The G336S Variant in the Human Neurofilament-M Gene Does Not Affect Its Assembly or Distribution: Importance of the Functional Analysis of Neurofilament Variants

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Abstract. The human neurofilament medium (hNFM) subunit is one of the 3 neurofilament (NF) polypeptides, which are the most abundant intermediate filament (IF) proteins in post-mitotic neurons. The formation of neurofilamentous aggregates is a pathological hallmark of many neurodegenerative diseases, including the Lewy bodies found in Parkinson disease (PD). A Gly336Ser (G336S) variant in the rod domain of hNFM has recently been described in a patient with early-onset autosomal-dominant PD. In this study, we have generated a mammalian expression vector encoding the variant hNFM cDNA and characterized its effects on the formation of heteropolymers of IFs in heterologous cell lines. We have also investigated the distribution of the (G336S) hNFM variant protein in neuronal CAD cells, as well as the effects of the variant on the distribution of other cellular organelles and proteins. Our results demonstrate that the G336S variant does not affect the formation of IF networks nor the distribution of the variant hNFM protein. Our data suggest that if the G336S variant is involved in the development of PD, it does not appear to be due to defects in the assembly and distribution of NFs.

Key Words: Assembly; Lewy bodies; Neurofilament; Parkinson disease; Transport.

INTRODUCTION

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease, affecting approximately 1% to 2% of the population aged 65 or older (1, 2). Alterations of neurofilaments (NFs), including aggregation, mislocalization, and aberrant axonal transport, have been described in a number of neurological and neurodegenerative diseases (3–8). A novel neurofilamentopathy with formation of NF inclusions and dementia has been described (9, 10) further supporting a potential role for NF disruption and inclusion formation in neurodegenerative disorders. Recently, a single-nucleotide change in the human neurofilament medium (hNFM) gene, resulting in a substitution of glycine at position 336 by serine (G336S), has been described in a French-Canadian patient with early-onset autosomal-dominant PD (11). Three siblings of the patient’s mother were heterozygous for the mutations, but did not have any symptoms of PD. The heterozygous mutation was not found in a control French-Canadian population. The location of the amino acid substitution in coil 2B of the rod domain of hNFM is only 3 amino acid residues downstream of the Gln333Pro mutation in the human neurofilament light (hNFL) gene linked to Charcot-Marie-Tooth (CMT) disease (12). The highly conserved rod domain of intermediate filaments (IFs) plays a crucial role in the assembly of NFs and the G336S substitution in hNFM could disrupt the formation of a NF network, similar to the CMT-linked hNFL mutations (13).

Although the authors acknowledged that this hNFM(G336S) polymorphism could be a rare variant not directly related to PD (11), several recent reports have described this hNFM(G336S) variant as involved in the development of PD (14, 15). In the absence of further genetic data, it is important to perform a functional analysis of the effects of this amino acid substitution on the assembly of NF networks and its distribution in neuronal cells. We therefore characterized the effects of the variant hNFM(G336S) on the formation of IF networks and on the axonal transport and intracellular distribution of the variant hNFM protein, since disruption of intracellular transport pathways could be a common mechanism implicated in the disease. Our results demonstrate that the hNFM(G336S) variant does not affect the assembly of NF networks or the transport of NFs in neuronal cells. Furthermore, we found that neurofilamentous networks containing the hNFM(G336S) variant did not have increased sensitivity to the effects of toxins linked to Parkinson disease.

MATERIALS AND METHODS

Construction of Expression Vectors

Cloning of the wild-type (wt) hNFM cDNA has been described previously (13). We generated the cytomegalovirus-driven pCI-hNFM(G336S) construct using standard site-directed mutagenesis techniques. The primers used were hNFM(G336S)-F (5'-CTAGAGTCGGTGCGCAGCACCAAGGAGT-3') and hNFM(G336S)-R (5'-GACTCTTGTTGCTGCGACCCGACTCTAG-3'). Constructs in which hNFM was fused to the enhanced green fluorescent protein (EGFP) were generated. The pEGFP-hNFM (wt) and pEGFP-hNFM(G336S) fusion constructs were generated by subcloning the cDNAs for wt and hNFM(G336S) into the EcoRI site of the pEGFP-C1 fusion construct.
vector (Clontech, Palo Alto, CA). All the final constructs were sequenced to ensure that the cDNAs were in frame and to confirm that the G336S variant was successfully introduced.

The bicistronic Cdk5-p35 construct was generated by digestion of the pGBT-Cdk5 construct with BamHI (17). The 0.7-kb fragment was blunt-ended before being ligated in the 4.0-kb pCI vector that was digested with SmaI and dephosphorylated with alkaline phosphatase. The pAS2-1-p35 construct was digested with NotI + BamHI to release p35(wt), which was then blunt-ended and cloned into the blunt-ended pCI plasmid digested with SmaI. The pCI-Cdk5-p35 bicistronic construct was generated by digesting the pCI-p35(wt) clone with BglII + BamHI, which released a 2.7-kb fragment containing the cytomegalovirus immediate early enhancer/promoter, an intron, the p35 cDNA, and the SV40 late poly A. This fragment was then cloned into the pCI-Cdk5 construct digested with BamHI. The construct encoding the wild-type human amyloid precursor protein (APP1-695) was a kind gift from Dr. Tae-wan Kim (Colorado University).

