Published in final edited form as:

Acta Neuropathol. 2005 October ; 110(4): 360–368.

Neuronal intranuclear inclusions are ultrastructurally and immunologically distinct from cytoplasmic inclusions of neuronal intermediate filament inclusion disease

Sabrina Mosaheb and Julian R. Thorpe

Electron Microscope Division, Sussex Centre for Advanced Microscopy, School of Life Sciences, University of Sussex, Brighton, UK

Lida Hashemzadeh-Bonehi

Biochemistry Group, School of Life Sciences, University of Sussex, Brighton, UK

Eileen H. Bigio

Department of Neuropathology, Northwestern University Medical School, Chicago, Illinois, USA

Marla Gearing

Department of Pathology and Laboratory Medicine and Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, Georgia, USA

Nigel J. Cairns

Department of Pathology and Immunology, Washington University School of Medicine, Campus Box 8118, 660 South Euclid Avenue, St. Louis, MO 63110, USA E-mail: cairns@wustl.edu Tel.: +1-314-3627420 Fax: +1-314-3624096

Abstract

Abnormal neuronal cytoplasmic inclusions (NCIs) containing aggregates of α -internexin and the neurofilament (NF) subunits, NF-H, NF-M, and NF-L, are the signature lesions of neuronal intermediate filament (IF) inclusion disease (NIFID). The disease has a clinically heterogeneous phenotype, including fronto-temporal dementia, pyramidal and extrapyramidal signs presenting at a young age. NCIs are variably ubiquitinated and about half of cases also have neuronal intranuclear inclusions (NIIs), which are also ubiquitinated. NIIs have been described in polyglutamine-repeat expansion diseases, where they are strongly ubiquitin immunoreactive. The fine structure of NIIs of NIFID has not previously been described. Therefore, to determine the ultrastructure of NIIs, immunoelectron microscopy was undertaken on NIFID cases and normal aged control brains. Our results indicate that the NIIs of NIFID are strongly ubiquitin immunoreactive. However, unlike NCIs which contain ubiquitin, α -internexin and NF epitopes, NIIs contain neither epitopes of α -internexin nor NF subunits. Neither NIIs nor NCIs were recognised by antibodies to expanded polyglutamine repeats. The NII of NIFID lacks a limiting membrane and contains straight filaments of 20 nm mean width (range 11–35 nm), while NCIs contain filaments with a mean width of 10 nm (range 5–18 nm; *t*-test, *P*<0.001). Biochemistry revealed no differences in neuronal IF protein mobilities between NIFID and normal brain tissue. Therefore, NIIs of NIFID contain filaments morphologically and immunologically distinct from those of NCIs, and both types of inclusion lack expanded polyglutamine tracts of the triplet-repeat expansion diseases. These observations indicate that abnormal protein aggregation follows separate pathways in different neuronal compartments of NIFID.

Keywords

Neuronal intermediate filament inclusion disease; Ubiquitin; α -Internexin; Neurofilament; Frontotemporal dementia

Introduction

Neuronal intermediate filament (IF) inclusion disease (NIFID) has a clinically heterogeneous phenotype, including frontotemporal dementia, pyramidal and extrapyramidal signs presenting at a young age [1,4,12,17]. Macroscopically, there is atrophy of the frontal and anterior temporal lobes and the caudate nucleus is frequently affected. Microscopically, there are the stereotypical features of all frontotemporal lobar degenerations (FTLDs): neuronal loss, microvacuolation, and reactive gliosis in affected areas. Abnormal neuronal cytoplasmic aggregates of α -internexin and the three neurofilament (NF) subunits, NF-H, NF-M, and NF-L are the signature lesions of NIFID [1,4,6,7,12,13]. Neuronal cytoplasmic inclusions (NCIs) of NIFID are variably ubiquitinated and, in about half of cases, there are neuronal intranuclear protein inclusions (NIIs), which are also ubiquitinated [4,12,17]. The fine structure of NIIs of NIFID has not previously been described.

NIIs are not unique to NIFID as they have been described in several neurodegenerative diseases including: polyglutamine-repeat expansion diseases (Huntington's disease, spinocerebellar ataxias, and neuronal intranuclear inclusion disease), synucleinopathy (multiple system atrophy), frontotemporal lobar degeneration with motor neuron disease-type inclusions (FTLD-MND-type), and amyotrophic lateral sclerosis (ALS) with neuronal intranuclear protein hyaline inclusions. [16,19,21,22,23,27,28,29,30,33]. Nuclear inclusions are not specific to neurodegenerative diseases as Marinesco bodies and intranuclear rodlets are also found in the aging brain [18,34]. However, these inclusions have also been associated with age-related motor dysfunction [34].

