptides were localized using streptavidin coupled to Alexa Fluor 488 (1:200 dilution; Invitrogen) in double-labeling experiments with anti-A2B5, -CNP, or -MBP Ab revealed by appropriate anti-Ig coupled to Alexa Fluor 568. Similarly the cells uptaking TRITC-TUB were characterized throughout their differentiation stage using the same Abs.

In addition, for cellular localization of NF, TUB*, and peptides by confocal microscopy, nuclei were counterstained by DAPI (4′,6′-diamidino-2-phenylindole (Kubista et al., 1987)). Cultures were visualized using a Leica TCS SP8 (Service Commun d'Imagerie et d'Analyse Microscopique, University of Angers).

Sampling and statistical analysis

Each experiment was run three times – with different batches of NF, TUB*, and peptide preparations – in triplicate for each of the treatments (control, NF, TUB*, and different dilutions of the TBS-, or scramble, peptides). Similarly three comparative experiments were performed in triplicate for NF, TUB*, or peptides in association with markers of endocytosis, or inhibitors of endocytosis (methyl cyclodextrin, dynasore). Counting of labeled cells was performed on 20 optic fields distributed randomly on each coverslip; at least 10^3 cells were counted for each type of treatment. Results were averaged (mean ± SD) and compared with appropriate tests (Mann and Whitney test, Student's *t* test adapted to small samples, variance analysis (ANOVA, *F* test)).

Ethics

The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Formal approval to conduct the experiments described has been obtained from the Regional Ethics Committee for the use of laboratory animals. This implies that all efforts were made to minimize the number of animals used and their suffering.

RESULTS

NF, TUB and TBS-peptides are uptaken by cells of the OL lineage

The uptake of NFP5 fractions could not be detected in OL cultures by confocal microscopy (not shown). On the contrary, putative internalization of cytoskeleton proteins (NFP2 and TUB*) and of biotinylated TBS-peptides was observed by confocal microscopy in A2B5+ OLP, differentiated (CNP+) OL, and MBP+ mature OL (Figs. 1–5). Confocal microscopy of double immunostained slides clearly demonstrated the presence of these peptides and proteins inside cells of the OL lineage. In addition orthogonal images demonstrating that added proteins and peptides were in the same plane as the DAPI-labeled nuclei confirmed the internalization of these molecules (Figs. 1, 4, 5 and 8).

The uptake of NF fractions P2 was observable; it labeled OLP (A2B5+ cells) (Fig. 1) and MBP+ OL (Fig. 1). The internalization of axon cytoskeleton proteins was also demonstrated for TUB*, which was identified inside the cytoplasm of OLP and mature OL (Fig. 2).

Contrary to NF fractions, NF subunits did not alter OL growth when added separately. Neither the proliferation of OLP (BrdU+/A2B+ cells), nor their differentiation or maturation was modified versus control following treatment for 20–72 h with the three subunits NFL,