Cell Lines, Culture Conditions, and Transient Transfections

We used the human adrenocarcinoma cell line SW13 Vim', (devoid of all cytoplasmic IFs) or the parental cell line SW13 Vim', expressing an endogenous vimentin network (18) for the experiments to study IF assembly. Dr. Robert Evans (University of Colorado) generously provided us with both cell lines. For studies on intracellular distribution and axonal transport of hNFM(G336S), we used the neuronal cell line CAD (19), generously provided by Dr. Dona Chikaraishi (Duke University). Both SW13 cell lines were maintained in DMEM media supplemented with 5% fetal bovine serum and a 1% antibiotic solution in a humid atmosphere at 37°C and 5% CO₂. CAD cells were maintained in DMEM-F12 medium with 8% fetal bovine serum and 1% antibiotic solution at 37°C and 5% CO₂.

Transfection experiments were carried out using the Lipofectamine Plus kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. For immunofluorescence analysis the cells were grown on 18-mm glass coverslips. After transfection and the total cell lysates were collected in sample buffer, boiled, and run in 10% SDS-PAGE gels.

Indirect Immunofluorescence Microscopy

Immunostaining was carried out as previously described (13). Briefly, slides were incubated first in blocking solution containing 10% normal goat serum, followed by incubation at room temperature with primary antibodies. The slides were washed in 1 × PBS and incubated with the corresponding fluorescently labeled Alexa-Fluor-488 and −594 secondary antibodies (Molecular Probes, Eugene, OR). The slides were washed again with 1 × PBS and mounted for visualization using a Nikon Eclipse 800 microscope and a Snap II digital camera. Images were processed using Adobe Photoshop and the figures prepared using Adobe Illustrator.

Antibodies

We used monoclonal mouse anti-NFL antibody (clone NR4, Sigma, St. Louis, MO), monoclonal mouse anti-NFM antibody (clone NN18, Sigma), monoclonal mouse anti-vimentin antibody (clone V9, Sigma), polyclonal rabbit anti-NFL and anti-NFM antibodies (20), polyclonal rabbit anti-vimentin antibody (clone 1274), polyclonal rabbit anti-tubulin antibody (kindly provided by Dr. Gregg Gundersen, Columbia University), monoclonal mouse anti-tubulin antibody (clone 2–28–33, Sigma), monoclonal anti-EGFP antibody (Clontech) and polyclonal anti-Cdk5 antibody (clone C-8, Chemicon, Temecula, CA).

Analysis of the Effect of the hNFM(G336S) Variant on the Formation of IF Networks

Analysis of the assembly characteristics of the hNFM(G336S) variant protein displayed increased sensitivity to parkinsonian toxins that have been previously linked to the development of PD, SW13 Vim' cells were co-transfected with wild-type hNFL plus either wild-type or hNFM(G336S). The cells were incubated without the toxins 24 h after transfection to allow the formation of normal NF networks, and were then treated with 30 µM paraquat (PQ), 1 µM or 3 µM of 1-methyl-4-phenylpyridium (MPP⁺), and 15 nM, 30 nM, or 100 nM rotenone. MPP⁺ was diluted in H₂O, and rotenone was diluted in 100% ethanol. The cells were treated with the toxins for 24 h, 48 h, or 72 h before being fixed and stained as described.

Both mitochondrial complex I inhibitors used in our studies (MPP⁺ and rotenone, a kind gift of Dr. Miquel Vila, Columbia University) have been reported to have toxic effects on the microtubule (MT) network at high concentrations (21). We have investigated the effect of the highest dose used of the 3 drugs on the MT network of SW13 Vim' cells co-transfected with wild-type hNFL plus either hNFM(wt) or hNFM(G336S). For analysis of the effects of the toxins on the MT networks, the cells were treated with PD toxins for 24 h before being fixed and immunostained.

Live Cell Imaging of CAD Cells Transfected with pEGFP-hNFM(G336S)

The EGFP-hNFM(G336S) construct was transfected in CAD cells, and the cells were subsequently differentiated in serum-free media for different periods of time (24 h to 72 h). A Nikon Eclipse TE300 inverted microscope with a SPOT digital camera was used to acquire images of EGFP fluorescence and phase-contrast microscopy using a ×20 objective at different times post-transfection in the same live cell cultures.


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Effects on Mitochondrial Distribution Induced by Overexpression of hNFM in CAD Cells

In order to investigate whether the overexpression of hNFM would affect the distribution of mitochondria in neuronal cells, CAD cells were differentiated in serum-free media for 96 h before transfection with pCI-hNFM(wt) or pCI-hNFM(G336S). At 24 h post-transfection, mitochondria were labeled with the 400 nM of the specific mitochondrial dye MitoTracker Red (Molecular Probes) for 60 min, washed with PBS, fixed with 4% paraformaldehyde in 1 × PBS, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, and stained with an anti-NFM monoclonal antibody. The mitochondrial dye stock was prepared by dissolving the dye in dimethyl sulfoxide.

Effects of the hNFM(G336S) Variant on the Axonal Targeting of APP

We co-transfected pCI-hNFM(wt) or pCI-hNFM(G336S) with a plasmid encoding the wild-type sequence of human APP1±695. The cells were differentiated in serum-free media for 96 h prior to transfection, fixed at 24 h post-transfection and co-stained with an anti-NFM monoclonal antibody plus an anti-APP polyclonal antibody (a kind gift of Dr. Tae-wan Kim, Columbia University).

Influence of the hNFM(G336S) Variant on the Axonal Distribution of NF Protein Kinases

For these experiments we cultured CAD cells overnight in complete media and then co-transfected them with constructs encoding either wild-type or hNFM(G336S) and constructs encoding Cdk5-p35. After transfections, the cells were allowed to differentiate and extend processes in serum-free media for 72 h before being fixed and immunostained following standard procedures with a monoclonal anti-NFM antibody plus a polyclonal rabbit anti-Cdk5 antibody.