NIIs are the signature lesions of the trinucleotide repeat expansion diseases and their significance has been reinforced by transgenic mouse models. These have shown that NIIs containing the pathological protein leads to neurodegeneration [23]. However, evidence suggests that NIIs may also play a protective role [9,14,25]. For example, a mouse model of spinocerebellar ataxia type 7, a CAG trinucleotide repeat disorder, develops nuclear inclusions which contain ubiquitinated abnormal ataxin protein [2,35]. This mouse model was also manipulated to express a marker protein which accumulated in neurons vulnerable to developing pathology. The presence of NIIs was inversely correlated with accumulation of the marker, suggesting that NII numbers are inversely correlated with neuronal degeneration. These results, along with those of other studies, suggest that aggregation of toxic protein, or protein fragments, in nuclear inclusions may reduce the soluble cellular pool of the harmful species, thereby protecting the cell [2,9,14,20,25]. Although it is unknown whether NIIs result from toxic or protective mechanisms, NIIs and NCIs are the signature lesions of NIFID [4]. Identification of the molecules which aggregate as NIIs or NCIs will help to elucidate the mechanisms of pathogenesis in NIFID and these mechanisms may also parallel pathogenesis in other neurodegenerative diseases with abnormal neuronal protein aggregates. Here we describe, for the first time, the ultrastructure of neuronal intranuclear inclusions of NIFID and compare both the structure and immunoelectron profile of their filaments with those of cytoplasmic inclusions.

Materials and methods

Brain tissue collection, processing, and neuropathological assessment

Fresh brain tissue was either rapidly frozen by contact with an aluminium plate on solid CO₂ or formalin-fixed and paraffin wax-embedded. Brain tissues were obtained from four clinically and neuropathologically well-characterised cases of NIFID from the United States (three cases) and the United Kingdom (one case) and four age-matched normal controls were obtained from the Centre for Neurodegenerative Disease Research, University of Pennsylvania, USA (Table 1). All of the disease cases displayed the pathological features previously described in NIFID

[1,4,12,17]. Tissue was removed according to the Institutional Review Board (IRB) guidelines and informed consent of the next-of-kin was obtained for brain autopsy. Frontal lobe tissue (middle/superior frontal gyrus) was used for analysis as this region contains relatively high numbers of neuronal cytoplasmic and nuclear inclusions and was available for both immunoelectron microscopic and biochemical studies. Frontal lobe tissue from a mouse expressing N171 huntingtin with 82 glutamine repeats [26] was kindly donated by Dr. D. Rubinsztein (University of Cambridge, UK), and used as a positive control for anti-polyglutamine repeat expansion antibodies.

Histology and immunohistochemistry

Tissue blocks were taken from the middle frontal gyrus. Histological stains included haematoxylin and eosin. Antigen retrieval was performed by heating sections in a solution of 0.5% ethylenediaminetetraacetic acid (EDTA) in 100 mmol/L TRIS, pH 7.6 at 100°C for 10 min. Immunohistochemistry (IHC) was undertaken on 6- to 10- μ m-thick sections prepared from formalin- (cases NIFID1, 3, 4) or 4% paraformaldehyde- (case NIFID2) fixed, paraffin wax-embedded tissue blocks using the avidin-biotin complex detection system (Vector Laboratories, Burlingame, CA) and the chromogen 3,3'-diaminobenzidine (DAB); sections were counter-stained with haematoxylin. Antibodies used included those that recognise ubiquitin, expanded polyglutamine repeats, and epitopes of class IV neuronal IF proteins including phosphorylation-dependent and non-phosphorylation-dependent anti-neuronal IF antibodies (Table 2). Anti-neuronal IF antibodies used in this study are well characterised and have been used previously to demonstrate epitopes of neuronal IFs in NIFID [4,6,7].

Transmission electron microscopy

Frozen brain tissue stored at -70°C was brought to -20°C . The grey matter was identified, dissected, and samples placed directly into a cold (4°C) solution of 4% formaldehyde and 0.1% glutaraldehyde (vacuum-distilled) in phosphate buffered saline (PBS). The following procedures were carried out at 4°C . After 18 h of fixation, the samples were rinsed thoroughly in PBS then dehydrated in an ethanol series and embedded in Unicryl resin (British BioCell International, Cardiff, UK) as previously described [32].

Serial thin sections were collected onto formvarcoated transmission electron microscopy (TEM) support grids and immunogold labelled using an established methodology [31]. Briefly, a modified PBS, pH 8.2, containing 1% BSA, 500 $\mu\text{l/l}$ Tween 20, 10 mM Na EDTA and 0.2 g/l NaN_3 (PBS+) was used for all dilutions of antibodies and secondary gold probes. All sections were blocked in normal goat serum (1:10 in PBS+) for 30 min at room temperature. The sections were then incubated overnight at 4°C with the antibody of interest (see Table 2) and in the case of the polyclonal antibodies, a concurrent protein-matched, non-immune rabbit serum control incubation. After rinsing (3×2 min) in PBS+, sections were incubated in the appropriate secondary antibody gold probe [10-nm gold particle-conjugated goat anti-mouse IgG (GaM10) or anti-rabbit IgG (GaR10) 1:10 in PBS+] for 1 h at room temperature. Sections were subsequently rinsed in PBS+ (3×10 min) and distilled water (4×5 min) and post-stained in 0.5% uranyl acetate for 90 min. For ultrastructural analysis, non-immunolabelled sections were collected onto bare TEM support grids and stained with uranyl acetate as above and with lead citrate (10 min). Thin sections were examined in a Hitachi 7100 TEM at 100 kV. Images were acquired digitally with an axially mounted Gatan Ultrascan 1000 CCD camera (Gatan, UK). Ultrastructural measurements were carried out using software provided with the camera and Student's *t*-test was used to compare mean fibril and granular dimensions.

Sequential biochemical fractionation

Grey and underlying white matter was dissected from the middle frontal gyrus and weighed; fractions were dissolved in detergents of increasing strength as previously described [7].