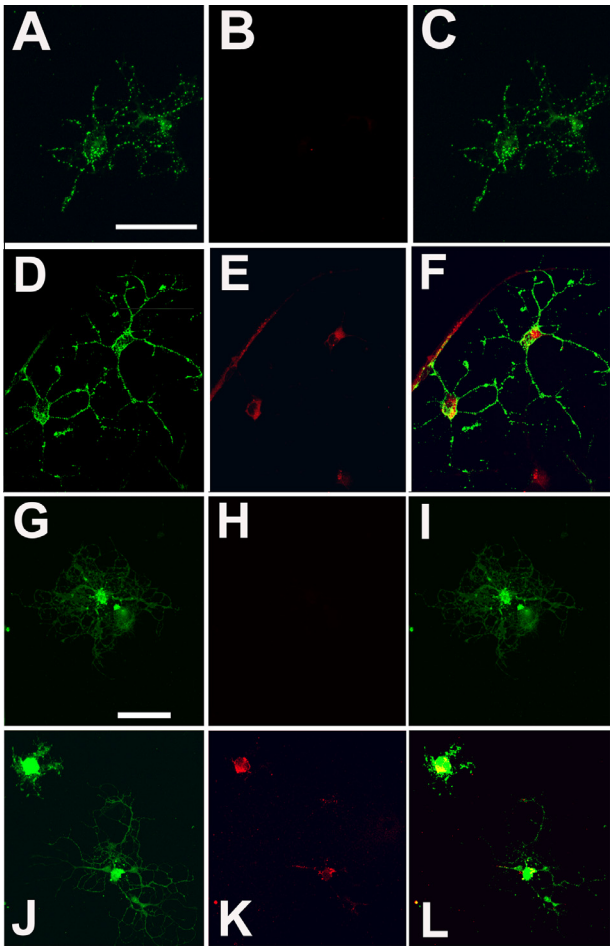

Fig. 2. Visualization by confocal microscopy of TRITC-labeled tubulin (red, E, F, K, L) uptake by A2B5 + OL progenitors (A-F), and mature MBP + OL (G-L) (green). (A-C) and (G-I) correspond to control cells, and (D-F) and (J-L) to OL treated with TRITC-labeled TUB. Right fields (C, F, I, L) correspond to merged photographs. Bar = 50 μm in (A-F) and (G-L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NFM, or NFH separately. Concomitant treatment with the three subunits was also ineffective at either low or high concentrations. Finally, they were not detectable in OL by immunocytochemistry at these concentrations (not shown).

However, internalization of NFL-TBS.40–63 and KER-TBS.1–24 biotinylated peptides was observed in OLP (Fig. 5), in differentiated cells (Figs. 3–5) and in mature MBP+ OL (Fig. 5). Although KER-TBS.1–24 has no significant effect on OL (Fressinaud and Eyer, 2014), both NFL-TBS.40–63 and KER-TBS.1–24 (10 μM) labeled 50% of all cells of the OL lineage (olig2+) cells, whereas the random sequence peptide NFL-SCR was detected in only 20% of them (Fig. 4E) ($P < 0.01$). Several parameters were shown to influence the uptake, namely the type of peptides and their concentration. As could be expected, the uptake – determined by the number of cells labeled – was dependent on peptide concentration for TBS-peptides. At 1 μM around 25% of CNP+ cells had incorporated the TBS-peptides, whereas 75% were labeled at 50 μM. Typically, NFL-TBS.40–63 labeled the same percentage of cells as KER-TBS.1–24 (Fig. 3B). On the contrary, NFL-SCR labeled only 25% of CNP+ OL at this concentration (50 μM) versus 70% and 85% for NFL-TBS.40–63 and KER-TBS.1–24 respectively (Fig. 4E). In addition, biotinylated NFL-SCR peptides could be observed outside most of the cells. This extracellular location was confirmed by orthogonal images as well as 3D reconstruction images obtained by confocal microscopy (Fig. 4B–D).

More specifically, the cellular uptake was dependent on the type of molecule: TBS-peptide or protein, and on the differentiation stage of OL (Fig. 5E). So, NFP2 and TUB* labeled around half of OLP, versus only 25% for TBS-peptides. In addition, mature (MBP+) OL labeling varied from 70% for TUB* to 50% for TBS-peptides, and 30% for NFP2. TBS-peptides labeled preferentially differentiated OL (CNP+) (75% of the cells).

This preferential uptake of NFP2 and TUB* by OLP might explain at least in part the differences previously recorded between NF and TUB – which promote OLP proliferation and differentiation (Fressinaud et al., 2012) – and TBS-peptides whose biological activity is more restrained, affecting mainly differentiation (Fressinaud and Eyer, 2014) (see Discussion).

Mechanism of entry of NF and TBS-peptides into OL

The mechanism by which TBS-peptides enter OL was first explored by comparison of their localization to that of labeled-cholera toxin subunit B (CTXB), a marker of endocytosis (Rodal et al., 1999; Duchardt et al., 2007) (Fig.6). Confocal microscopy revealed that TBS-peptides co-localized with CTXB. In addition, pretreatment with methyl β cyclodextrin totally prevented CTXB, as well as TBS-peptides entry into OL (Fig. 6). Methyl β cyclodextrin