RESULTS

The G336S Variant Does Not Affect the Ability of hNFM to Assemble into IF Networks

We used an in vitro overexpression system using cytomegalovirus-driven constructs in SW13 Vim⁻ and SW13 Vim⁺ cells to investigate the effects of the hNFM (G336S) variant on the assembly of homopolymeric and heteropolymeric IF networks. Parallel experiments with the pCI-hNFM(wt) construct were conducted as controls. We first analyzed the exogenous expression of wild-type and hNFM(G336S) proteins by Western blotting to ensure that the proteins produced were the correct size (not shown).

As seen in Figure 1, neither wild-type hNFM (Fig. 1A) nor hNFM(G336S) (Fig. 1B) self-assembled in SW13 Vim⁻ cells that are devoid of a cytoplasmic IF network. Both wild-type and hNFM(G336S) readily co-assembled with wild-type hNFL when co-transfected in SW13 Vim⁻ cells (not shown, but the phenotypes were identical as those seen in Fig. 7). We carried out a time-course experiment in which either pCI-hNFM(wt) or pCI-hNFM(G336S) were co-transfected with pCI-hNFL(wt) and fixed at 24 h, 48 h, or 72 h post-transfection. Immunostaining was carried out with polyclonal anti-NFM antibody plus monoclonal anti-vimentin antibody to demonstrate that filament formation in the absence of endogenous vimentin expression in SW13 Vim⁻ was not due to the occasional Vim⁺ cell, and with monoclonal anti-NFL antibody plus polyclonal anti-NFM antibody, to show colocalization of the 2 exogenously expressed proteins. The results confirmed that both wild-type and the G336S variant of hNFM co-assembled with wild-type hNFL at all post-transfection times studied, and that the hNFM proteins always colocalized with hNFL (not shown).

Alterations in the stoichiometry of the NF subunits have been shown to affect the formation of a normal NF network. Therefore, we co-transfected wild-type or hNFM(G336S) with hNFL(wt) at 2:1, 1:1, and 1:2 ratios in SW13 Vim⁺ cells. The cells were fixed at 48 h post-transfection and stained with polyclonal anti-NFM plus monoclonal anti-vimentin antibodies, or with monoclonal anti-NFL plus polyclonal anti-NFM antibodies. Heteropolymeric filaments with similar morphologies were formed in all cases, and the exogenously expressed hNFL(wt) and hNFM(G336S) proteins colocalized perfectly (not shown). These results indicate that hNFM(G336S) can form normal heteropolymeric NFL/NFM filamentous networks over a range of concentrations.

Moreover, when either of the hNFM cDNAs was overexpressed in SW13 Vim⁺ cells, exogenously expressed wild-type (Fig. 1C, E) and G336S (Fig. 1D, F) hNFM incorporated into the endogenous vimentin network, and co-staining with polyclonal anti-NFM (Fig. 1E, F) and monoclonal anti-vimentin (Fig. 1C, D) antibodies demonstrated perfect colocalization. In summary, the G336S variant in the rod domain of hNFM does not appear to have any deleterious effect on the assembly of NFM/NFL or NFM/vimentin heteropolymers.

We further investigated whether the hNFM(G336S) variant is phenotypically identical to wild-type hNFM by conducting transient co-transfections experiments in SW13 Vim⁻ cells with mutant hNFL constructs (not shown). SW13 Vim⁻ cells were co-transfected with pCI-hNFL(wt) or pCI-hNFL(G336S) plus pCI-hNFL(Q333P), pCI-hNFL(P8R) or pCI-hNFL(Q333P), fixed at 48 h post-transfection and immunostained. In all cases the phenotypes observed were indistinguishable between wild-type and hNFM (G336S). Co-staining with monoclonal anti-NFL and polyclonal anti-NFM antibodies demonstrated perfect colocalization of hNFM and hNFL proteins, independently of the assembly phenotype. All transfected cells were negative for expression of endogenous vimentin.

These results confirm that the hNFM(G336S) variant can assemble normally with hNFL(wt) over a wide range of protein concentrations and periods of time. Therefore,
the hNFM(G336S) variant appears phenotypically identical to wild-type hNFM in the formation of heteropolymeric neurofilaments with hNFL.

**Normal Axonal Targeting of the hNFM(G336S) Variant in Cultured Neuronal CAD Cells**

Although the hNFM(G336S) variant did not appear to affect the formation of NF networks, we wanted to investigate the possibility that it could affect its intracellular distribution in a neuronal cell line. We used the central nervous system CAD cell line, which readily differentiates upon serum withdrawal, extending processes that resemble dendrites and axons. We initially transfected pCI-hNFM(wt) and pCI-hNFM(G336S) in CAD cells prior to differentiation. Following transfections, CAD cells were allowed to differentiate in serum-free media for 17 h (not shown) or 48 h (Fig. 2) before being fixed and immunostained with anti-NFM monoclonal antibody and anti-tubulin polyclonal antibody. Tubulin staining was used to assess the morphology of CAD cells.

The distributions of both wild-type and hNFM (G336S) proteins were similar in the transfected cells. Some cells displayed intense hNFM immunostaining in the cell bodies, with fainter hNFM immunoreactivity in the neurites. In other cells, the anti-NFM immunostaining was predominantly neuritic. The staining for hNFM in neuronal processes was in general more homogeneous throughout the short neurites, but in all cases there was a tendency for increased anti-NFM staining in the distal ends of neurites (Fig. 2A, C). It is interesting to note that in spite of the elevated levels of expression of hNFM in some cells (Fig. 2B, D), there were no signs of neuritic degeneration. Our data demonstrate that there are no clear differences in the distributions of wild-type and hNFM (G336S) proteins in CAD cells.