Briefly, tissue was homogenised in Hi-Salt (50 mM TRIS) buffer containing 10 mM EDTA, 5 mmol/l MgSO₄, 0.75 M NaCl, 0.02 M NaF, 0.5 mM PMSF, and a cocktail of protease inhibitors, and centrifuged at 25,000 g for 30 min at 4°C. Supernatants were saved as the HS fraction and pellets were washed by re-extraction in HS buffer. Resulting pellets were subjected to two sequential extractions in 10 ml/g Triton-X (TX) buffer containing Hi-Salt, 1% Triton X-100, and protease inhibitors and centrifuged as for the HS fraction. Supernatants were saved as the TX fraction. Pellets were homogenised in RIPA buffer containing 250 mM TRIS, 750 mM NaCl, 25 mM EDTA, 2.5% sodium deoxycholate, 0.5% sodium SDS and 5% NP40 adjusted to pH 8.0, and centrifuged and pelleted as above. The supernatants were saved as the RIPA fraction. Pellets were resuspended in 2% SDS in 50 mM TRIS with protease inhibitors, and centrifuged as above but at 15°C. Supernatants were preserved as the SDS fraction. Pellets were resuspended by sonication in 70% formic acid and centrifuged at 25,000 g for 1 h at 4°C. Myelin precipitate was removed prior to the samples being vacuum dried and saved as the FA fraction. Protein concentration was determined using the Coomassie protein assay (Pierce, Rockford, IL) and bovine serum albumin as a standard. SDS sample buffer (10 mM TRIS, pH 6.8, 1 mM EDTA, 40 mM dithiothreitol, 1% SDS, 10% sucrose) was added to samples of HS, TX, RIPA, and FA, and sample buffer without SDS (10 mM TRIS, pH 6.8, 1 mM EDTA, 40 mM dithiothreitol, 10% sucrose) was added to SDS-soluble samples, followed by heating at 100°C for 5 min.

Western blot analysis

Prior to electrophoresis, aliquots were mixed with an equal volume of 2× Laemmli sample buffer [15], boiled at 100°C for 10 min and equal amounts of protein were then separated by 7.5% SDS-PAGE. The proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membrane in buffer containing 25 mM TRIS, 191.8 mM glycine and 20% methanol. Membranes were blocked in PBS containing 3% powdered skimmed milk and 2% bovine serum albumin (BSA) for 1 h at room temperature and incubated in primary antibody overnight at 4°C. After six 10 min washes in PBS-Tween, the membranes were incubated for 2 h at room temperature in the appropriate secondary antibody conjugated to horseradish peroxidase (HRP). The membranes were then washed six times in PBS-Tween and immunolabelled proteins were visualised by enhanced chemiluminescence reagents using SuperSignal West-Pico substrate (Perbio Science Ltd., UK). Antibodies were used at the following dilutions: monoclonal mouse anti-glyceralde-hyde-3-phosphate dehydrogenase (anti-GAPDH; Abcam Ltd., Cambridge, UK) 1:10,000, SMI 31 1:10,000; SMI 32 1:5,000 and goat anti-mouse-HRP 1:5,000.

Results

NIIIs of NIFID are intensely ubiquitinated

In all of the four cases of NIFID, both NCIs and NIIs were readily detected in the frontal lobes by ubiquitin IHC (Fig. 1), but no ubiquitin-positive neuronal or glial inclusions were seen in any of the control cases. The density of cytoplasmic inclusions was generally greater in the superficial layers, the area of most severe neuronal loss, as was the density of NIIs. The ratio of NIIs to NCIs varied considerably between cases from about 1:50 to less than 1:300. Generally, the NIIs were more intensely labelled by anti-ubiquitin antibodies than were the cytoplasmic inclusions. Previously, we have shown that some, generally compact, NCIs are only weakly or unstained by ubiquitin IHC [1,6]. With ubiquitin IHC, the NIIs appeared round, sometimes elongated, occasionally occupying more than 50% of the nuclear volume. Single or multiple inclusions occupied a single nucleus, as has been previously described [4], and NIIs could be found in isolation or in combination with cytoplasmic inclusions.

NIIIs of NIFID are filamentous and morphologically distinct from cytoplasmic inclusions

NCIs, being more numerous than NIIs, were readily identified by TEM. Generally, two types of cytoplasmic inclusion could be identified: tightly arrayed filaments of a compact cytoplasmic inclusion (CCI) and loosely aggregated filaments of a cytoplasmic inclusion (LACI), the latter was previously described as a Pick body-like inclusion, the most common morphological type in NIFID [4,12,17]. NIIs varied in size and shape and sometimes filled almost the entire nucleus. As with light microscopy, both round and elongated inclusions were observed. At low magnification, the NIIs could be distinguished by an apparent disruption in the usually uniform nuclear appearance (Fig. 2A). High magnification revealed that NIIs contained both granular and filamentous material (Fig. 2B), the filaments being the predominant component; there was no limiting membrane. The mean diameter of the granules was 32 nm (range 13–52 nm), significantly wider than the average filament width of 20 nm (range 11–35 nm; *t*-test, $P < 0.001$), suggesting that granules are distinct from filaments and not simply filaments in cross-section. The filaments of the NIIs were randomly oriented and appeared to be shorter than those of CCIs. However, in thin sections, filament curvature and/or orientation may spuriously reduce apparent length.