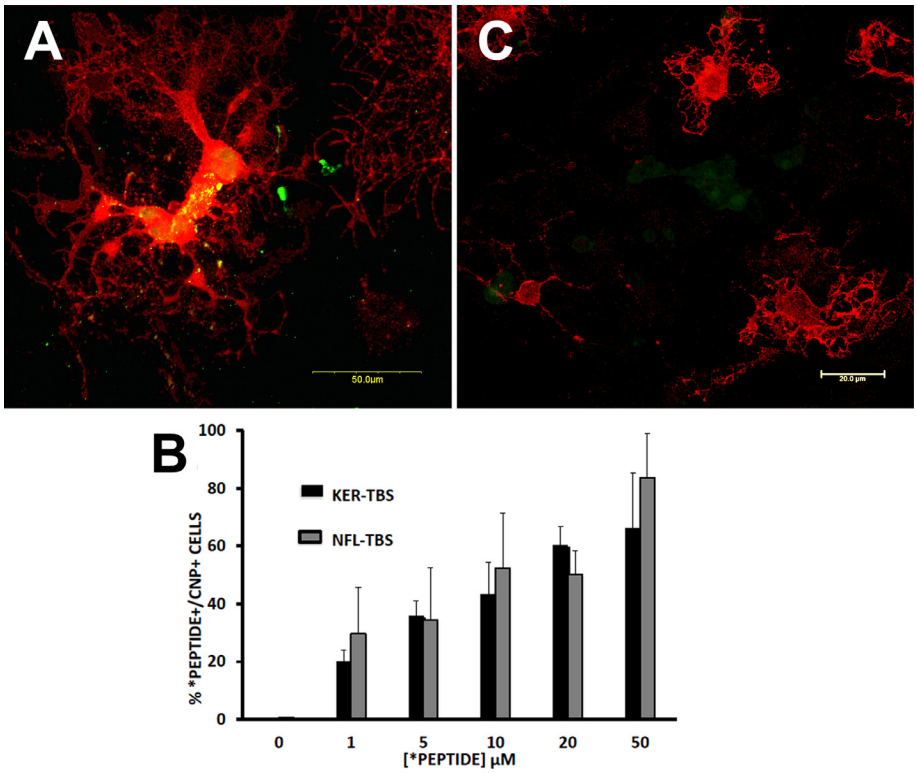

Fig. 3. Intracellular localization of biotinylated NFL-TBS.40–63 (50 μM concentration) revealed by streptavidin coupled to Alexa-fluor 488 (green) in OL cultures labeled for CNP (A) coupled with Alexa-fluor 568 (red). On the contrary there is no uptake of NFL-scramble peptide (50 μM concentration, biotinylated peptide revealed by Alexa fluor 488) by CNP + OL (C). Confocal microscopy, merger of fields. Bar = 50 μm. in (A) and 20 μm in (C). (B) the uptake of TBS-peptides (NFL, gray bars; KER, black bars) is concentration dependent. Results as percentage (mean ± SD) of total CNP + OL labeled by the peptides ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