**Further Characterization of the hNFM(G336S) Variant Using EGFP-hNFM Constructs**

We generated N-terminal fusion constructs of pEGFP-C1 and hNFM(wt) or hNFM(G336S) and analyzed their assembly characteristics in SW13 Vim− and SW13 Vim+ cells. The use of EGFP-fused at the N-terminus of rat NFM has been previously used successfully as a reporter in axonal transport studies (22). We first confirmed that the full-length fusion proteins were expressed by Western blotting using anti-NFM and anti-EGFP antibodies (not shown).

**Assembly Characteristics of the pEGFP-hNFM(G336S) in Non-Neuronal Cells:** We first determined that EGFP-hNFM assembled into a normal IF network. Both p-EGFP-hNFM(wt) and pEGFP-hNFM(G336S) were transfected into SW13 Vim− and SW13 Vim+ cells. As expected, EGFP-hNFM(G336S) in SW13 Vim− cells was unable to self-assemble as demonstrated by staining with polyclonal anti-NFM (Fig. 3A) and monoclonal anti-EGFP (Fig. 3B) (note the punctate staining pattern). These results also confirmed that the exogenously expressed fusion proteins could be detected with either anti-NFM or anti-EGFP antibodies. When pEGFP-hNFM(G336S) was expressed in SW13 Vim+ cells, the transfected protein incorporated into the endogenous vimentin filament network, as demonstrated with staining using monoclonal anti-EGFP (Fig. 3C) and polyclonal anti-vimentin antibodies (not shown). Similar results were obtained with pEGFP-hNFM(wt). These experiments confirm that EGFP-hNFM retains the assembly characteristics of hNFM and can be used as a reporter to study axonal transport of hNFM proteins.

**Distribution and Transport of EGFP-hNFM(G336S) in Neuronal CAD Cells:** After demonstrating the assembly capabilities of the EGFP-hNFM, we investigated its distribution and transport in CAD cells. We initially transfected CAD cells grown for 24 h in complete media and transfected them with wild-type or G336S pEGFP-hNFM. After transfections, the cells were differentiated in serum-free media and fixed and immunostained at 17 h (not shown) and 48 h post-transfection (Fig. 3D, E). As described before, the phenotypes observed depended upon the amounts of exogenous hNFM protein expressed. While higher levels of expression resulted in the accumulations of the EGFP-hNFM proteins in the cell body (Fig. 3D, see cells on the lower left), lower levels of expression were associated with staining in both the cell bodies and the neuronal processes of CAD cells (Fig. 3D, see cells on top and right). The EGFP-hNFM proteins had a tendency to accumulate in the distal processes of the cells (Fig. 3D, arrows), similar to what we observed for the untagged hNFM proteins (Fig. 2B, D). The accumulation of EGFP-hNFM protein did not appear to affect the morphology of the neurites, as shown by staining.

**Fig. 1.** The G336S variant does not affect the ability of hNFM to assemble into IF networks. Transfection of pCI-hNFM(wt) (A, C, E) or pCI-hNFM(G336S) (B, D, F) into SW13 Vim− (A, B) and SW13 Vim+ (C–F). Cells were fixed at 48 h post-transfection and immunostained with anti-vimentin monoclonal antibody plus anti-NFM polyclonal antibodies. Neither wild-type nor hNFM (G336S) can self-assemble in the absence of an endogenous vimentin network (A, B; the absence of staining using anti-vimentin antibodies is not shown). Transfection experiments in SW13 Vim+ cells demonstrate the incorporation of the exogenous hNFM proteins into the endogenous vimentin network (C–F), with colocalization of vimentin (C–D) and hNFM (E–F). Images were taken at ×60. Scale bar = 25 μm.
using anti-tubulin antibody (Fig. 3E, arrows). Results from these experiments indicate that the EGFP-hNFM(G336S) protein has the same subcellular distribution and neuritic targeting patterns as the EGFP-hNFM(wt) protein.

To study the transport of the EGFP-hNFM(G336S), we carried out imaging of live cells after transfection of neuronal CAD cells. CAD cells were transfected and allowed to differentiate in serum-free media, and the cells were imaged at 24 h (not shown), 48 h (Fig. 3F), and 72 h (Fig. 3G) post-transfection for both EGFP fluorescence and phase-contrast microscopy (not shown) using an inverted microscope. Although higher levels of EGFP fluorescence were always observed in the cell bodies of CAD cells, EGFP-hNFM(G336S) was normally targeted to the neuronal processes (Fig. 3F, G). Within the neurites, EGFP expression was brighter in the terminal segments of the processes, and the expression of EGFP was also high in the varicosities typical of differentiated CAD cells. This pattern of EGFP expression could be observed more clearly at 72 h post-transfection (Fig. 3G). Composite images of EGFP fluorescence and phase-contrast demonstrated that transfected cells appeared healthy (not shown). Occasionally, we saw some cells expressing very high amounts of EGFP-hNFM. These cells (Fig. 3G, asterisk) had an abnormal morphology, including neuritic degeneration and somatic swelling, due to the high levels of expression, and accumulation of exogenous proteins. The same phenomenon has previously been observed after overexpression of high levels of NFs (23).

