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Heterogeneity in the properties of NEFL mutants causing Charcot–Marie–Tooth disease results in differential effects on neurofilament assembly and susceptibility to intervention by the chaperone-inducer, celastrol

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ABSTRACT

Aberrant aggregation of neurofilament proteins is a common feature of neurodegenerative diseases. For example, neurofilament light protein (NEFL) mutants causing Charcot–Marie–Tooth disease induce misassembly of neurofilaments. This study demonstrated that mutations in different functional domains of NEFL have different effects on filament assembly and susceptibility to interventions to restore function. The mouse NEFL mutants, NEFL^{Q333P} and NEFL^{P8R}, exhibited different assembly properties in SW13-cells, cells lacking endogenous intermediate filaments, indicating different consequences of these mutations on the biochemical properties of NEFL. The p.Q333P mutation caused reversible misfolding of the protein. NEFL^{Q333P} could be refolded and form coil–coiled dimers, *in vitro* using chaotropic agent, and in cultured cells by induction of HSPA1 and HSPB1. Celastrol, an inducer of chaperone proteins, induced HSPA1 expression in motor neurons and prevented the formation of neurofilament inclusions and mitochondrial shortening induced by expression of NEFL^{Q333P}, but not in sensory neurons. Conversely, celastrol had a protective effect against the toxicity of NEFL^{P8R}, a mutant which is sensitive to HSBP1 but not HSPA1 chaperoning, only in large-sized sensory neurons, not in motor neurons. Importantly, sensory and motor neurons do not respond identically to celastrol and different chaperones are upregulated by the same treatment. Thus, effective therapy of CMT not only depends on the identity of the mutated gene, but the consequences of the specific mutation on the properties of the protein and the neuronal population targeted.

1. Introduction

Neurofilaments (NFs) are the class of intermediate filaments (IFs) exclusively expressed in mature neurons in both the central and peripheral nervous systems. They are composed of NEFL, NEFM and NEFH, the low, medium and high molecular weight neurofilament proteins (Perrot et al., 2008). The three NF subunits display the structural organisation typical of IF proteins, consisting of a conserved central α-helical "rod" domain and variable amino-terminal "head" and carboxyl-terminal "tail" domains. The head domain has multiple phosphorylation sites which are involved in either regulation of NF assembly, interaction with other cytoskeletal elements and organelles, or transport of NF oligomers (Giasson et al., 1996; Nakamura et al., 2000; Sihag and Nixon, 1989, 1991). NEFM and NEFH have particularly long tail domains important for neurofilament spacing and stability (Barry et al., 2010; Leermakers and Zhulina, 2010; Rao et al., 2012). The α-helical rod domain of the NF subunits contains long stretches of heptad repeats which align in a head-to-tail fashion to form coiled–coil dimers. The dimers undergo antiparallel association to form tetramers which assemble into rope-like 10 nm filaments that are insoluble in non-ionic detergents such as Triton X-100 (Herrmann and Aebi, 2000).

NF proteins are commonly misassembled in neurodegenerative diseases (Perrot et al., 2008), including subtypes of the peripheral sensory-motor neuropathy, Charcot–Marie–Tooth disease (CMT) caused by mutations in *NEFL* (CMT2E and CMT1F), CMT1F showing demyelinating features in addition to axonopathy (De Jonghe et al., 2001; Mersiyanova et al., 2000). Several mutations in the

Abbreviations: NF, neurofilaments; IF, intermediate filament; NEFL, neurofilament light protein; NEFH, neurofilament heavy protein; NEFM, neurofilament medium protein; CMT, Charcot–Marie–Tooth disease; BN-PAGE, Blue Native Polyacrylamide Gel Electrophoresis; PDVF, polyvinylidene fluoride; DRG, dorsal root ganglia; HSP, heat shock protein.