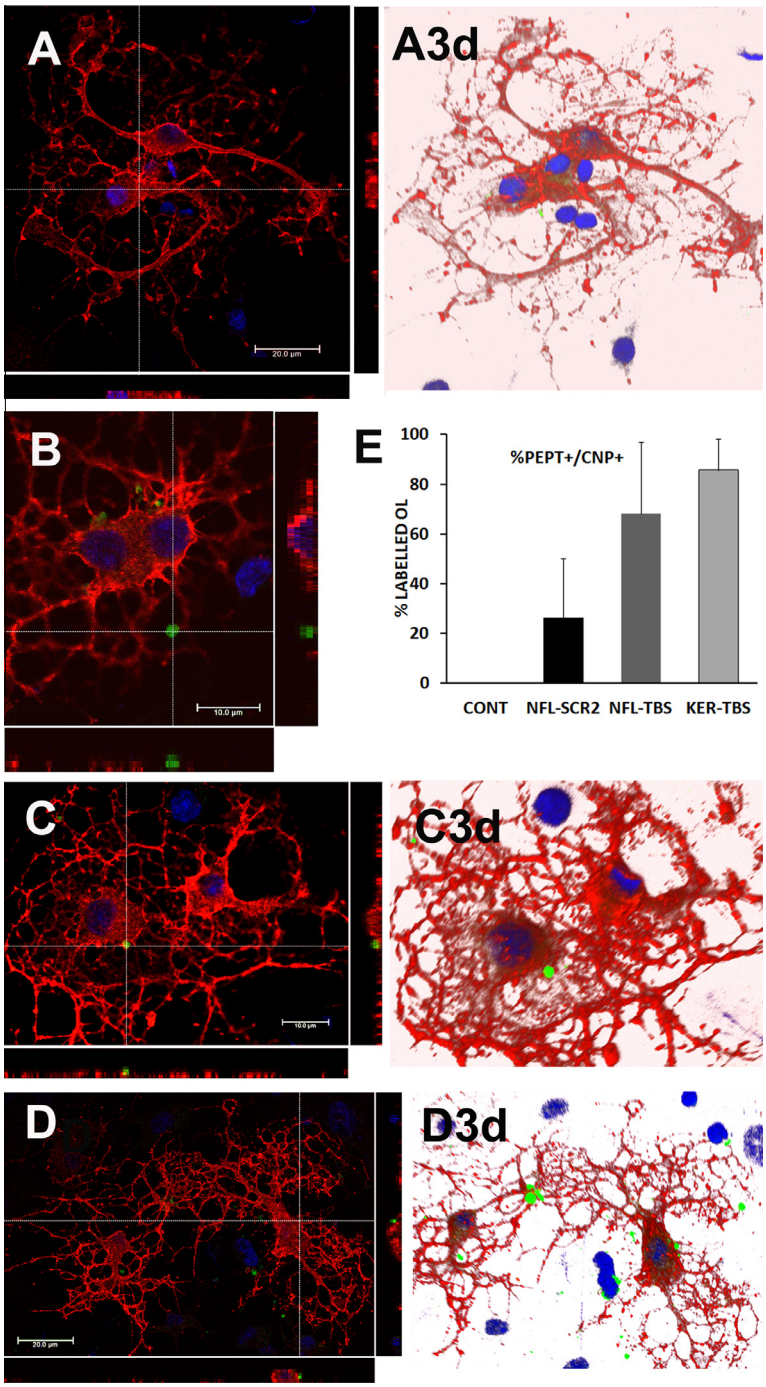

Fig. 4. Localization of biotinylated NFL-scramble peptide revealed by streptavidin coupled to Alexa fluor 488 (green) in OL cultures labeled for CNP coupled to Alexa-fluor 568 (red) (B-E). Nuclei counterstain: DAPI (blue). Top panels (A, A3d) represent untreated cultures (control, only CNP and DAPI staining are observable). In cultures treated with the NFL-scramble peptide (B-D), most of the cells are surrounded by the peptide, however they do not incorporate it. Confocal microscopy, merger of fields; orthogonal images are presented below and on the right of each photograph (A-D), right panels (A3d-D3d) display 3D images of the corresponding fields. Bar = 10–20 μm as specified. (E) Quantification of TBS-peptides (gray bars) and NFL-SCR (black bar) uptake in CNP + OL. Results as percentage (mean ± SD) of differentiated CNP + OL that have incorporated active TBS-peptides compared to NFL-SCR. Only TBS-peptides are significantly ($p < 0.01$) uptaken by OL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is known to alter lipid rafts within the plasma membrane, and inhibits clathrin- and caveolin-dependant endocytosis (Rodal et al., 1999). Together, these results show that TBS-peptides penetrate within these cells by endocytosis.

The involvement of clathrin-coated pit endocytosis in internalization of TBS-peptides and NFP2 was further confirmed by the demonstration of partial inhibition of the process by dynasore cotreatment. Dynasore is known as an inhibitor of dynamin, a GTPase involved in clathrin-dependent endocytosis (Henley et al., 1998; Kirchhausen et al., 2008). Concomitant treatment of cultures with NFP2, or peptides, and dynasore resulted in

