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Triton-soluble phosphovariants of the high molecular weight neurofilament subunit from NB2a/d1 cells are assembly-competent

Implications for normal and abnormal neurofilament assembly

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Abstract

NB2a/d1 cells incorporate neurofilaments (NFs) containing extensively phosphorylated high (NF-H) molecular weight subunits into the Triton-insoluble cytoskeleton of axonal neurites elaborated during differentiation with dibutyryl cAMP. However, immunocytochemical and biochemical analyses demonstrate the constitutive expression and extensive phosphorylation of a sizeable pool of (200 kDa) NF-H. We examined by cell-free analyses whether or not this Triton-soluble NF-H pool was assembly-competent in cell-free analyses. Triton-soluble fractions from ^{35}S-radiolabeled NB2a/d1 cells were incubated with dissociated mouse CNS Triton-insoluble cytoskeletons that had been dissociated by treatment with 6 M urea. Following overnight dialysis to remove urea, low-speed centrifugation to sediment Triton-insoluble cytoskeletons resulted in the co-sedimentation of radiolabeled NF-H, indicating that Triton-soluble NF-H was capable of association with Triton-insoluble structures. Triton-soluble, extensively phosphorylated NF-H from NB2a/d1 cells was also capable of co-assembling with purified NF-L. Following high-speed centrifugation (100,000 × *g* for 1 h) to sediment any oligomeric assemblies, the Triton-soluble fraction from NB2a/d1 cells was mixed with purified NF-L that had been solubilized by 6 M urea. Following overnight dialysis to remove urea, high-speed centrifugation sedimented both NF-L and Triton-soluble NF-H from NB2a/d1 cells, demonstrating that Triton-soluble NF-H variants are assembly-competent. These data suggest that NF-H variants represent precursors for NF assembly, and indicate that their assembly within NB2a/d1 cells must be under temporal and spatial regulation.

Key words: Neurofilament; Axonal outgrowth; Cytoskeleton; Phosphorylation; Neurofibrillary pathology

1. Introduction

Neurofilaments (NFs) are comprised of a 'backbone' subunit that is roughly analogous to other intermediate filaments, and two higher molecular weight 'associated' proteins, termed NF-L, NF-M and NF-H, for 'low', 'middle' and 'high' molecular weight subunits that migrate at 70, 145 and 200 kDa on SDS-gels, respectively. NF subunit assembly, and the interaction of NFs with each other and with other axonal constituents, is regulated by multiple phosphorylation events (for review, see [1]). Unlike other intermediate filament species, in which dynamic behavior has long been recognized, NFs have been classically viewed as relatively static structures. However, in recent years, divergent analyses contributed to a more complex picture of dynamics (for review, see [2]).

The developmental appearance, phosphorylation and deposition of NFs is complex. A 'two-stage' developmental pattern of neurofilament expression has been described in which NFs comprised of only NF-L and NF-M appear during initial neuritogenesis and are gradually replaced during embryonic development by NFs containing NF-H isoforms and additional extensively phosphorylated isoforms of NF-M [3]. These early-appearing NF proteins might form relatively unstable structures, since NFs are not commonly seen ultrastructurally until well after all three subunits are detected immunochemically [4]. The onset of NF-H expression temporally coincides with the stabilization of neuronal circuitries; accordingly, the presence of NF-H may be required to mediate certain axonal events, such as slowing of cytoskeletal transport and growth of axonal caliber [3]. Although the subsequent conversion of NFs comprised of NF-M and NF-L to a three-subunit NF is fairly generalized [5], whether this is achieved by replacement of the entire existing NF network, or by incorporation of NF-H subunits into 'vacant' sites along pre-existing 'immature' neurofilaments, or by a combination of both mechanisms, remains an open question.