By TEM, CCIs, corresponding to hyaline conglomerate inclusions by light microscopy, contained dense arrays of tightly packed neuronal IFs (Fig. 2C, D) while LACIs appeared as loosely aggregated filaments decorated by granular material (Fig. 2E, F). These two types of NCIs have previously been reported [4] and may represent different stages in the evolution of cytoplasmic neuronal IF inclusions in NIFID. Both CCIs and LACIs lacked limiting membranes. CCIs are composed of filaments with a mean diameter of 9.9 nm (range 5–18 nm), significantly thinner than the average NII filament width of 20 nm (*t*-test, $P < 0.001$). Unlike the apparently random orientation of filaments of the NIIs and LACIs, those within CCIs tended to be similarly oriented and often ran parallel to each other in swirling patterns (Fig. 2D).

Filaments of NIIs contain ubiquitin epitopes, but lack neuronal IF and expanded polyglutamine repeat epitopes

Previously, we have shown that the NCIs of NIFID contain epitopes of class IV neuronal IF proteins and are ubiquitinated to varying degrees [4,6,7]. To further characterise inclusion content, we have used immunogold labelling TEM; a semi-quantitative assessment of the immunolabelling density of inclusions by each antibody is summarised in Table 3. The most striking labelling of the NIIs was observed with antibodies to ubiquitin epitopes. Although NIIs, CCIs and LACIs were ubiquitin positive (Fig. 3A–C), the NIIs were the most robustly labelled. A concurrent control incubation in protein-matched non-immune rabbit serum exhibited minimal labelling (data not shown). These observations confirm our previous IHC studies with the light microscope [4,6,7]. Unlike the filaments of CCIs, the filaments of NIIs were not labelled by either antibodies to α -internexin or antibodies to phosphorylated NF epitopes (Fig. 3D, G). Conversely, filaments of CCIs were heavily labelled by neuronal IF protein antibodies (Fig. 3E, H). Filaments of LACIs contained only negligible quantities of α -internexin epitopes and were weakly labelled with phosphorylated NF antibodies (Fig. 3F, I). Neither the NIIs nor the CCIs were labelled by anti-non-phosphorylated NF antibodies; however, very low levels of non-phosphorylated NF epitopes were detected in LACIs (data not shown). Anti-polyglutamine repeat epitopes of NIIs were clearly labelled by the 1C2 antibody in tissue from the frontal lobe from a mouse expressing N171 huntingtin with 82 glutamine repeats (data not shown). As with previous studies using IHC with the light microscope [4,6,7], none of the inclusion types (NII, CCI or LACI) contained filaments that were labelled by the 1C2 antibody, indicating that expanded polyglutamine repeat epitopes are absent from the pathological inclusions of these cases of NIFID.

NF proteins of NIFID have the same electrophoretic mobility as those of normal brain

In neurodegenerative diseases, misfolded proteins in neuronal or glial inclusions are characteristically post-translationally modified. To determine whether neuronal IF proteins in the inclusions were insoluble, sequential extraction of proteins in buffers of increasing protein solubilization strengths were performed on samples from diseased brains. Western blot analysis of fractions from NIFID frontal cortex revealed the presence of NFs and α -internexin in the SDS-soluble fraction, but not in more soluble fractions (data not shown). Thus, unlike pathological aggregates of insoluble tau in Alzheimer's disease (AD), and α -synuclein in Parkinson's disease (PD), the inclusions of NIFID were comparatively soluble, and this difference in solubility may account for the absence of extracellular neuronal IF aggregates following cell death in this disease.

Previously, we have shown that α -internexin, although a major component of the NCIs of NIFID, does not appear to be modified, as detected by Western blots [7]. Here we have investigated the mobility of NF proteins in the four cases of NIFID examined here. The relative mobilities of phosphorylated NF proteins and protein fragments, that were recognised by the SMI 31 antibody, were similar in both NIFID and age-matched normal brain tissue (Fig. 4). A low molecular mass band (approximately 40 kDa) was detected only after long exposure (1 h) in the NIFID samples and this most likely is a breakdown product of NF-L. The mobilities of non-phosphorylated NF proteins (as detected by SMI 32 antibody) in NIFID were indistinguishable from the controls (data not shown).

Discussion

We describe here, for the first time, the ultrastructure and immunoelectron microscopic profile of NIIs of NIFID. NIIs and NCIs were found in the frontal lobes of all four cases examined, but in none of the controls. Previous studies have indicated that the NCIs of NIFID are disease specific and not a by-product of normal ageing [4,6,7]. Nuclear inclusions are of variable shape, usually round, and often occupy the larger part of the nucleus. They are typically much larger than the nucleolus, which is often seen in an eccentric position within the nucleus. Single or multiple forms may occupy a single nucleus, and NIIs may be found in isolation or in combination with a cytoplasmic inclusion. NIIs are found, typically, at much lower densities than cytoplasmic inclusions. The average filament width of an NII is 20 nm (range 11–35 nm), significantly broader than those of the CCI (9.9 ± 0.35 nm; *t*-test, $P < 0.001$), indicating that NII filaments are morphologically distinct from those of the CCIs.