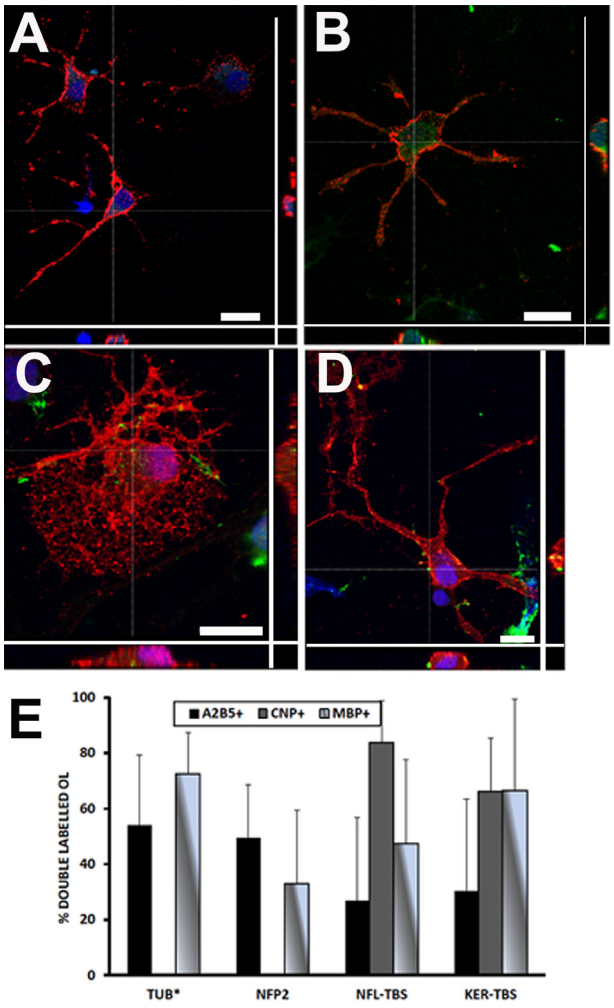

Fig. 5. The uptake of NFP2, TUB*, and TBS-peptides depends on the stage of differentiation of OL cells. Visualization by confocal microscopy (nuclei counterstain by DAPI, merged images and orthogonal images). Biotinylated NFL-TBS.40.63 (A-C) and KER-TBS.1–24 (D) are labeled by streptavidin coupled to Alexa-fluor 488 (green). Note that only part of OL progenitors (A2B5 + cells (red), A-B) have taken up NFL-TBS.40.63, since some cells are negative (no green fluorescence (A)), whereas others are positive (B). A more important proportion of mature OL (MBP + cells (red), C-D) demonstrate uptake for NFL-TBS.40.63 (C) and KER-TBS.1–24 peptides (D). Quantification of endocytosis (mean ± SD) for TUB*, NFP2 and TBS-peptides depending on the stage of differentiation of the cells (E) ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a 50% decrease in the number of cells labeled for the tested peptides (Figs. 7A and 8G) (whereas DMSO alone had no effect). In addition, numerous OL surrounded by extracellular NFP2 or peptides were observed by confocal microscopy after dynasore treatment (Figs. 7 and 8).

On the contrary, TUB* internalization was not altered significantly by dynasore treatment (decreased by 15% for MBP+ OL) (Figs. 6B and 7G). This difference between NFP2 and TUB* did not appear to rely on the stage of differentiation of OL since in the absence of dynasore TUB* uptake by mature MBP+ cells or OLP was similar to that of NFP2.

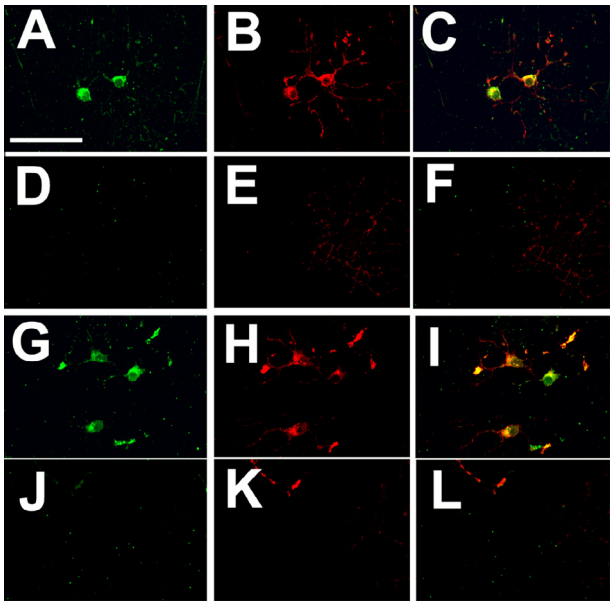

Fig. 6. TBS-peptides penetrate into OL cells by endocytosis as choleara toxin. Visualization by confocal microscopy. Biotinylated NFL-TBS.40.63 (A-F) and KER-TBS.1–24 (G-L) peptides are labeled by streptavidin coupled to Alexa-fluor 488 (green). They colocalized with TRITC-labeled cholera toxin (red, B, H), a known marker of endocytosis. When OL are pretreated by cyclodextrin (D-F, J-L), a known inhibitor of endocytosis, both peptides and cholera toxin uptake by OL are abolished (F, L). Merger of corresponding fields (C, F, I, L) are presented on the right; note colocalization of TBS-peptides and cholera toxin labeling in C and I. Bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DISCUSSION