The ease of manipulation of cultured neurons and continuous neuronal cell lines has been useful for eluci-

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dating certain aspects of synthesis and assembly of cytoskeletal proteins [6–8]. NB2a/d1 cells, in particular, have been useful for studies of the dynamics of NF phosphorylation and assembly. Following differentiation with dbcAMP, NB2a/d1 cells elaborate axonal neurites and at this time incorporate NF subunits within the Triton-soluble cytoskeleton. As observed in neurons in situ [9], extensively phosphorylated NF subunits are segregated within the cytoskeleton of axons, while only hypophosphorylated NF subunits are incorporated into the perikaryal cytoskeleton [10,11]. However, these cells contain substantial Triton-soluble pools of NF subunits, including extensively phosphorylated isoforms of NF-H, prior to elaboration of axonal neurites [11,12]. Pulse-chase analyses have suggested, but not confirmed, that at least some of this Triton-soluble NF-H may represent precursors for NF assembly [12], and that their phosphorylation before assembly and axonal transport is a function of their extended residence time within perikarya [13,14]. The presence of a significant pool of Triton-soluble extensively phosphorylated NF-H within perikarya [11,12] raises the question as to how these subunits are normally prevented from depositing into perikaryal cytoskeletons; the importance of such regulation is underscored by the rapid accumulation of phosphorylated NFs within perikaryal cytoskeletons following neurotoxin exposure [11,15–23]. To address this issue, in the present study we conducted cell-free analyses under conditions that promote NF assembly to determine whether or not these Triton-soluble NF-H subunits are assembly-competent.

2. Experimental

2.1. Culture and differentiation of NB2a/d1 cells

NB2a/d1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics. To induce the outgrowth of axonal neurites [10], the medium was replaced with 1 mM dibutyryl cyclic AMP (dbcAMP) 24 h after plating and incubation was continued for 72 h. All cell culture reagents were from Sigma Chemical Co., St. Louis, MO.

2.2. Radiolabeling

Metabolic labeling of proteins was carried out as described previously [12]. Briefly, two cultures in 10 cm^2 plates were incubated for 15 min with methionine-deficient Eagle's medium (Irvine Scientific, Irvine, CA) containing 10% FCS and 2 mM glutamine, followed by 15 min incubation in the same medium containing 5 mCi [^{35}S]methionine:cysteine Translabel (ICN Radiochemicals, Irvine, CA), after which cultures were immediately harvested.

Incorporation of [^{32}P]orthophosphate was carried out as described [12]. Two cultures were rinsed with phosphate-free Eagle's minimal essential medium (Irvine Scientific, Irvine, CA) containing 10% FCS and 2 mM glutamine, then incubated for 2 h in the same medium containing 100 μCi [^{32}P]orthophosphate (specific activity 1,000 Ci/mmol; New England Nuclear, Boston, MA) after which cultures were immediately harvested.

2.3. Fractionation of cells into Triton-insoluble cytoskeleton

Following 3 days of dbcAMP treatment, cells were fractionated into Triton-insoluble and -soluble fractions according to the procedure of Chiu and Norton [23], as previously utilized for NB2a/d1 cells [11]. Cells were rinsed with cold Tris-buffered saline (TBS; pH 6.8), then scraped from the plate with a rubber policeman in cold 1% Triton X-100 in 50 mM Tris-HCl (pH 6.8), 5 mM ethylene diamine tetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 50 μg/ml leupeptin. The cells were homogenized in a glass-Teflon homogenizer, and centrifuged for 10 min at 13,000 × g at 4°C. Sedimented material, defined as the (Triton-insoluble) cytoskeleton, was resuspended in 1% sodium dodecyl sulfate (SDS) in the above buffer. The Triton-insoluble fraction was also isolated by these methodologies from mouse brain [11].

2.4. Immunoprecipitation

Immunoprecipitation of fractions from two radiolabeled cultures was carried out as described [12]. In all experiments, the entire fractions were used to permit comparison of relative amounts of radiolabel in each fraction. The Triton-soluble supernatant was made 1% SDS. The fractions were diluted with 4 vols. of 60 mM Tris-HCl (pH 7.6), 190 mM NaCl, 6 mM EDTA, and 1.25% Triton X-100 and incubated overnight at 4°C with reciprocal shaking with 1:100 dilutions of the following anti-neurofilament subunit antisera raised in this laboratory: a rabbit polyclonal antisera (H3) directed specifically against NF-H (H3). The specificity of these antisera has been previously reported [24]. Samples were then incubated at room temperature with 10 mg of protein A–Sepharose complex (Sigma Chem. Co.) for 2 h with reciprocal shaking, followed by extensive washing with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and 0.02% SDS. Elution of immunoprecipitated proteins was carried out by heating at 100°C for 1 min with 100 μl of 125 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol, 2% SDS, 5% β-mercaptoethanol and 10% glycerol.

