Differential subcellular localization of phosphorylated neurofilament and tau proteins in degenerating neurons of the human entorhinal cortex

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A panel of novel monoclonal antibodies was tested on the human entorhinal cortex for the recognition of age- and disease-related changes of neurofilament proteins (NF). Several antibodies identified phosphorylated NF-H subunit, which occurred preferentially in an those aged between 60 and 80 years and were localized in degenerating neurons. Such neurons also contained neurofibrillary tangles, but neurofilament aggregates did not co-localize with tangles, nor did the quantity nor the number of NF-positive neurons correlate with the severity of Alzheimer's disease. This points to a susceptibility of NF in a subset of neurons for phosphorylation- and metabolically related morphological changes during neurodegeneration. NeuroReport 14:000–000 © 2003 Lippincott Williams & Wilkins.

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INTRODUCTION

In neurons, the major cytoskeletal elements are neurofilaments (NF), which are part of class IV intermediate filaments. They are composed of the triplet proteins of high, medium and low mol. wt, NF-H, NF-M and NF-L respectively. They form the structural backbone of neurons by polymerizing into filaments and interact with other filaments and organelles [1]. Post-translational modifications, such as phosphorylation, are known to modulate their functions and aberrant modifications may occur in neurodegenerative diseases [2,3]. Recently, great emphasis has been placed on the pathology of a microtubule-associated protein tau and its phosphorylation. Tau proteins are components of paired helical filaments (PHF) which in turn indicate structural changes of the cytoskeleton and which are used to evaluate the extent of Alzheimer's disease (AD) [4–6]. Other structural and metabolic proteins are likely to undergo similar age- and disease-related post-translational changes. Neurofilament proteins have long been found implicated in AD pathology [7]. A monoclonal antibody was described that detects an age-related phosphorylation epitope in NF, but the biochemistry of such phosphorylation remains poorly understood [8]. Amyotrophic lateral sclerosis is an age-related neurological disease characterized by neurofilament accumulation in axons, which is followed by degeneration of motor neurons [9]. Monoclonal antibodies against phosphorylation sites of abundant structural proteins, among them neurofilaments, may therefore provide valuable tools to analyze age- and disease-related changes in the structural organization of neuronal and glial cells [4].

Here we have set out to characterize a panel of novel monoclonal antibodies against neurofilaments and to test whether they can be used to identify early events in neurodegeneration. We have chosen to perform our study on the human entorhinal cortex (EC). This brain area constitutes an ideal tissue for investigating incipient changes of the Alzheimer's type [10].

MATERIALS AND METHODS

Tissues: Parts of the human EC were removed at autopsy and either immediately frozen at −80°C for biochemistry or immediately fixed with 4% paraformaldehyde for 48 h, rinsed with PBS, and kept at 4°C until immunohistochemistry. For the comparative study autopsy human brain tissues were obtained from six controls without known

cognitive deficits and Braak stages between I and III and five demented cases with histologically verified AD, Braak stages IV–VI [10] (details in figure legends). For the initial screening of the novel NF antibodies, we used human brain tissue (temporal lobe) obtained from a 68-year-old male with mild AD (Braak stage III) and a post-mortem delay of 5 h. The tissue used for the characterization of antibodies was used either in its native form or after dephosphorylation with alkaline phosphatase [11].

Antibodies: A novel series of antibodies against intermediate filaments were generated. A Balb/c mouse was immunized with a pellet of pig brain cold-stable proteins after depolymerization of microtubules [12] and this material was used for initial screening. The immunization protocol, the technique for the fusion of spleen cells with mouse myeloma cells Sp2/0, the screening, cloning and subcloning methods and ascites production have been described previously [13–15]. The antibodies have been screened for their reactivity with purified bovine neurofilament proteins and were tested for their cross-reactivity with neurofilaments of other species such as rat and cat (unpublished data). Preliminary data with several of these antibodies showing identification of only a subset of axons in cat brain has been presented previously in poster form [16]. Immunoglobulin subclasses were determined using a mouse monoclonal antibody isotyping kit Isostrip (Roche). Several previously characterized [5,11,17] anti-tau monoclonal antibodies TAU-1, AT8 and AD2, two polyclonal antibodies against tau No. 5 [11] and a commercial anti-tau polyclonal antibody (Sigma T6402, lot 99H4806, Buchs, Switzerland) were used.