Like CCIs and LACIs, the NIIs of NIFID are ubiquitinated, but the intensity of staining, as seen by IHC, is usually much more robust in the NIIs. Previously, we and others have shown that neuronal IF protein epitopes are found within NCIs of NIFID [1,4,6,7,12,13,17]. Our IHC studies [3,4,6,7] and the immunogold labelling TEM described here failed to detect epitopes to neuronal IF proteins within the filaments of NIIs of the frontal lobe. In one study, a small subset of NIIs in the granule cells of the dentate fascia, containing epitopes of NFs has been reported, but no NF epitopes were seen in NIIs of the Purkinje cells of the cerebellum, indicating that NIIs of NIFID are neuropathologically heterogeneous [12]. Thus, in addition to being structurally distinct, the NIIs are also immunologically distinct from the cytoplasmic inclusions of NIFID. This variable immunolabelling profile of inclusions in different cellular compartments is an enigmatic feature of this disease.

NIIs are not unique to NIFID as they have been described in polyglutamine-repeat expansion diseases, including Huntington's disease, spinocerebellar ataxias, and neuronal intranuclear inclusion disease (NIID) [16,19,29]. The association between NIIs and this group of diseases has led to the hypothesis that they are a common neuropathological feature of triplet-repeat disorders with polyglutamine-repeat expansions [10]. As in NIFID, the NIIs of triplet-repeat

disorders are ubiquitinated, but those of triplet-repeat disorders also contain aggregates of pathological protein with a polyglutamine-repeat expansion [11]. In a small subset of NIIs in one study, epitopes of polyglutamine tracts have been reported in the Purkinje cells of the cerebellum, indicating that NIIs may be neuropathologically heterogeneous [12]. Using an anti-polyglutamine expansion antibody (1C2), we were unable to detect polyglutamine expansion tracts in NIIs or NCIs (CCIs and LACIs) in the frontal lobes by either IHC or immunoelectron microscopy. Recently, NIIs in a case of ALS with NIIs were reported to be ubiquitinated and 1C2 negative, but labelled with anti-ataxin 3 (A3C-1) antibody, and contained 10- to 12-nm-diameter filaments reminiscent of polyglutamine intranuclear hyaline inclusions [27]. In contrast, in NIFID, filaments of NIIs were 20 nm in diameter, but lacked neuronal IF epitopes. Intranuclear inclusions have also been reported in another FTLN, i.e. FTLN-MND-type [33], and the NIIs of NIFID most closely resemble the morphology of those of FTLN-MND-type. Neither in NIFID nor in FTLN-MND-type (data not shown) were polyglutamine expansion epitopes present within the NIIs or NCIs. Thus, the distribution, morphology, and immunological profile of NIIs of NIFID are distinct from those of the polyglutamine repeat expansion diseases and ALS, but resemble the immunohistochemical and morphological appearance of NIIs in FTLN-MND-type.

The role played by NIIs in neuronal degeneration is not fully understood. NIIs may result from a cytoprotective mechanism or they may be deleterious to neuronal function [2,9,14,16,19, 23,35]. The formation of NIIs in polyglutamine repeat diseases is thought to result from proteins containing polyglutamine tract expansions of more than approximately 40 glutamine residues becoming destabilised and substrates for proteolysis. They are then proteolytically cleaved in the cytoplasm and the fragments are transported to the nucleus, where they become ubiquitinated and targeted for proteasomal degradation [22,23,24]. These protein fragments may be more toxic and more prone to aggregation than the full-length protein [24]. NIIs may cause neuronal dysfunction or cell death by several mechanisms. For example, NIIs may impair transcription and mRNA processing [24]. The ubiquitinated NIIs of some polyglutamine repeat disorders recruit the 20S proteasome [8,22,24], which may reduce the ability of the proteasome to turnover short-lived proteins. The consequential build-up of these proteins, which are normally efficiently cleared by the proteasome, may be detrimental to the cell. Additionally, the slow aggregation of misfolded and associated chaperone proteins is likely to obstruct intranuclear, cytoplasmic, nuclear-cytoplasmic and axonal transport of proteins essential for maintenance of normal neuronal function.

Recently, we described α -internexin as a major component of the NCIs, which are also immunopositive for NFs, the pathological hallmark of NIFID [4,6,7]. To determine whether the proteins identified within NIFID inclusions are post-translationally modified, a characteristic feature of pathological proteins in neurodegenerative diseases [5], we used biochemical methods to investigate the mobility of soluble neuronal IF proteins. We have shown previously that the mobility of soluble α -internexin from frozen brain homogenate, as detected by Western blot, is indistinguishable between cases of NIFID and age-matched controls [7]. This observation suggests that α -internexin is not post-translationally modified. Western blotting data in this study confirm that the mobilities of both phosphorylated and nonphosphorylated NFs are similar in disease and age-matched controls. Taken together these results indicate that neuronal IF proteins are not abnormally post-translationally modified in NIFID. Nonetheless, the presence of aggregates of α -internexin and hyperphosphorylated NFs within the cytoplasm is abnormal and likely to impair normal cellular function.