2.5. In vitro assembly analyses

The ability of Triton-soluble H-200 to associate with Triton-insoluble structures was examined by mixing 100 μg of the dbcAMP-treated Triton-soluble fraction that had been radiolabeled with [^{35}S]methionine as described above. NB2a/d1 cells with 100 μg of Triton-insoluble mouse CNS cytoskeletons that had been solubilized by incubation with 6 M urea. This mixture was dialyzed overnight against 40 vols. of 50 mM Tris-HCl (pH 6.8), 5 mM EDTA, 2 mM PMSF and 50 μg/ml leupeptin, to remove urea and allow NF reassembly. The mixture was made 1% Triton and centrifuged (13,000 × g, 10 min). The resulting pellet and supernatant were subjected to SDS-gel electrophoresis and autoradiographic analyses as described below.

The ability of Triton-soluble H-200 to co-assemble with purified NF-L was also examined. For these experiments, the above Triton-soluble fraction was further centrifuged at 100,000 × g for 1 h to insure sedimentation of any remaining NFs or oligomeric NF subunit assemblies [12,13]. The resulting clarified Triton-soluble supernatant was incubated with 1 mg each of purified dissociated NF-L and NF-M in 6 M urea (obtained from ICN Biochemicals, Costa Mesa, CA) and utilized as previously described [25], dialyzed overnight against 40 vols. of assembly buffer (10 mM phosphate buffer, pH 6.8, 1 mM EGTA, 0.5 mM ATP, 1 mM PMSF and 1 mM $MgCl_2$) [26], followed by a 1 h incubation at 37°C, then sedimented at 100,000 × g for 1 h. The resulting pellets and supernatants were subjected to SDS gel electrophoresis and immunoblot analysis.

2.6. Gel electrophoresis, autoradiography and immunoblotting

Samples received an equal volume of 2 × Laemmli treatment buffer, and were then boiled for 5 min and electrophoresed on 7% polyacrylamide SDS-gels. Gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose in a Hoefer Transphor apparatus. Replicas were immunostained as described with H3 or a polyclonal antiserum raised in this laboratory against NF-L [25]. Gels containing radiolabeled samples were stained with Coomassie brilliant blue, dried on a Hoefer slab gel dryer (Hoefer Scientific, San Francisco, CA) and autoradiographs were obtained with X-Omat AR-5 film.

3. Results and discussion

Immunoprecipitation and immunoblot analyses of NB2a/d1 cells demonstrate the presence of extensively

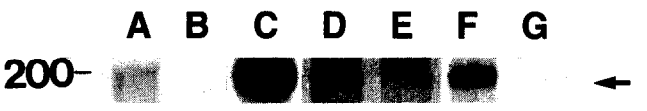

Fig. 1. Extensively phosphorylated NF-H exists in Triton-soluble and -insoluble fractions in NB2a/d1 cells. Immunostaining of a nitrocellulose replica of the high-speed supernatant derived from the Triton-soluble fraction of NB2a/d1 cells with a monoclonal antibody (SMI-31) directed against phosphorylated epitopes of NF-H demonstrates the presence of extensively phosphorylated NF-H in this fraction (lane A); dephosphorylation of the sample with alkaline phosphatase prior to electrophoresis eliminates this immunostaining (lane B). Immunoprecipitation with a polyclonal antiserum directed against extensively phosphorylated NF-H (lanes C–G) demonstrates the presence of extensively phosphorylated (200 kDa) NF-H in Triton-soluble and -insoluble fractions of NB2a/d1 cells. Lane C is an autoradiograph of material immunoprecipitated from the high-speed supernatant derived from the Triton-soluble fraction of ^{32}P-labeled NB2a/d1 cells. Lanes D–G are regions of Coomassie-stained gels, and lanes F and G depict the corresponding autoradiographs, of material immunoprecipitated from Triton-soluble and -insoluble fractions derived from NB2a/d1 cells following a brief (15 min) pulse-labeling with [^{35}S]methionine; Coomassie staining demonstrates the presence of extensively phosphorylated NF-H in Triton-soluble (D) and -insoluble (E) fractions, while the corresponding autoradiograph reveals the association of virtually all of the radiolabel with Triton-soluble NF-H. The 200 kDa region of all samples is indicated on the left of the figure, and the migratory position of NF-H is indicated on the right (arrow). In all samples, the supernatant derived from high-speed centrifugation of the Triton-soluble fraction is shown. Only the relevant region of gels is presented.