Taken together, the data obtained with imaging of live cells confirmed that the hNFM(G336S) variant did not appear to affect the intracellular distribution and neuritic targeting of hNFM. They also further validated the use of EGFP-hNFM proteins to study the intracellular transport of NF subunits.

Overexpression of hNFM Does Not Affect the Distribution of Mitochondria in CAD Cells

Alterations in the intracellular distribution of mitochondria have been observed in neurodegenerative disorders associated with accumulation of NFs. We used our neuronal culture model to investigate whether the hNFM(G336S) variant had any effects on the intracellular distribution of mitochondria.

For these experiments, neuronal CAD cells were differentiated for 96 h in serum-free media before being transfected with pCI-hNFM(wt) or pCI-hNFM(G336S). At 24 h post-transfection, mitochondria were labeled with the fluorescently labeled dye MitoTracker Red before being fixed and immunostained using anti-NFM antibodies. Again, no differences were observed between cells transfected with hNFM(wt) (Fig. 4A, B) and cells transfected with hNFM(G336S) (Fig. 4C, D). Staining of hNFM was more prominent in the cell body (Fig. 4A, C), and there was a tendency of increased hNFM staining in the distal segments of the neurites (Fig. 4A, arrow). Mitochondrial labeling was also very intense in the cell body (Fig. 4B, D), and although mitochondrial labeling was seen throughout the neurites, it was more intense in the distal ends (Fig. 4B, D, arrows) and in the varicosities (Fig. 4D, long arrows). Even when overexpression of hNFM resulted in accumulations in the cell bodies, the distribution of mitochondria to the processes was not affected. When focal accumulations of exogenous hNFM in the cell body were observed (wild-type or G336S, the images shown for illustrative purposes in Fig. 4C correspond in this case to hNFM[G336S]), accumulations of hNFM did not affect the labeling of mitochondria in the cell body (Fig. 4D, wide arrow). When these focal accumulations of hNFM protein were observed in the processes (Fig. 4C, long arrow), they did not affect the distribution of mitochondria throughout the neurite, although the mitochondrial labeling was stronger in the area corresponding to accumulated hNFM protein.

In summary, overexpression of either wild-type or hNFM(G336S) did not affect the distribution of mitochondria in neuronal CAD cells in culture, and we did not observe any differences between cells transfected with hNFM(wt) and cells transfected with hNFM(G336S).

Normal Axonal Targeting of hAPP Protein in CAD Cells Co-Transfected with hNFM

The hAPP protein has been widely used as a marker of axonal transport and has been shown to accumulate in the axons of neurons in certain neurodegenerative states and after traumatic head injury (24). To study the effects of the G336S variant on hAPP distribution, we carried out transient co-transfections of hAPP with either...
pCI-hNFM(wt) or pCI-hNFM(G336S) constructs in neuronal CAD cells.

CAD cells were differentiated in serum-free media for 96 h and then co-transfected with hAPP and either wild-type or hNFM(G336S). At 24 h post-transfection, the cells were fixed and immunostained using monoclonal anti-NFM and polyclonal anti-APP antibodies. Exogenous overexpression of hNFM proteins did not affect the distribution and transport of hAPP (Fig. 5). The immunostaining for hAPP was very strong in the cell bodies and was also observed throughout the cell processes (Fig. 5A, B). A certain degree of colocalization of both proteins in the processes could be observed. When the cells did not have very long processes, we could often observe staining for both proteins throughout the neurites (Fig. 5C, D). The images in Figure 5 were overexposed to see the staining for both hNFM and hAPP in the fine processes of the cell (Fig. 5A, B). As observed previously, hNFM displayed a tendency to accumulate in the distal ends of the processes (Fig. 5A, C, arrows). These experiments demonstrated that overexpression of wild-type or hNFM(G336S) did not alter the subcellular distribution of the hAPP protein, suggesting that the hNFM(G336S) variant does not appear to have any deleterious effects on fast anterograde transport.

hNFM(G336S) Does Not Affect the Transport of Cyclin-Dependent Kinase-5 (Cdk5)

Aberrant hyperphosphorylation of NFM has been observed in a number of pathological conditions. Phosphorylation of NFM occurs on the lysine-serine-proline (KSP) repeats in the tail domain. These repeats are phosphorylated by a number of kinases, including Cdk5 and the members of the mitogen-activated family of protein kinases. NFs and these protein kinases have been isolated together from cytoskeletal complexes. Previous studies in our laboratory demonstrated phosphorylation of rat NFH by Cdk5 (15). Thus, we decided to explore the possibility that the hNFM(G336S) variant would result in altered distribution of Cdk5, which could potentially result in the aberrant phosphorylation and disruption of the NF networks. To ensure co-expression of Cdk5 and p35, its specific activator, in the same cells we generated a bicistronic pCI-Cdk5-p35 construct. We have previously shown that co-transfection of Cdk5 and p35 from separate constructs results in kinase activity (17).

Neuronal CAD cells were transfected with the bicistronic pCI-Cdk5-p35 construct and either wild-type or hNFM(G336S) constructs, and differentiated for 72 h before fixation and immunostaining using anti-NFM monoclonal plus anti-Cdk5 polyclonal antibodies. Staining for Cdk5 demonstrated its predominant localization in the cell body (Fig. 6B, D). We also observed nuclear staining using anti-Cdk5 antibodies. Some Cdk5 immunostaining was seen in neuronal cell processes, albeit not at very high levels and with a tendency to accumulate in the distal ends of neurites (Fig. 6B, D, arrows). Even in cells with focal cytoplasmic accumulations of exogenously expressed hNFM (Fig. 6C, big arrow), the cytoplasmic staining of Cdk5 was not altered. However, increased Cdk5 immunoreactivity in the cell processes (Fig. 6D, arrows) correlated well with increased accumulation of hNFM in the neurites (Fig. 6C, arrows). Similar results were obtained when a dominant-negative Cdk5 construct was used for transient transfections instead of the Cdk5-p35 bicistronic construct (not shown).