We have previously demonstrated that NF fractions, as well as NFL-TBS.40–63 synthetic peptides, act as proremyelinating agents *in vitro* (Fressinaud et al., 2012; Fressinaud and Eyer, 2013, 2014). Nevertheless, the cell penetration mechanism of these proteins and peptides remains elusive. Namely, no membrane receptor and no intracellular signaling pathway has been identified yet. Since NF are released in the CSF during MS (Lycke et al., 1998; Teunissen et al., 2005), and their concentration correlates with disease severity (Gresle et al., 2011; Kuhle et al., 2011), it is possible that these proteins act on OL and modulate remyelination *in vivo*. This could also occur with peptides resulting from NF degradation during demyelination (Jain et al., 2009). Phagocytosis of neuronal debris has been observed in association with axonal damage in MS (Huizinga et al., 2012), and proteolysis alterations occur during autoimmune encephalomyelitis (an experimental model for MS) (Jain et al., 2009). Thus it was important to clarify how extracellular NF, or peptides derived from NF, and other cytoskeleton proteins such as TUB – the expression of which is also altered in MS lesions (e.g. Fressinaud et al., 2005) – could penetrate and regulate the growth of OL lineage cells.

To determine which subunit (H, M or L) of NF fractions could display their biological activity recorded *in vitro*, we first completed previous studies with the analysis of the effect of the three purified NF subunits on OL. Contrary

to what was observed for NFL-TBS.40–63 synthetic peptides, neither NFL nor the other NF subunits, alone or in combination, were effective in our conditions, even at high concentrations. This might be due to properties differing from the native heteropolymer after purification procedures (e.g. modification of the phosphorylation sites, alterations to the three dimensional structure). In addition the fate of these subunits could not be determined since they could not be localized by confocal microscopy *in vitro*. Similarly, NFP5 labeling could only just be identified within OL as a very thin punctuated intracytoplasmic labeling. Therefore the study was subsequently performed with NFP2 only.

Localization of TUB*, NFP2 and NFL-TBS.40–63 into immature and mature cells could be observed under standard conditions. Most, but not all the cells, were labeled by these proteins and peptides. Confocal microscopy with orthogonal analysis confirmed that labeling was intracellular for TUB*, NFP2, NFL-TBS.40–63 and KER-TBS.1–24. Their localization was mostly observed in the perinuclear cytoplasm, whereas processes were less frequently stained. Meticulous observation was unable to distinguish a specific morphology or type of differentiation associated with labeling, i.e., OL with a greatly differentiated morphology as well as those with less pronounced processes were labeled. Interestingly, NFL-SCR which displays no activity on OL (Fressinaud and Eyer, 2014), was only uptaken by less than a quarter of the cells.

Since NF and NFL-TBS.40–63 peptides have no sequence homology with typical ''cell-penetrating peptides'' (Prochiantz, 2008), the involvement of endocytosis in their internalization by OL was analyzed. This process has been recognized in glioblastoma cell lines for NFL-TBS.40–63 (Lépinoux-Chambaud and Eyer, 2013). Nevertheless this paradigm is far from OL cultures since in this cell line peptides were captured by tumoral, cycling and proliferating cells. On the contrary, in our cultures non-proliferating OL (i.e. differentiated and mature OL) mostly were labeled by the proteins and peptides we tested. In our cultures colocalization of TBS-peptides with cholera toxin B subunit, and inhibition of labeling by pretreatment with methyl cyclodextrin demonstrated that NFL-TBS.40–63 peptides were also internalized in OL by endocytosis (Rodal et al., 1999; Duchardt et al., 2007). In addition, since dynasore is known as an inhibitor of dynamin, a GTPase involved in clathrin-dependent endocytosis (Henley et al., 1998; Kirchhausen et al., 2008), the inhibition by 50% of internalization by concomitant treatment with dynasore demonstrated that endocytosis of NFL-TBS.40–63 and NFP2 proceeds mainly, although not exclusively, through clathrin-coated vesicles. Similar results were observed with KER-TBS.1–24. Thus clathrin-dependent endocytosis of NFP2 and TBS-peptides by OL was confirmed by using two different inhibitors of this process. On the contrary, TUB* uptake was not affected by dynasore treatment, pointing out different uptake pathways between several axon cytoskeleton proteins.