phosphorylated (200 kDa) NF-H in both the Triton-soluble and insoluble fractions of NB2a/d1 cells (Fig. 1) (see also [11,12]). High-speed centrifugation (100,000 × *g*, 1 h) sediments some but not all of these NF-H variants, indicating that while some of them exist in an oligomeric form, a significant percentage of these Triton-soluble extensively phosphorylated NF-H subunits are present as monomers (Fig. 2). That these Triton-soluble NF-H variants are phosphoproteins is confirmed by behaviors identical to that of extensively phosphorylated NF-H isolated from Triton-insoluble cytoskeletons, including their incorporation of [^{32}P]orthophosphate, their migration at 200 kDa (which is a consequence of extensive phosphorylation) [10,27], and the loss of immunoreactivity with phosphate-dependent monoclonal antibodies and antisera following treatment with alkaline phosphatase (Fig. 1). The association of [^{35}S]methionine radiolabel with Triton-soluble 200 kDa NF-H before Triton-insoluble subunits indicates that the soluble subunits represent newly synthesized protein rather than older subunits released by NF degradation (Fig. 1), and further indicate, by their migration at 200 kDa, that NF-H can undergo extensive phosphorylation without prior assembly into NFs (see also [12]).

We examined whether or not these Triton-soluble NF-H variants were assembly-competent by cell-free analyses using mouse CNS cytoskeletons and purified NF-L. Triton-insoluble cytoskeletons from mouse brain were solubilized in 6 M urea and mixed with the Triton-soluble fraction from dbcAMP-treated NB2a/d1 cells that had been radiolabeled with [^{35}S]methionine. Following overnight dialysis to remove urea, low-speed centrifugation (13,000 × *g*, 10 min) in the presence of 1% Triton reassembly and sedimentation of mouse CNS cytoskeletons (Fig. 3). The presence of radiolabel associated with NB2a/d1 proteins facilitated the selective monitoring of the distribution of NF-H from the Triton-soluble fraction of these cells. When the mixture was dialyzed overnight to remove urea and allow reassembly of mouse cytoskeletons, selective co-sedimentation of the NF-H Triton-soluble fraction of NB2a/d1 cells was observed (Fig. 2).

Our previous studies indicated that some of the Triton-soluble isoforms exist in oligomeric assemblies, and pulse-chase radiolabeling studies suggest that the bulk of radiolabeled NF subunits appear transiently in this high-speed sedimentable fraction before their ultimate arrival in the Triton-insoluble cytoskeleton [12]. Indeed, electron microscopic analyses of the Triton-soluble fraction and material sedimented from this fraction by high-speed centrifugation reveals the presence of NFs that react with SMI-31 (to be published). It is not unexpected that the assembly of subunits into initially Triton-soluble NFs would precede their incorporation into the Triton-insoluble cytoskeleton, since the Triton-insoluble cytoskeleton itself is only an operational definition, and this fraction is sedimentable following relatively low-speed centrifugation (i.e. 13,000 × *g*, 10 min) due to extensive cross-linking and interactions among is constituent polymers. However, it must be kept in mind that co-sedimentation of initially Triton-soluble subunits, be they in a monomeric form or assembled into (Triton-soluble) NFs, with an excess of Triton-insoluble cytoskeletons under cell-free conditions demonstrates only the otherwise undefined association of these Triton-soluble subunits with Triton-insoluble structures and does not con-

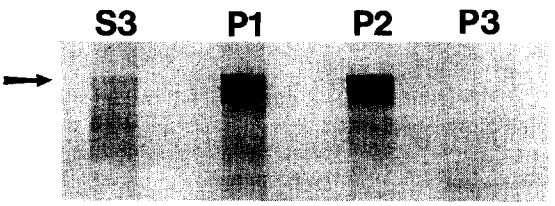

Fig. 2. Triton-soluble extensively phosphorylated NF-H exists in monomeric and oligomeric forms. Nitrocellulose replica of fractions derived from NB2a/d1 cells immunostained with H3 antiserum. Cells were homogenized in the presence of 1% Triton and subjected to low-speed centrifugation to sediment Triton-insoluble cytoskeletons (first pellet: 'P1'). The Triton-insoluble cytoskeleton contains significant levels of extensively phosphorylated NF-H (arrow indicates 200 kDa). The resulting Triton-soluble supernatant was subjected to two subsequent high-speed centrifugations (100,000 × *g*, 1 h each). Substantial levels of extensively phosphorylated NF-H was also recovered in the second ('P2') pellet, but none was observed in the third ('P3') pellet. The final supernatant resulting from the three centrifugations ('S3') contains extensively phosphorylated NF-H.

