Neuroscience 298 (2015) 42–51

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

C. FRESSINAUD [a,b]* **AND J. EYER** [b]

[a] *LUNAM, Neurology Department, University Hospital, Angers, France*

[b] *LUNAM, Neurobiology and Transgenesis Laboratory – UPRES EA 3143, University Hospital, Angers, France*

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.

*Correspondence to: C. Fressinaud, Neurology Department, University Hospital, 4 rue Larrey, 49933 Angers Cedex 9, France. Tel: +33-02-44-68-84-92; fax: +33-02-41-35-35-94.

E-mail address: catherine.fressinaud@univ-angers.fr (C. Fressinaud).

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.

http://dx.doi.org/10.1016/j.neuroscience.2015.04.003

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)