Electrophoresis and Western blots: Brain homogenate proteins (50 μg/slot) were separated by 3.6–15% SDS polyacrylamide gel electrophoresis and either stained with Coomassie Brilliant Blue or electrically transferred to nitrocellulose filters and immunostained with monoclonal antibodies. Proteins were detected with peroxidase-conju-

gated secondary antibodies followed by 4-chloro-1-naphthol coloration [18].

Dephosphorylation: Brain tissue was homogenized in dephosphorylation buffer (50 mM Tris–HCl, pH 8.2, 135 mM NaCl, 1 mM EDTA) including protease inhibitors (10 mM phenylmethyl sulphonyl fluoride, 30 μg/ml each of leupeptin, antipain and pepstatin, and 15 μg/ml E64) and with phosphatase inhibitors in control samples. Aliquots of 100 μg proteins were incubated with 50 IU alkaline phosphatase (Boehringer Mannheim, molecular grade) for up to
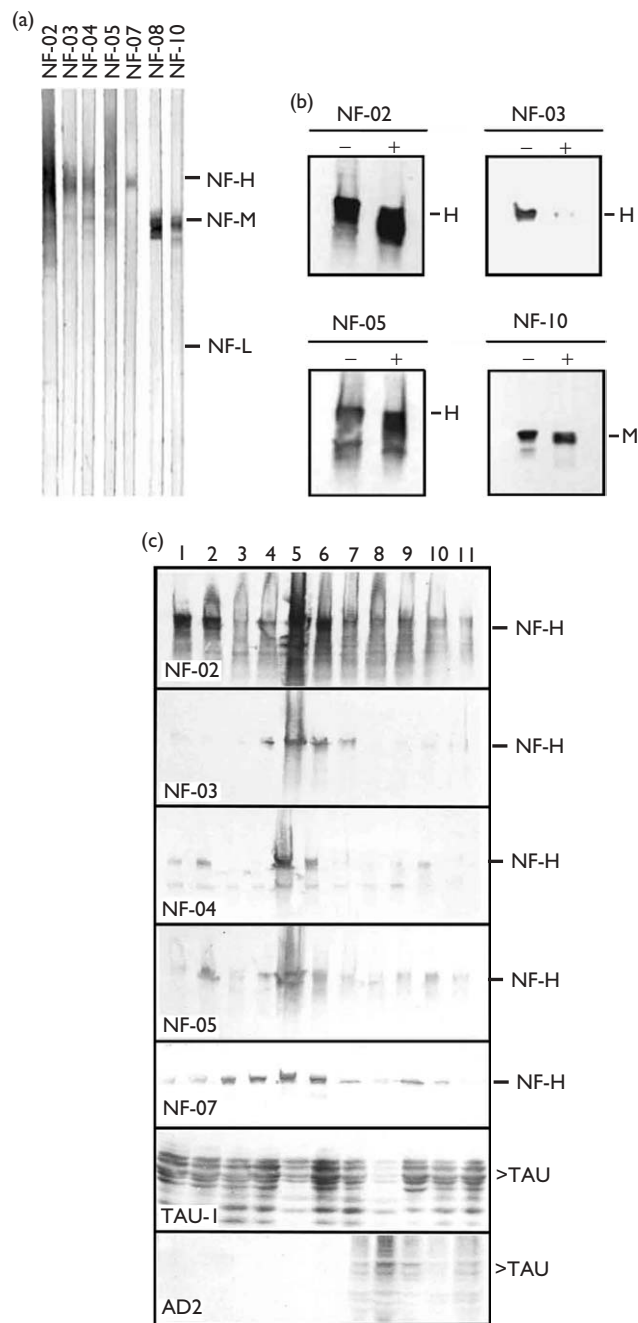
Fig. 1. Screening of novel antibodies for reactivity with NF subunits. The individual monoclonals have been numbered NF-02 to NF-08 and NF-10. (**a**) Western blots containing human brain homogenate (68-year-old male with mild AD) were stained. Location of NF subunits is indicated to the right. (**b**) Blots with native, untreated (−) brain tissue and dephosphorylated (+) tissue were used. Examples of immuno-reactions of four antibodies are shown. Antibody NF-03 showed reduced reactivity on dephosphorylated tissue. Reactivity of the antibody with a phosphorylated NF subunit is indicated at the bottom of (a) with the letter p for monoclonals NF-03, NF-04 and NF-07. (**c**) Entorhinal cortex tissues from controls: cases 1-6 had no known cognitive changes: 1: 52-year-old female, 4 h postmortem delay; 2: 56-year-old male, 10 h postmortem delay; 3: 59-year-old male, 10 h postmortem delay; 4: 62-year-old female, 7 h postmortem delay; 5: 78-year-old male, 11 h postmortem delay; 6: 79-year-old male, 5 h postmortem delay; and from histologically verified AD cases (7-11): 7: 79-year-old male, 10 h postmortem delay; 8: 84-year-old female, 13 h postmortem delay; 9: 90-year-old female, 2.5 h postmortem delay; 10: 94-year-old female, 5 h postmortem delay; 11: 94-year-old female, 8 h postmortem delay. Nitrocellulose blots were stained with different antibodies for neurofilament subunit NF-H with monoclonal antibodies NF-02, NF-03, NF-04, NF-05, NF-07, and for tau proteins with monoclonal antibodies TAU-1 and AD2.