firm that they have assembled into NFs. Indeed, such initially Triton-soluble subunits may have bound to microtubules and/or cross-linked with Triton-insoluble NFs [28–30]. However, the incorporation of NF subunits into the Triton-insoluble cytoskeleton in situ is subject to these same caveats. Accordingly, the demonstration of the ability of Triton-soluble NB2a/d1 NF-H to co-sediment with mouse CNS Triton-insoluble cytoskeletons fails to demonstrate any inherent inability for them to do so in situ, and therefore implies that the incorporation of these subunits into Triton-insoluble structures is somehow regulated within intact NB2a/d1 cells. Such regulation may mediate the normal segregation of Triton-insoluble extensively phosphorylated NFs within the axonal cytoskeleton within NB2a/d1 cells [10] and CNS [9], and disruption of this regulation may lead to the rapid accumulation of phosphorylated NFs within perikaryal cytoskeletons following neurotoxin exposure [11,15–23].

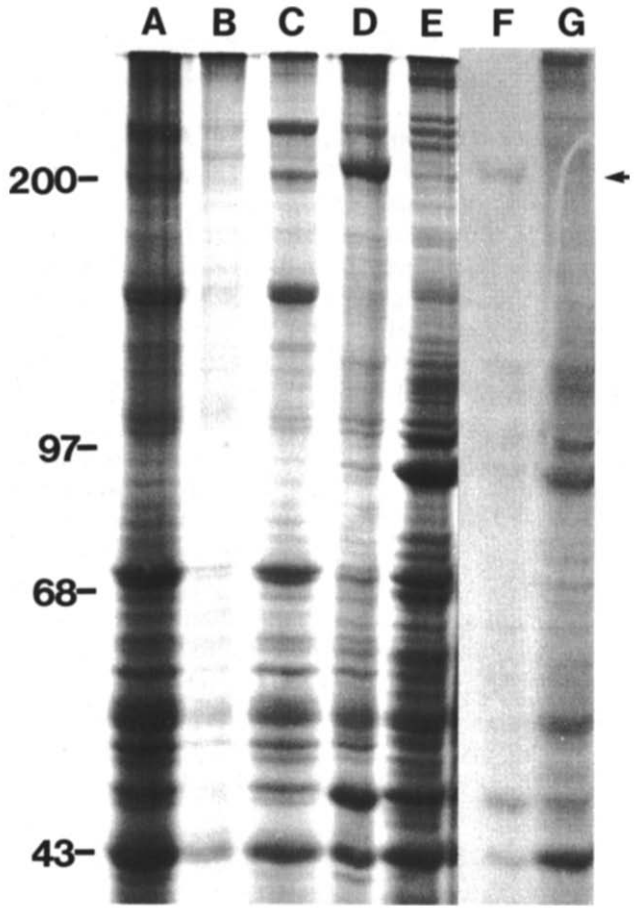

Fig. 3. Triton-soluble H-200 is capable of cell-free incorporation into Triton-insoluble structures. Lanes A–E are visualized by Coomassie staining. Lane A contains 100 μg of Triton-insoluble mouse CNS cytoskeleton. Neurofilament triplet proteins are apparent migrating at 200, 145 and 70 kDa, in addition to other cytoskeletal components. An equivalent amount of cytoskeletons was solubilized with 6 M urea and centrifuged (13,000 × g, 10 min), generating a low-speed pellet (B) and neurofilament-containing supernatant (C). This material was combined with 100 μg of Triton-soluble high-speed supernatant from [^{35}S]methionine-labeled NB2a/d1 cells and dialyzed to remove urea. The mixture was made 1% Triton and centrifuged (13,000 × g, 10 min). Lanes D and E depict the resulting pellet (D) and supernatant (E); note the reassembly of structures including neurofilament triplet proteins. Additional bands in D and E, not present in A–C, are derived from the NB2a/d1 soluble fraction. Lanes F and G depict autoradiographic analyses of material in D and E, in order to specifically monitor NB2a/d1 proteins. Note the selective pelleting of originally Triton-soluble H-200 from NB2a/d1.