Our results suggest that the hNFM(G336S) variant does not affect the intracellular distribution of one of its in vivo protein kinases, Cdk5. However, the potential exists for accumulations of Cdk5 within focal accumulations of hNFM in the neurites.

Fig. 3. Further characterization of the hNFM(G336S) variant using EGFP-hNFM fusion proteins. A–C: Assembly of pEGFP-hNFM in non-neuronal SW13 cells. pEGFP-hNFM(G336S) was transfected into SW13 Vim− (A, B) and SW13 Vim+ (C) cells. The cells were fixed at 48 h post-transfection and stained with monoclonal anti-vimentin plus polyclonal anti-NFM antibodies (A, the absence of vimentin staining is not shown), or with monoclonal anti-EGFP antibody (B, C). EGFP-hNFM(G336S) cannot self-assemble in the absence of an endogenous IF network (A, B), but readily incorporates into the endogenous vimentin network (C). Similar results are observed for EGFP-hNFM. These experiments demonstrate that both wild-type and (G336S) variant EGFP-hNFM have the same assembly characteristics as hNFM. Images were taken at ×60. Scale bar = 25 μm. D, E: Intracellular distribution of EGFP-hNFM in neuronal CAD cells. pEGFP-hNFM(G336S) was transfected into CAD cells; the cells were fixed after differentiating in serum-free media for 48 h post-transfection and co-stained with monoclonal anti-NFM antibody (D) and monoclonal anti-tubulin antibody (E). Exogenous EGFP-hNFM(G336S) protein distributed homogenously through the cell body and neurites (two cells on the right), with more intense staining in the distal segments of the neurites (D, arrows). Even when focal accumulation of EGFP-hNFM(G336S) was observed (two cells on the left), the staining with anti-tubulin demonstrated the normal morphology of neurites (E, arrows). Images were taken at ×40. Scale bar = 35 μm. F, G: Live cell imaging of neuronal CAD cells overexpressing pEGFP-hNFM. CAD cells were transfected with pEGFP-hNFM(G336S) and allowed to differentiate in serum-free media after transfection. At different times post-transfection images were taken from the same cultures. Shown are live images of cells at 48 h post-transfection (F) and at 72 h post-transfection (G). Only the EGFP images are shown. EGFP-hNFM(G336S) localized throughout the cell bodies and the neurites, with increased EGFP expression localized in the varicosities that are typical of differentiated CAD cells (G, F, arrows). EGFP fluorescence in transfected cells was progressively brighter with increasing times post-transfection. Very high levels of exogenous proteins were deleterious for the cells (G, cell with *). Transfection of pEGFP-hNFM(wt) resulted in the same phenotypes (not shown). Images were taken at ×20. Scale bar = 55 μm.
Fig. 4. Overexpression of hNFM(G336S) does not affect the intracellular distribution of mitochondria. pCI-hNFM(wt) (A, B) or pCI-hNFM(G336S) (C, D) were transfected in CAD cells that had been differentiated in serum-free media for 96 h. At 24 h post-transfection the cells were labeled with the mitochondrial-specific dye MitoTracker Red before being fixed and stained with monoclonal anti-NFM antibody. Images corresponding to staining with anti-NFM antibody (A, C) and labeling with MitoTracker Red staining (B, D) are shown. Mitochondria were much more intensely labeled in the cell body, and unevenly distributed throughout the neurites. Local accumulations of exogenously expressed hNFM protein were concomitant with increased mitochondrial labeling and focal colocalization, without affecting the mitochondrial labeling in the cell body or the neurites (B, D, arrows). Images were taken at ×40. Scale bar = 20 μm.
NF Networks Containing the hNFM(G336S) Variant Do Not Have Increased Sensitivity to the Effects of Toxins Linked to PD

A number of environmental neurotoxins have been linked epidemiologically and experimentally to the development of PD. Aggregation of NFs with other components in the Lewy bodies characteristic of PD suggest the possibility that these neurotoxins could induce the collapse and/or aggregation of NF networks. Even though the hNFM(G336S) variant can assemble...
Fig. 6. Expression of hNFM(G336S) does not affect the intracellular distribution of Cdk5. The bicistronic pCI-Cdk5-p35 construct was co-transfected with wild-type (A, B) or G336S (C, D) pCI-hNFM into CAD cells, which were allowed to differentiate in serum-free media for 72 h post-transfection before being fixed and stained using monoclonal anti-NFM antibody (A, C) plus polyclonal anti-Cdk5 antibody (B, D). Cdk5 immunostaining was very bright in the cell bodies, and the pattern was not disrupted when focal accumulations of exogenous hNFM protein in the cell soma were observed (C, big arrow). Faint immunostaining using an anti-Cdk5 antibody was observed in neurites. Increased staining of Cdk5 (B, D, arrows) correlated with focal accumulations of hNFM in the neurites for both wild-type and hNFM(G336S) (C, arrows). Images were taken at ×40. Scale bar = 25 μm.

normally, it is possible that it could result in the formation of NF networks that are more susceptible to the noxious effects of these neurotoxins.