24 h at 37°C. The reaction was stopped by adding phosphatase inhibitors and SDS-sample buffer [11].

Immunocytochemistry: Following preincubation in 3% fetal calf serum in Tris-buffer (50 mM Tris–HCl, pH 7.4, 122 mM NaCl), floating vibratome sections, cut in a frontal plane at 40 μm, were exposed overnight to monoclonal antibodies (hybridoma supernatants were used at a dilution of 1:10, ascites fluids were used at dilutions 1:1000–1:2000). Subsequently, tissue was exposed for 2 h to peroxidase-conjugated rabbit anti-mouse IgG (DAKO) diluted 1:100. Peroxidase conjugated secondary antibody was visualized with 4-chloro-1-naphthol. Sections were mounted and cover-slipped with semi-solid mounting fluid [18].

Confocal microscopy (Leica TCS NT) was used to discriminate neurofilaments and tau staining by using the polyclonal antibodies against tau and monoclonal antibody NF-03 against NF-H (1:1000 diluted) for the first incubation over night. After several rinses with Tris-buffered saline brain sections were incubated with goat anti-rabbit antibody conjugated with Texas red and goat anti-mouse antibody conjugated with Oregon green at a dilution of 1:50 (Molecular Probes). Excitation was obtained with an Argon-Krypton laser, with lines set at 488 nm for Oregon green excitation and 568 nm for Texas red excitation, the emitted light was filtered through appropriate filters (BF 530/30 for Oregon green LP 590 for Texas red). Images were taken with a ×40 objective, NA 1.2, with an electronic zoom of around 2-fold. Picture size is between 284 Ko and 1 Mbytes. For each field, a digitized series of optical sections at different planes of focus were collected; the sections were separated by 500–800 nm. Care was taken to use the full dynamic range of the photomultipliers using a special look up table (glowover-glowunder, Leica). The sections were processed using Adobe Photoshop software.