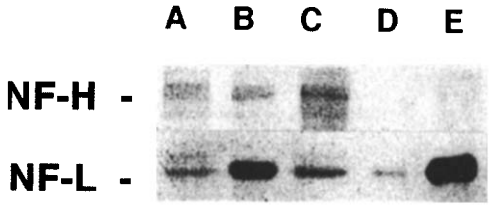

Fig. 4. Triton-soluble H-200 is capable of co-assembling with purified NF-L. Nitrocellulose replicas of the clarified Triton-soluble fraction from NB2a/d1 cells before and after mixing with purified NF-L. Both NF-H and NF-L are detected in the clarified Triton-soluble fraction from NB2a/d1 cells (lane A); this fraction also contains NF-M (not shown, see [12]). Purified NF-L, supplied by the manufacturer in 6 M urea, does not sediment (lane D), but remains in the supernatant (lane E). Following mixture with the clarified Triton-soluble fraction from NB2a/d1 cells (lane C) and overnight dialysis to remove urea, the majority of NF-L was sedimented (lane B). NF-H from NB2a/d1 cells was co-sedimented along with NF-L (e.g. compare lanes A and B).

We further addressed the assembly properties of Triton-soluble NF-H by sedimenting oligomeric assemblies by high-speed centrifugation (100,000 × g, 1 h), and then mixing the clarified Triton-soluble supernatant with urea-solubilized, purified NF-L. This mixture was then incubated under conditions that promoted NF assembly [26] and again subjected to high-speed centrifugation. We have previously demonstrated that repeated high-speed centrifugation of the Triton-soluble fraction of NB2a/d1 cells alone does not sediment additional NF-H (Fig. 2). However, when mixed with purified NF-L, the Triton-soluble NF-H from NB2a/d1 cells co-sedimented with NF-L following high-speed centrifugation (Fig. 4). These latter experiments demonstrate the ability of monomeric Triton-soluble NF-H to co-assemble into NFs.

These findings do not address the assembly competence of Triton-soluble extensively phosphorylated NF-H within NB2a/d1 cells; indeed, recent studies have indicated that NF assembly behavior within intact cells is quite distinct from that observed in cell-free analyses. While each of the NF-H subunits can form sedimentable structures under cell-free conditions [31,32], transfection and microinjection analyses demonstrate rapid incorporation of all 3 NF subunits into the endogenous intermediate filament (IF) network following their individual expression by transfection or microinjection [33–39]. However, transfection of each of the NF subunits into cells lacking an endogenous IF system demonstrates that NF subunits are obligate heteropolymers: NF-L cannot efficiently assemble without either NF-H or NF-M

[40,41]. Complex regulatory systems for NF assembly also exist in NB2a/d1 cells, as evidenced by the lack of significant co-localization of endogenous NF and vimentin filament networks [42] vs. the rapid co-localization of microinjected NF-H with vimentin filaments in these cells (Wang and Shea, to be published).

The presence of monomeric NF-H within the Triton-soluble fraction may be a reflection of insufficient NF-L to promote further NF assembly. This possibility is weakened by the observation of both NF-L and NF-M within the Triton-soluble fraction. However, NF-L within this fraction may be phosphorylated on the N-terminal assembly-regulatory sites [42] that are common to several IF types [43–49].

An additional possibility is that the presence of a sizeable pool of Triton-soluble NF-H in NB2a/d1 cells reflects over-expression of this subunit, which is indeed regulated independently from the other NF subunits [50]. This pool could also arise by insufficient degradation. That phosphorylation protects NFs against proteolysis [51,52] is consistent with the possibility that premature extensive phosphorylation could contribute to the aberrant accumulation of Triton-soluble NF-H. However, the results of the present study demonstrate that NB2a/d1 Triton-soluble NF-H variants are assembly-competent at least under cell-free conditions, and provide further evidence, suggested by in situ pulse-chase radiolabeling studies [12], that they may represent potential precursors for NF assembly. The physiological relevance of these subunits is further supported by the observation of significant levels of Triton-soluble extensively phosphorylated NF-H in the early postnatal mouse CNS and continuing at low levels into adult life [53,54]. The transient presence of large quantities of unassembled NF-H during development may, by incorporating into the existing NFs comprised of only NF-L and NF-M [3], facilitate the timely stabilization of axonal cytoskeleton, and its continued presence at lower levels may support continued exchange of NF-H subunits with existing NFs ([55]; for review, see [1]).

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