We transiently co-transfected hNFL(wt) with either hNFM(wt) or hNFM(G336S) subunits in SW13 Vim− cells, and cultured the cells for 24 h to allow enough time for the formation of heteropolymeric hNFL/hNFM networks. The cells were then treated with different concentrations of 3 PD-linked toxins: the herbicide PQ and the mitochondrial complex I inhibitors rotenone and MPP⁺. We fixed the cells at different times after treatment and analyzed the integrity of the NF networks by co-staining with anti-NFL and anti-NFM antibodies. We also investigated the potential effects of these toxins on the MT network.

We did not observe any differences with regard to the effects of PD toxins on networks containing wild-type hNFM or hNFM(G336S). Representative images corresponding to cells harboring NF networks containing the variant hNFM(G336S) protein are shown in Figure 7. Treatments carried out included 30 μM PQ, 1 μM and 3 μM MPP⁺, and 15 nM, 30 nM, and 100 nM rotenone,
Fig. 7. NF networks containing hNFM(G336S) protein are not disrupted by parkinsonian-mimetic toxins. SW13 Vim<sup>−</sup> cells were co-transfected with pCI-hNFL(wt) plus pCI-hNFM(wt) or pCI-hNFM(G336S). After transfections, the cells were allowed to form heteropolymeric NFL-NFM networks for 24 h before treatment with different doses of PQ, MPP<sup>+</sup>, and rotenone, for 24 h, 48 h, or 72 h. After these treatments, the cells were fixed and immunostained. No deleterious effects of the parkinsonian-mimetic toxins were observed in the heteropolymeric NF networks (either wild-type or hNFM(G336S) containing filaments) at any of the doses or treatment times that we used. Representative images are shown of SW13 Vim<sup>−</sup> cells co-transfected with hNFL(wt) and hNFM(G336S) proteins and treated with 30 μM PQ (A–D), 3 μM MPP<sup>+</sup> (E–H) or 100 nM rotenone (I–L) for 48 h (A, B, E, F, J, K) or 24 h (C, D, H, I, L, M), and stained with anti-NFL (A, E, I) plus polyclonal anti-NFM (B, F, J), or with anti-NFM (C, G, K) plus anti-tubulin (D, H, L) antibodies. Images were taken at ×60. Scale bar = 25 μm.

for a total of 24 h, 48 h, or 72 h. The images corresponding to 48 h treatments with the higher dose of each toxin tested are shown, including 30 μM PQ (Fig. 7A, B), 3 μM MPP<sup>+</sup> (Fig. 7E, F), and 100 nM rotenone (Fig. 7I, J). As we can see after immunostaining with monoclonal anti-NFL (Fig. 7A, E, I) plus polyclonal anti-NFM (Fig. 7B, F, J), cells in general displayed some bundled and some thinner filaments, probably related to the levels of expression of each individual protein, with perfect colocalization of both hNFL and hNFM proteins in all transfected cells. The same phenotypes were observed when treatment was carried out for 24 h or 72 h, and when hNFM(wt) was used for co-transfection with hNFL(wt) (not shown).

Both rotenone and MPP<sup>+</sup> have toxic effects on MTs, albeit at higher doses than those used in our study. We carried out parallel transfection experiments using the highest doses for each toxin tested: 30 μM PQ (Fig. 7C, D), 3 μM MPP<sup>+</sup> (Fig. 7G, H), and 100 nM rotenone (Fig. 7K, L). The cells were fixed after 24 h of treatment and stained with polyclonal anti-NFM (Fig. 7C, G, K) plus monoclonal anti-tubulin (Fig. 7D, H, L) antibodies. We observed a normal hNFL/hNFM heteropolymeric network and a normal MT network in all cases, confirming...
previous data showing that at these doses of the parkin-
sonian-mimetic toxins there are no deleterious effects on
the MT network.

The results obtained in these experiments do not sug-
gest a direct damaging effect of toxins linked to PD on
the NF networks, nor an increased susceptibility to PD-
linked toxins conferred by the hNFM(G336S) variant.

DISCUSSION

Alterations in NFs have been identified in a number
of neurological and neurodegenerative diseases (25, 26).
Mutations in the hNFL gene have been linked to CMT
disease (12, 27–30). Studies from our laboratory (13), as
well as others (31) have demonstrated a dominant nega-
tive effect of the CMT-linked hNFL mutations on the
assembly and transport of IFs (13, 31). These studies pro-
vide a rationale for the involvement of NF disorganiza-
tion on the development of neuropathy in this autosomal
dominant subset of CMT patients. Since NF subunits are
one of the major components of Lewy bodies seen in PD
and in other neurodegenerative diseases (32), a number
of studies have been performed to determine if there is a
link between mutations in NFs and PD. In one study, 328
sporadic and familial PD patients and 344 control indi-
viduals were screened for mutations in the hNFL gene,
but no mutations were found (33). A mutation in the
hNFL gene has been reported in an early-onset PD pa-
tient (11). The mutation had an incomplete penetrance
of 25%, and no further reports have confirmed the involve-
ment of this single-nucleotide polymorphism (SNP) in
PD. Without additional genetic data, the functional anal-
ysis of SNPs in NFs is particularly important in order to
determine which SNPs are pathogenic mutations, and
which appear to be uncommon variations and/or potential
risk factors (34). Within this context, an increased sus-
ceptibility to NF assembly disruption by the hNFL
(G336S) variant could result in increased susceptibility to
Lewy body formation and axonal degeneration.