RESULTS

Characterization of novel neurofilament antibodies: We have screened a panel of novel antibodies against neurofilament proteins for their subunit-specificity and their reactivity with phosphorylated and dephosphorylated proteins. NF-02, NF-04, NF-07, NF-08 and NF-10 are of IgG1 subtype, while NF-03 and NF-05 are of the IgM subclass and react with human NF-H or NF-M (Fig. 1a). Antibodies NF-03, NF-04 and NF-07 were directed against phosphorylated sites as tested in Fig. 1b and indicated on the bottom of the strips in panel a with the letter P. On SDS-PAGE, dephosphorylated proteins usually have less charge thus increasing their migration in electrophoresis gels and proteins are of smaller apparent mol. wt. Accordingly, antibodies that react with a phosphorylation-independent epitope react at the same intensity also with the dephosphorylated NF subunits. Autopsy tissue from the entorhinal cortex was obtained from six subjects without known cognitive changes and from five subjects with histologically verified AD. Samples were prepared for Western blots and stained with a panel of novel anti-NF antibodies and with antibodies for tau proteins (Fig. 1c). Antibodies NF-03 to NF-07 showed some differences to the general staining pattern of NF-02 and several of the 11 samples revealed no or little immunoreactivity. It is interesting that NF-03 to NF-07 showed
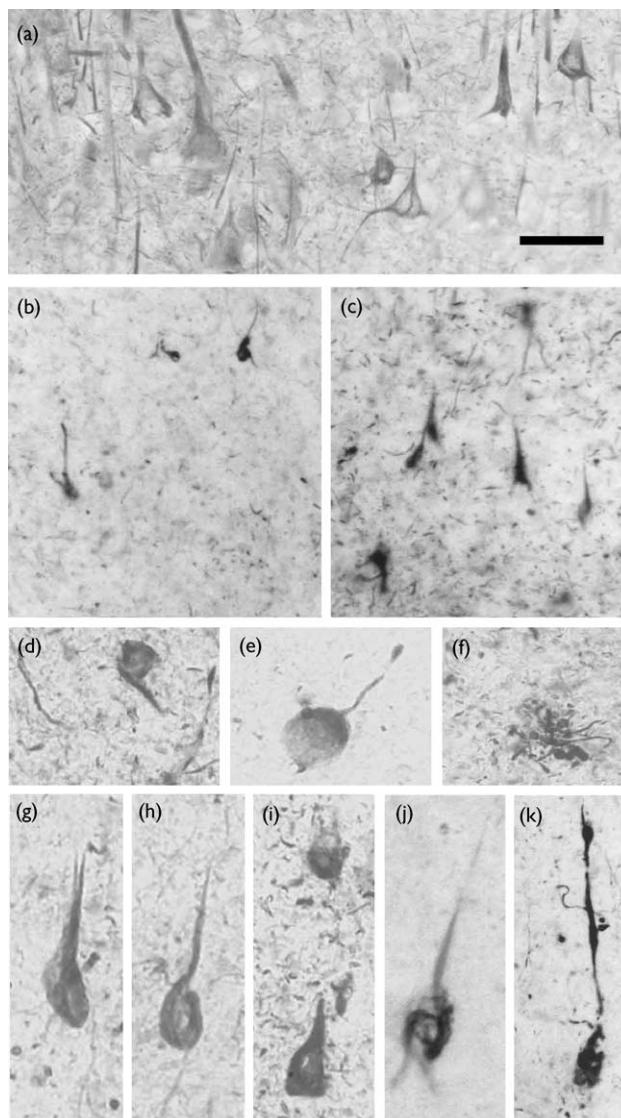
Fig. 2. Variety of neuronal staining is demonstrated with several antibodies. Normal NF distribution with antibody NF-05 is shown in a 79-year-old male (5 h postmortem delay) without neuropathological disorder (**a**). (**b,c**) Tissue from a 94-year-old female with mild AD; (**d**–**i**) tissue of an 84-year-old female with severe AD; (**j,k**) tissue of a 94-year-old female, with mild AD. (b) and (c) correspond to consecutive sections, which were stained with antibody NF-03 (b), and with antibody AT8 (c), a marker for AD-type phosphorylation of tau. Note that similar structures, mainly neuropilar threads and neuronal cell bodies, were stained by both antibodies. (d, f-j) show NF-03 staining, (e) represents NF-04 staining and (a) and (k) represent NF-05 staining. Bar = 20 μm (a,d,e,g-k) and 40 μm (b,c,f).

preferential staining of several samples form subjects in the 60–80 year age range. This staining was not proportional to that seen with NF-02. Because several of these antibodies are directed against phosphorylated epitopes, the extent of NF-phosphorylation may vary between samples, i.e. samples of case 1, 2, 3 or 11, show very low reactivity with some

antibodies. Reactivity of NF-08 and NF-10 against the NF-M subunit showed an equal staining pattern in all samples (not shown). This figure demonstrates that some NF antibodies reacted with a particular type of NF modification occurring preferentially in the group aged 60–80 years and irrespective of the severity of AD. Antibody TAU-1 indicated a variable immunoreactivity independent of the severity of the disease, while the AD2 antibody recognized a pathological form of tau exclusively in verified AD cases (samples 7–11).

Immunohistochemical detection of neurofilament and tau proteins: In Fig. 2a, a typical neurofilament staining is shown with antibody NF-05 in human entorhinal cortex of an old non-demented subject. Antibody NF-03 (Fig. 2b) was compared to phospho-tau distribution with antibody AT8 in the same area (Fig. 2a) of the entorhinal cortex of case 10. At first glance, twice as many neuronal elements were identified with AT8 against AD-type phosphorylated tau than with NF-03 against phosphorylated NF-H (Fig. 2b,c). Similarly shaped neurons were identified by both antibodies, which were characterized by dense immunoreactive material in the somatodendritic part. Furthermore, antibodies NF-03, NF-04 and NF-05 were used to stain EC tissues from several other cases. Many neurons with a particular neurofilament arrangement were observed (Fig. 2d–k). Antibody NF-03 revealed spike-like inclusions in neuronal somata and proximal dendrites. These bundles were almost filling the neuronal soma as shown in panels g and h, suggesting a considerable change in the organization of the neuronal cytoskeleton. Rarely, cells with a more globular shape were seen, as shown with antibody NF-04 in Fig. 2e (likely to represent a Cajal-Retzius cell since it was observed in the top cortical layer). Neurofilament immunoreactivity was also detected in senile plaques (Fig. 2f). Most of the neurofilament antibodies reacted with elements of senile plaques, however the staining intensity varied between antibodies. Antibody NF-03 also stained particular neurons with coiled intermediate filaments (Fig. 2j), while NF-05-stained fragmented intracellular NF material probably corresponding to a degenerating neuron (Fig. 2k). It is evident that many cells undergo changes in the aging entorhinal cortex, pointing to extensive degenerative events.