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head domain of NEFL (P8R, P8Q, P8L, P22S, and E89K) and in the rod domain (N97S, A148V, Q333P and E397K) have been identified (De Jonghe and Jordanova, 2011). These mutants are impaired in their ability to co-assemble with other subunits to form normal IF networks in adrenocarcinoma cells lacking endogenous IF (SW13-), in catecholaminergic differentiated cells (CAD) and in motor neurons; instead they aggregate and form cytoplasmic inclusions (Brownlees et al., 2002; Perez-Olle et al., 2002, 2004; Tradewell et al., 2009). Foci of misassembled NFs have been observed in nerves of a patient carrying the p.L286P mutation, suggesting that the aberrant aggregation property of NEFL mutants is relevant to pathogenesis of CMT2E and CMT1F (Fabrizi et al., 2007). However, in motor and sensory neurons, NEFL mutants also integrate into existing filamentous structures prior to their disassembly and the formation of large NF inclusions (Tradewell et al., 2009). Interestingly, abnormalities in mitochondrial morphology, reminiscent of CMT2A caused by mutation in mitofusin 2 (MFN2), precede the formation of NF inclusions in cultured motor neurons suggesting that other early events are important in CMT pathogenesis (Tradewell et al., 2009).

We previously reported that the formation of cytoskeletal and mitochondrial abnormalities induced by expression of mutants affecting the NEFL head ($NEFL^{P8R}$) and rod domain ($NEFL^{Q333P}$) had mutant-specific sensitivity to upregulation of specific stress-inducible chaperones using gene transfer (Tradewell et al., 2009). This raises the question of how similar are the aggregates formed by different NEFL mutants and if they would be amenable to drug therapies that induce expression of endogenous chaperone genes. Celastrol is a plant-derived triterpene that induces expression of the stress-inducible heat shock protein (HSP), HSPA1 (a.k.a. HSP70), including in motor neuron *in vivo* (Kiaei et al., 2005). Celastrol differentially induced expression of HSPA1 and HSPB1 in motor and sensory neurons in dissociated cultures of murine spinal cord-dorsal root ganglia (DRG), and differentially affected the assembly properties of $NEFL^{Q333P}$ and $NEFL^{P8R}$ in these neuronal subtypes, findings that have implications for treatment of CMT with chaperone-inducers.

2. Materials and methods

2.1. Antibodies

Mouse and goat anti-myc antibodies (clone 4E10 and sc789, Santa Cruz Biotechnology Inc.), mouse monoclonal anti-NEFL (clone NR4, Sigma–Aldrich, 1:1000), anti-NEFM (clone NN18, Sigma–Aldrich, 1:1000) and anti-NEFH (clone N52, Sigma–Aldrich, 1:1000), and rabbit polyclonal anti-GAPDH (Abcam 1:1000), HSPA1 (Stressgen 1:200) and HSPB1 (Santa Cruz Biotech, 1:200) were used in this study. The following secondary antibodies from Jackson ImmunoResearch Laboratories were used: donkey Cy3-conjugated anti-mouse IgG, Cy3-anti-rabbit or Cy3-anti-goat IgG; Cy2-conjugated anti-mouse IgG, and HRP-conjugated anti-mouse IgG or anti-rabbit IgG.

2.2. Plasmid construction and biochemical procedures

The following plasmid constructs were used: cDNAs encoding murine myc-tagged $NEFL^{wt}$, $NEFL^{Q333P}$, and $NEFL^{P8R}$ subcloned into pcDNA4.1 (Tradewell et al., 2009); pRC-CMV-NEFM and pRC-RSV-NEFM as described elsewhere (Giasson and Mushynski, 1997) and pOCT-dsRed in pcDNA3 (courtesy of Dr. H. McBride, McGill University, Montreal, QC, Canada).

2.2.1. Assembly of NEFL with other NF subunits

To evaluate NF assembly in cells, SW13-cells, which do not express endogenous intermediate filament proteins, were co-transfected with plasmids encoding wt or mutant NEFL plus NEFM or NEFH using lipofectamine 2000 (Invitrogen). Cells were lysed in buffer containing 25 mM Tris and 2 M urea and the samples were analysed by a semi-native version of BN-PAGE, in which 2 M urea is included in the gel in order to break up NF, but retain small oligomeric forms of NF proteins (Athlan and Mushynski, 1997).