Although α -internexin is a major component of the NCIs of NIFID, it is not specific because it is also a minor component of the pathological inclusions of other neurodegenerative diseases [6]. Abnormal perikaryal accumulations of NF proteins also occur in AD, PD and MND [5]. However, NFs may be chaperone proteins and not the primary pathological protein in most of

these disorders. It is possible that neuronal IF proteins are also chaperone proteins in NIFID and that aggregates of these proteins may be protective against the upstream effects of an, as yet unknown, pathological protein

Cytoplasmic aggregates of NFs in AD, PD, and MND may occur in response to progressive neuronal dysfunction as a result of several mechanisms: impaired axonal transport, post-translational modification of protein (e.g. tau in AD, α -synuclein in PD), environmental toxins, and stress. Cytoplasmic NF aggregates may provide neuroprotection by acting as a phosphorylation sink. In this way, NFs might absorb the effects of deregulated phosphorylation events and, in doing so, minimise the phosphorylation of those proteins rendered toxic by inappropriate post-translational modification [20]. It is possible that neuronal IF protein accumulation in NIFID results from some toxic insult, but no evidence exists for any exposure to environmental toxins [4].

The presence of NIIs in NIFID that are ubiquitinated, but do not contain neuronal IF protein epitopes, indicates that NIIs form independently from cytoplasmic inclusions and are morphologically and immunologically distinct. If accumulations of neuronal IF proteins in NIFID are secondary to the effects of some other toxic molecule, or event, the NIIs of NIFID may occur prior to, independently of, or in combination with, the cytoplasmic inclusions. Further investigations are now underway to determine other molecules within NIIs and NCIs of this disease. The identification of novel ubiquitinated molecules within the inclusions will throw new light on the pathogenesis of NIFID.

Acknowledgements

We thank the staff of the Alzheimer's Disease Research Center Neuropathology Laboratory, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, USA, for technical support; Dr. David Rubinsztein, University of Cambridge, UK, for provision of transgenic mouse brain tissue; Drs Evelyn Jaros and Robert H. Perry, Newcastle General Hospital, Newcastle-upon-Tyne, UK, for human brain tissue, and the families of patients whose generosity made this research possible. Support for this work was provided by grants from the Wellcome Trust, UK, (GR066166AIA) to J.R.T. and N.J.C., and the National Institute on Aging of the National Institutes of Health (P50 AG05681 and P01 AG03991 to N.J.C. and ES12068 to M.G.). These data were presented as a preliminary report at the Annual Meeting of the American Association of Neuropathologists, 2005.

References

1. Bigio EH, Lipton AM, White CL, Dickson DW, Hirano A. Frontotemporal and motor neurone degeneration with neurofilament inclusion bodies: additional evidence for overlap between FTD and ALS. *Neuropathol Appl Neurobiol* 2003;29:239–253. [PubMed: 12787321]
2. Bowman AB, Yoo S-Y, Dantuma NP, Zoghbi HY. Neuronal dysfunction in a polyglutamine disease model occurs in the absence of ubiquitin-proteasome system impairment and inversely correlates with the degree of nuclear inclusion formation. *Hum Mol Genet* 2005;14:679–691. [PubMed: 15661755]
3. Cairns NJ, Armstrong RA. Spatial correlations between the neuronal inclusions, swollen achromatic neurons, and glial cells in neuronal intermediate filament inclusion disease (NIFID). *J Neural Transm* 2005;112:473–480. [PubMed: 15375676]
4. Cairns NJ, Grossman M, Arnold SE, Burn DJ, Jaros E, Perry RH, Duyckaerts C, Stankoff B, Pillon B, Skullerud K, et al. Clinical and neuropathologic variation in neuronal intermediate filament inclusion disease. *Neurology* 2004;63:1376–1384. [PubMed: 15505152]
5. Cairns NJ, Lee VM, Trojanowski JQ. The cytoskeleton in neurodegenerative diseases. *J Pathol* 2004;204:438–449. [PubMed: 15495240]
6. Cairns NJ, Uryu K, Bigio EH, Mackenzie IR, Gearing M, Duyckaerts C, Yokoo H, Nakazato Y, Jaros E, Perry RH, et al. α -Internexin aggregates are abundant in neuronal intermediate filament inclusion disease (NIFID) but rare in other neurodegenerative diseases. *Acta Neuropathol* 2004;108:213–223. [PubMed: 15170578]