A common receptor for NFL-TBS.40–63 could be present in both OL and glioblastoma cells since endocytosis of this peptide in glioblastoma cells has also been observed (Lépinoux-Chambaud and Eyer, 2013). In glioblastoma cells several endocytosis

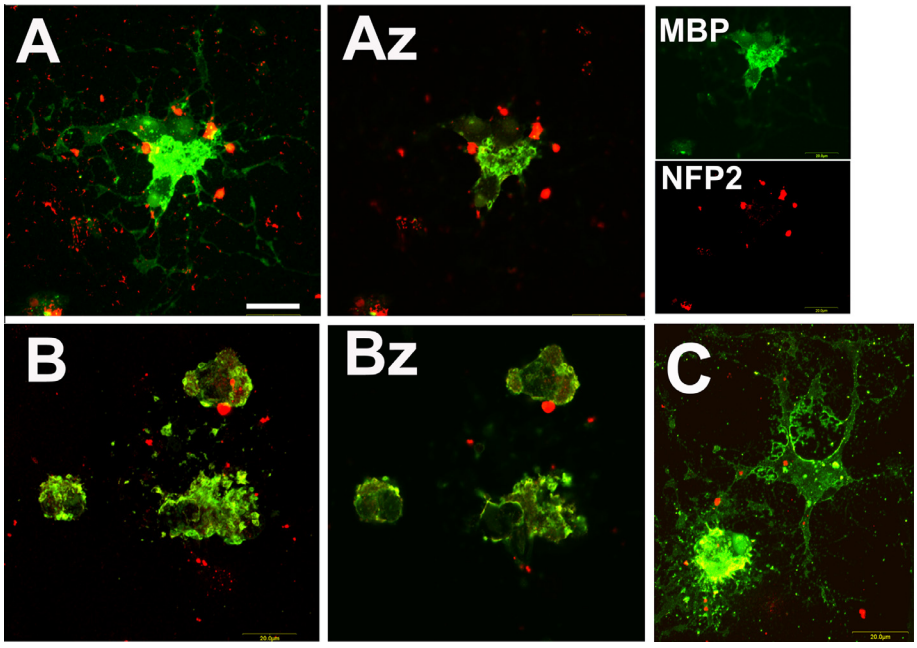

Fig. 7. Inhibition of NFP2 endocytosis by dynasore. Confocal microscopy of OL cultures treated concomitantly with dynasore and NFP2 (A, Az), or TUB* (B, Bz). Merged images (A, B) and corresponding z images (Az, Bz) of OL labeled by MBP (alexa fluor 488, green), and NF (alexa fluor 568, red) or TRITC-TUB* (red). Single channel images corresponding to (Az) are presented on the right for MBP and NFP2. Dynasore treatment results in inhibition of NFP2 uptake (Az), while TUB* endocytosis is not significantly suppressed by dynasore (Bz) (see text). In the absence of dynasore, DMSO alone has no effect on protein uptake (C). Bar = 20 μm (A–C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