The main aims of our study were i) to investigate
whether the G336S variant in the hNFL gene disrupts
the assembly of the NF network, ii) to investigate whether
this hNFL variant has a deleterious effect on neuronal
morphology and intracellular neuronal distribution, and
iii) to determine if the NF network composed of the G336
variant is more susceptible to toxins associated with PD.
Using heterologous cell lines, we characterized the as-
sembly of the hNFL(G336S) variant as compared with
hNFL(wt). We did not observe any differences regarding
the formation of heteropolymeric hNFL/hNFM networks
in SW13 Vim− cells, even when different ratios of NFs
were transfected and when analyzed at different times
post-transfection. Normal filaments were also formed
when the hNFM(G336S) variant was exogenously ex-
pressed in SW13 Vim− cells, which express endogenous
vimentin. These results indicate that it is unlikely that the
hNFM(G336S) variant affects the formation or mainte-
nance of heteropolymeric NF and IF networks, which has
been the most striking phenotype of NFL mutant proteins
linked to CMT2 (13, 31).

There is increased evidence for alterations of axonal
transport in neurodegenerative diseases (35), and specific
accumulations of NFM and NFH subunits in the proximal
axon have been implicated in the development of diabetic
neuropathy (36). We investigated the effects of the
G336S variant on the transport of hNFM using a neuronal
cell culture system and we did not observe any changes
in distribution of the G336S variant when compared to
the wild-type hNFM.

Mitochondrial dysfunction is thought to be one of the
main players in a wide range of neurodegenerative con-
ditions (37). A relationship between increased expres-
sion of NFs, neuronal degeneration and mitochondrial dys-
function has also been suggested in the Wobbler natural
mutant mouse, an animal model for degenerative disease
of the lower motoneurons with increased expression lev-
els of NFM (38–41). Furthermore, our laboratory has
shown that overexpression of α-internexin, another neu-onal IF led to neuronal loss in aged mice and altered
distribution of mitochondria, some of which were trapped
in neurofilamentous aggregates (42). In this study, we
showed using the mitochondrion-specific dye MitoTracker
Red, which labels only mitochondria with active mem-
brane potentials, demonstrated that exogenous overex-
pression of hNFM (wild-type or G336S) did not affect
the distribution of mitochondria in neuronal cells.

Human APP is transported along the axon in a kinesin-
dependent manner (43) and is considered to be a marker
for fast anterograde transport (44). APP has been shown
to accumulate when axonal transport is disrupted, and the
accumulation of APP is therefore used as a marker for
fast axonal transport defects (24). Our results suggest that
overexpression of wild-type and G336S variant hNFM in
neuronal CAD cells did not affect the intracellular distri-
bution of APP.

Hyperphosphorylation of NFs is thought to cause NF
disorganization and accumulation in some neurodegen-
ergative conditions. The protein kinase Cdk5 has been di-
rectly linked to the regulation of intracellular transport
(45) and of fast anterograde axonal transport (46). Cdk5
not only phosphorylates NFM (47), but has also been
found to colocalize with deposits of hyperphosphorylated
NFs in PD patients (48). We found that specific staining
for Cdk5 was stronger in focal accumulations of exoge-
nously expressed hNFM. These results correlate with pre-
vious data indicating that both NFs and some of the ki-
nases and phosphatases responsible for their phosphor-
ylation and dephosphorylation are found together
in cytoskeletal complexes in rat brain (49). Mislocal-
ization of Cdk5 resulting from the altered targeting of
hNFM(G336S) could affect axonal transport; however,
our data suggest that mislocalization of Cdk5 does not result from overexpression of the hNFM(G336S).

To investigate the possibility that the hNFM(G336S) variant could be involved in PD by other mechanisms other than altered NF network formation and altered distribution, we evaluated whether cells expressing heteropolymeric NF networks including hNFM(G336S) were more sensitive to toxins linked to PD. We used 3 parkinsonism-inducing environmental toxins, namely PQ, MPP⁺ and rotenone (50, 51), but we did not observe any differences between NF networks containing hNFM(wt) or the hNFM(G336S) variant. At the doses of toxins we used there were no obvious effects on the NF networks. Thus, the hNFM(G336S) polymorphism does not appear to confer increased sensitivity to parkinsonian toxins to the NF networks.

A recent study (34) reported a mutation analysis of hNFM as an interesting candidate for the development of PD, since chromosome 8p (containing both hNFL and hNFM) was previously linked to patients with family histories of PD. The authors did not find any PD patients or controls with the hNFM(G336S) variant, and indicated that the importance of this variant remains to be determined. They identified 2 new single nucleotide substitutions, G697R and del829V, plus the known A475T and P725Q substitutions. Both A475T and G697R were identified as polymorphisms based on the frequency with which they were found in the normal general population. The P725Q substitution was identified in a sporadic PD patient, while del829V did not co-segregate with PD. The authors concluded that NFM does not appear to play a major role in PD, and advocated for the functional analyses of these nucleotide substitutions to be carried out.

In summary, we have presented results of the functional analysis of the hNFM(G336S) genetic variant, which has been reported as involved in the development of PD. Our results show the lack of obvious deleterious effects of the hNFM(G336S) variant on the NF network and its susceptibility to alterations mediated by parkinsonian-mimetic toxins. Therefore, the G336S nucleotide variation in hNFM could be a very rare polymorphism and not linked to PD or, possibly, other unknown mechanisms or co-factors need to be involved in order for the hNFM(G336S) variant to become important in the pathogenesis of the disease. Our study also emphasizes the need for functional analysis of genetic variants of human NFs in order to determine their potential contribution to the development of PD and other neurodegenerative diseases.

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