7. Cairns NJ, Zhukareva V, Uryu K, Zhang B, Bigio E, Mackenzie IRA, Gearing M, Duyckaerts C, Yokoo H, Nakazato Y, et al. α -Internexin is present in the pathological inclusions of neuronal intermediate filament inclusion disease. *Am J Pathol* 2004;164:2153–2161. [PubMed: 15161649]
8. Cummings CJ, Mancini MA, Antalfy B, DeFranco DB, Orr HT, Zoghbi HY. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat Genet* 1998;19:148–154. [PubMed: 9620770]
9. Cummings CJ, Reinstein E, Sun YL, Antalfy B, Jiang YH, Ciechanover A, Orr HT, Beaudet AL, Zoghbi HY. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron* 1999;24:879–892. [PubMed: 10624951]
10. Davies SW, Beardsall K, Turmaine M, DiFiglia M, Aronin N, Bates GP. Are neuronal intranuclear inclusions the common neuropathology of triplet-repeat disorders with polyglutamine-repeat expansions? *Lancet* 1998;351:131–133. [PubMed: 9439509]
11. DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, Aronin N. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* 1997;277:1990–1993. [PubMed: 9302293]
12. Josephs KA, Holton JL, Rossor MN, Braendgaard H, Ozawa T, Fox NC, Petersen RC, Pearl GS, Ganguly M, Rosa P, et al. Neurofilament inclusion body disease: a new proteinopathy? *Brain* 2003;126:2291–2303. [PubMed: 12876145]
13. Josephs KA, Uchikado H, McComb RD, Bashir R, Wszolek Z, Swanson J, Matsumoto J, Shaw G, Dickson DW. Extending the clinicopathological spectrum of neurofilament inclusion disease. *Acta Neuropathol* 2005;109:427–432. [PubMed: 15754170]
14. Klement IA, Skinner PJ, Kaytor MD, Yi H, Hersch SM, Clark HB, Zoghbi HY, Orr HT. Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell* 1998;95:41–53. [PubMed: 9778246]
15. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
16. Liebermann AP, Robitaille Y, Trojanowski JQ, Dickson DW, Fischbeck KH. Polyglutamine-containing aggregates in neuronal intranuclear inclusion disease. *Lancet* 1998;351:884. [PubMed: 9525376]
17. Mackenzie IRA, Feldman H. Neurofilament inclusion body disease with early onset frontotemporal dementia and primary lateral sclerosis. *Clin Neuropathol* 2004;23:183–193. [PubMed: 15328884]
18. Marinesco G. Sur la présence des corpuscles acidophiles paranucleolaires dans la cellule du locus coeruleus. *Compt Rend Acad Sci* 1902;135:1000–1002.
19. Michalik A, Van Broeckhoven C. Pathogenesis of polyglutamine disorders: aggregation revisited. *Hum Mol Genet* 2003;12:R173–R186. [PubMed: 14504263]
20. Nguyen MD, Larivière R, Julien JP. Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. *Neuron* 2001;30:134–147.
21. Nishie M, Mori F, Yoshimoto M, Takahashi H, Wakabayashi K. A quantitative investigation of neuronal cytoplasmic and intranuclear inclusions in the pontine and inferior olivary nuclei in multiple system atrophy. *Neuropathol Appl Neurobiol* 2004;30:546–554. [PubMed: 15488031]
22. Perutz MF. Glutamine repeats and neurodegenerative diseases: molecular aspects. *Trends Biochem Sci* 1999;24:58–63. [PubMed: 10098399]
23. Ross CA. Intranuclear neuronal inclusions: a common pathogenic mechanism for glutamine-repeat neurodegenerative diseases? *Neuron* 1997;19:1147–1150. [PubMed: 9427237]
24. Rubinsztein DC, Wytenbach A, Rankin J. Intracellular inclusions, pathological markers in diseases caused by expanded polyglutamine tracts? *J Med Genet* 1999;36:265–270. [PubMed: 10227391]
25. Saudou F, Finkbeiner S, Devys D, Greenberg ME. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 1998;95:55–66. [PubMed: 9778247]
26. Schilling G, Becher MW, Sharp AH, Jinnah HA, Duan K, Kotzuk JA, Slunt HH, Ratovitski T, Cooper JK, Jenkins NA, et al. Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum Mol Genet* 1999;8:397–407. [PubMed: 9949199]

27. Seilhean D, Takahashi J, El Hachimi KH, Fujigasaki H, Lebre AS, Biancalana V, Durr A, Salachas F, Hogenhuis J, H de, et al. Amyotrophic lateral sclerosis with neuronal intranuclear protein inclusions. *Acta Neuropathol* 2004;108:81–87. [PubMed: 15114487]
28. Spillantini MG, Crowther RA, Jakes R, Cairns NJ, Lantos PL, Goedert M. Filamentous α -synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neurosci Lett* 1998;251:205–208. [PubMed: 9726379]
29. Takahashi J, Fukuda T, Tanaka J, Minamitani M, Fujigasaki H, Uchihara T. Neuronal intranuclear hyaline inclusion disease with polyglutamine-immunoreactive inclusions. *Acta Neuropathol* 2000;99:589–594. [PubMed: 10805106]
30. Takahashi J, Tanaka J, Arai K, Funata N, Hattori N, Fukuda T, Fujigasaki H, Uchihara T. Recruitment of nonexpanded polyglutamine proteins to intranuclear aggregates in neuronal intranuclear hyaline inclusion disease. *J Neuropathol Exp Neurol* 2001;60:369–376. [PubMed: 11305872]
31. Thorpe J. The application of LR gold resin for immunogold labelling. *Methods Mol Biol* 1999;117:99–110. [PubMed: 10327401]
32. Thorpe JR, Rulten S, Kay JE. Binding of a putative and a known chaperone protein revealed by immunolabelling transmission electron microscopy: a suggested use of chaperones as probes for the distribution of their target proteins. *J Histochem Cytochem* 1999;47:1633–1640. [PubMed: 10567447]
33. Woulfe J, Kertesz A, Munoz DG. Frontotemporal dementia with ubiquitinated cytoplasmic and intranuclear inclusions. *Acta Neuropathol* 2001;102:94–102. [PubMed: 11547957]
34. Woulfe J, Gray D, Prichett-Pejic W, Munoz DG, Chretien M. Intranuclear rodlets in the substantia nigra: interactions with Marinesco bodies, ubiquitin, and promyelocytic leukemia protein. *J Neuropathol Exp Neurol* 2004;63:1200–1207. [PubMed: 15581187]
35. Yoo SY, Pennesi ME, Weeber EJ, Xu B, Atkinson R, Chen S, Armstrong DL, Wu SM, Sweatt JD, Zoghbi HY. SCA7 knockin mice model human SCA7 and reveal gradual accumulation of mutant ataxin-7 in neurons and abnormalities in short-term plasticity. *Neuron* 2003;37:383–401. [PubMed: 12575948]

Fig 1.