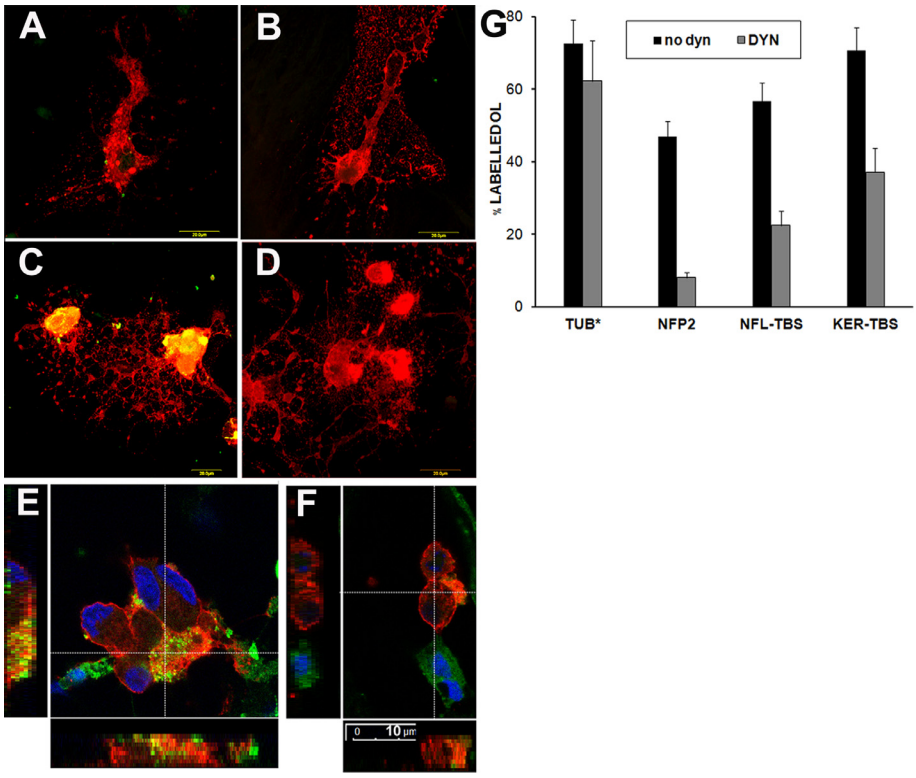

Fig. 8. Endocytosis of TBS-peptides is inhibited by dynasore. Biotinylated NFL-TBS.40.63 (A-B, E-F) and KER-TBS.1–24 (C-D) are labeled by streptavidin coupled to Alexa-fluor 488 (green). They are taken up (A, C, E) by OL labeled with CNP (red, A-F), whereas this process is abolished by dynasore (B, D, F). Bar = 20 µm in A-D, 10 µm in E-F. (G) The normal uptake (black bars) of NFP2 and TBS-peptides is strongly impaired by dynasore treatment (gray bars). On the contrary TUB* endocytosis is not significantly decreased by dynasore. Results in percentage (mean ± SD) of cells colabeled for proteins (TUB*, NFP2) and MBP, or for TBS-peptides (NFL-TBS.40.63 and KER-TBS.1–24) and CNP ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathways are involved, depending mainly on the cell line and on peptide concentration (Lépinoux-Chambaud and Eyer, 2013). Therefore, one or a combination of several receptors for the NFL-TBS.40–63 peptide could be shared between these different cells. Several experimental approaches are presently used in our laboratory to identify them.

The endocytosis of axon cytoskeleton proteins by OL is coherent with previous reports showing that OL are able to internalize various particles, including nanoparticles (e.g. Salgado et al., 2010) and other types of molecules such as ferritin via clathrin-dependent receptor-mediated endocytosis (Hulet et al., 2000). Pathogens such as the Borna disease virus (fatal immune-mediated neurological disease in horse and sheep) (Clemente and de la Torre, 2009), and the human polyoma JC virus (agent of progressive multifocal leukoencephalopathy) (Pho et al., 2000) can also penetrate glial cells through the same pathway.

Interestingly, a similar neuron/OL transfer of protein via endocytosis – i.e., α synuclein release by neurons and uptake by OL – appears as a major process of disease propagation during multisystem atrophy. This pathological phenomenon is also inhibited partially by dynasore, demonstrating the involvement of dynamin/clathrin-mediated endocytosis (Kisos et al., 2012; Reyes et al., 2014). Thus, dynamin could interfere in the endocytosis of several axon cytoskeleton proteins in OL, at least under pathologic conditions. In addition, α synuclein has been demonstrated to alter OLP differentiation and maturation (Ettle et al., 2014). So, extracellular neuronal protein uptake by OL through endocytosis could occur in several diseases or circumstances, and have opposite effects on their differentiation (promoting for NF, inhibiting for α synuclein) depending on the pathological process.

CONCLUSION

Our results demonstrate that NF, NFL-TBS.40–63 peptides, and other cytoskeleton proteins from neurons such as TUB, are internalized in most cells of the OL lineage *in vitro*. This process occurs through endocytosis, and mainly by clathrin-coated pits for NFP2 and peptides. We hypothesize that such a phenomenon could occur *in vivo* in demyelinating disease such as MS during which NF are released in the CSF. Whether *in vivo* NF or NFL-TBS.40–63, peptides have the same proremyelinating effects as observed *in vitro* is under current research in the laboratory.

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