Buffers containing urea have been used to solubilise IF and to generate intermediate oligomeric forms (Carden and Eagles, 1986) or in dialysis for limited reassembly of IF proteins from concentrated solutions of subunits in guanidine or urea (Cohlberg et al., 1987, 1995; Hatzfeld and Weber, 1990; Quinlan et al., 1986; Steinert, 1990). Reassembly of NFs *in vitro* from urea-containing buffers has been well documented (Liem and Hutchison, 1982; Zackroff et al., 1982). Dimers produced by dialysis against 2 M urea of mixtures of NEFL with NEFM or NEFH are considered physiologically relevant assembly intermediates (Athlan and Mushynski, 1997). Ability of $NEFL^{wt}$ or mutants to form hetero-oligomers with NEFM or NEFH was assessed using the technique of Athlan and Mushynski (Athlan and Mushynski, 1997).

To produce recombinant NEFL, SW13-cells were transiently cotransfected with pcDNA4.1-NEFL, encoding carboxy-terminal His-myc-tagged NEFL (Tradewell et al., 2009), plus plasmids encoding $NEFL^{wt}$, $NEFL^{Q333P}$ or $NEFL^{P8R}$. The cells were lysed in buffered urea (25 mM Tris, 8 M urea, pH7.3) and proteinase inhibitor cocktail (Sigma–Aldrich). The lysate was then centrifuged at 14,000 × *g* and the supernatant was incubated with cobalt beads (Novagen) for an affinity purification of His-tagged $NEFL^{wt}$, $NEFL^{Q333P}$ or $NEFL^{P8R}$. The column was washed with Tris–urea buffer and 10 mM imidazole to remove non-specific binding and the NEFL proteins were eluted with Tris–urea buffer complemented with 100 mM imidazole and 1 mM EDTA. Homo-oligomerisation of NEFL was promoted by dialysis for 2 h (MWCO 5 kDa, Millipore) against 25 mM Tris with 1 M urea (Athlan and Mushynski, 1997) and quantified by separation on semi-native BN-PAGE containing 1 M urea, transferring to PDVF membrane and Western analysis with antibody to myc (Fig. 2A). Stability of homo-oligomers was determined by increasing the concentration of urea by adding concentrated urea to 10 μl samples to achieve final concentration of 1.2–3.6 M (Athlan and Mushynski, 1997). Band intensity of NEFL dimers or monomers was measured using ImageJ (http://rsbweb.nih.gov/ij/) and expressed as a percentage of the dimer or monomer amount initially obtained with a 1 M urea dialysis – note that exposure of Western blots to detect dimers, monomers, or polymers (as shown in Fig. 2) was adjusted in order to remain in linear ranges of band intensity. The effect of urea on the dynamic assembly of NEFL was plotted on a graph in order to determine the maximal urea concentration to destabilise polymerised NEFL, standard deviations were omitted on the graph for figure clarity. The experiment was repeated three times and representative blots are shown in Fig. 2. PVDF membranes (Invitrogen) were stripped following the manufacturer's recommendation using a glycine stripping buffer (0.2 M glycine, 0.1% SDS, 1%Tween pH2.2)

2.3. Cell culture

The adrenocarcinoma cell line SW13-, which lacks intermediate filaments, was propagated in DMEM supplemented with 5% foetal calf serum (Hedberg and Chen, 1986).

Primary cultures of dissociated spinal cord and dorsal root ganglia (DRG) were prepared as previously described (Roy et al., 1998) according to a protocol approved by the McGill University Animal Care Committee. Briefly, spinal cords with DRG were removed from E13 mouse embryos, dissociated, plated on coverslips coated with polylysine and Matrigel (Invitrogen), and cultured in modified N3 medium as previously described (Roy et al., 1998). Cultures were used in experiments after 3–6 weeks. Motor neurons and DRG