A, B NCIs and NIIs in the frontal lobe of NIFID. **A** Numerous pleomorphic NCIs and a neuron containing both cytoplasmic and intranuclear inclusions (*asterisk*). **B** A high-power micrograph of the neuron marked with an *asterisk* in **A**. The nucleolus is in an eccentric position and the inclusion is round and occupies a large fraction of the nucleus and is more intensely stained than the cytoplasmic inclusion (*NII* neuronal intranuclear inclusion, *NCI* neuronal cytoplasmic inclusion, *NIFID* neuronal intermediate filament inclusion disease). Ubiquitin immunohistochemistry; bars **A** 50 μ m; **B** 10 μ m

Fig 2.

A–F Transmission electron micrographs of NIIs and NCIs of NIFID. **A** Low-magnification image of an NII (*asterisk* inclusion; *n* nucleolus). **B** Ultrastructure of filaments and granules (*arrows*) of the NII. **C** Low-magnification image of a CCI (*asterisk* inclusion; *n* nucleolus). **D** Filaments of a CCI. **E** Low-magnification image of an LACI (*asterisk* inclusion; *n* nucleolus). **F** LACI contains filaments and granular material (*arrows*) (CCI compact cytoplasmic inclusion, LACI loosely aggregated cytoplasmic inclusion). Bars **A, C, E** 2 μ m; **B, D, F** 100 nm

Fig 3.

A–I Immunoelectron micrographs of filaments within NIIs and NCIs. **A–C** Both NIIs and NCIs contain filaments with ubiquitin epitopes. **A** Intense labelling of filaments of an NII with anti-ubiquitin antibodies. **B, C** Ubiquitin epitopes are also present within filaments of a CCI (**B**) and an LACI (**C**). **D** NIIs are not labelled by α -internexin antibodies. **E** In contrast to NIIs, CCIs are intensely α -internexin immunopositive. **F** LACIs are sparsely labelled by α -internexin antibodies. **G** NII filaments are not labelled above background levels by antibodies recognising phosphorylated NF epitopes (SMI 31). **H** In contrast, filaments within CCIs are heavily labelled by SMI 31. **I** LACIs are weakly immunopositive positive with anti-phosphorylated NF antibodies (*NF* neurofilament). Bars 100 nm

Fig 4.

Biochemistry of soluble NF proteins of NIFID and normal aging. Soluble NF proteins from brain homogenates of NIFID (NIFID 1–4) and normal brain (NL 1–4) have the same electrophoretic mobility in 7.5% polyacrylamide gel and visualized by Western blot analysis with anti-phosphorylated NF antibodies (SMI 31). The *middle panel* shows short exposure of film and the *lowest panel* long exposure (1 h) so that the full range of proteins and degradation products are visualised. Brain homogenates were loaded on the gel with equal protein concentration as demonstrated by the ubiquitously expressed protein, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (*upper panel*)

Table 1

Summary of demographic information of cases (*NL* neuropathologically normal, *NIFID* neuronal intermediate filament inclusion disease, *PMI* postmortem interval)

Case	Gender	Age at onset (years)	Disease duration (years)	Age at death (years)	Brain weight (g)	PMI (h)	Reference
NIFID 1	F	41	3	41	904	15	[4]
NIFID 2	M	48	4	52	1,310	24	[4]
NIFID 3	F	52	2.7	54	813	24	[4]
NIFID 4	M	56	4	60	1,250	24	[1,4]
NL 1	F	-	-	67	1,100	5.5	-
NL 2	M	-	-	60	1,500	14	-
NL 3	F	-	-	36	1,280	6	-
NL 4	M	-	-	65	1,346	26	-

Table 2

Characteristics of the antibodies used in this study (*IHC* immunohistochemistry, *IEM* immunoelectron microscopy)

Antigen	Poly/ monoclonal	Source	Clone	Dilution (IHC) [reference]	Dilution (IEM)
α -Internexin	Monoclonal	Cambridge Bioscience, UK	2E3	1:400 [4]	1:250
Phosphorylated neurofilament-H	Monoclonal	Sternberger Monoclonals Inc., USA	SMI 31	1:1,000 [4]	1:100
Non-phosphorylated neurofilament-H	Monoclonal	Sternberger Monoclonals Inc., USA	SMI 32	1:1,000 [4]	1:100
Polyglutamine	Monoclonal	Chemicon International Inc., USA	1C2	1:1,000 [4]	1:1,000
Ubiquitin	Polyclonal	DakoCytomation, Denmark	-	1:1,000	1:8

Table 3

Immunoelectron microscopic profile of NIIs and NCIs of NIFID. Semi-quantitative grading of immunolabelling: – unlabelled, +/- negligible, + mild, ++ moderate, +++ heavy, ++++ robust (*NII* neuronal intranuclear inclusion, *NCI* neuronal cytoplasmic inclusion, *CCI* compact cytoplasmic inclusion, *LACI* loosely aggregated cytoplasmic inclusion)

Antigen	NII	CCI	LACI
Phosphorylated neurofilament-H	–	+++	+
Non-phosphorylated neurofilament-H	–	–	+
α -Internexin	–	++++	+/-
Polyglutamine	–	–	–
Ubiquitin	++++	++	+++