Double staining and analysis by confocal microscopy of entorhinal cortex with anti-tau in red and anti-NF in green of a case with severe AD showed that neurons which contain tau-positive tangles may also contain neurofilament bundles (Fig. 3). No statistical analysis was performed, but one-third of 20 cells contained NF-immunoreactivity and tau-positive structures (Fig. 3a). In senile plaques, several
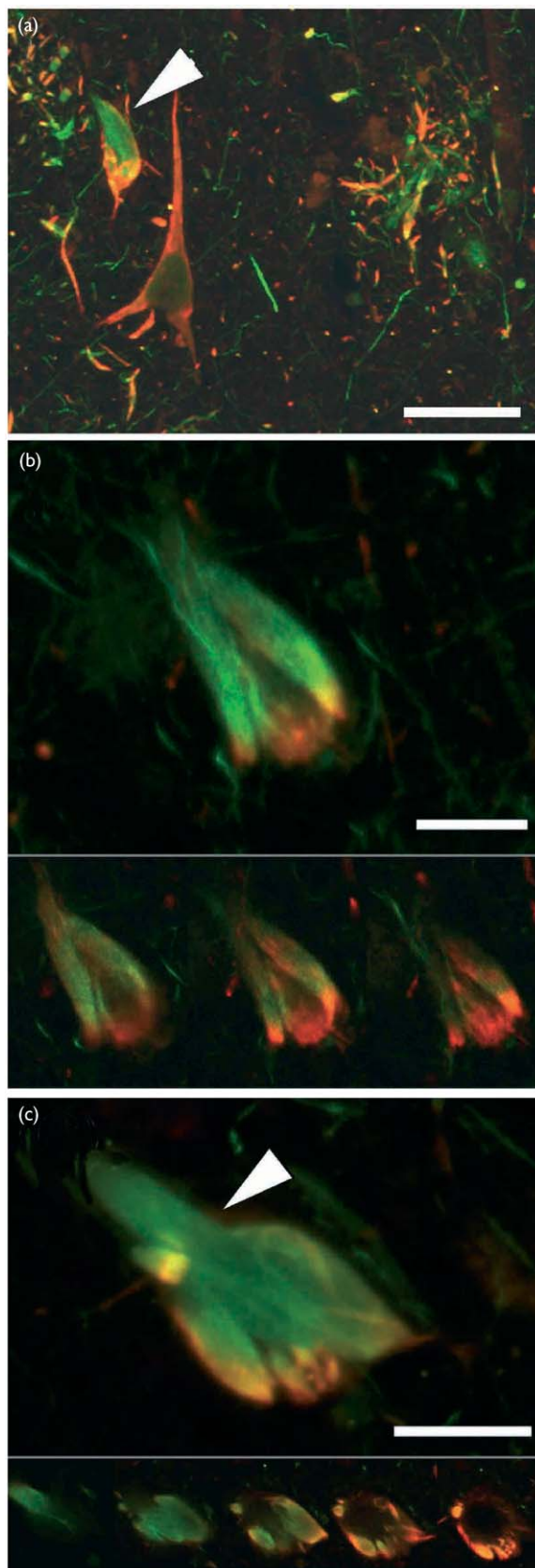
Fig. 3. Entorhinal cortex of a 84-year-old female with severe AD, (number 8 in Fig. Ic), was used for double-labeling experiments with polyclonal anti-tau (in red) and monoclonal anti-NF-H, NF-03 (in green). Secondary antibodies anti-rabbit Ig conjugated to Texas red and anti-mouse Ig conjugated to Oregon green were used to visualize staining by confocal microscopy. Note that there is little overlap of tau and NF staining in form of a yellow color. Cells with a thickness of I6 and 25 µm, respectively, were scanned with a separating distance of 500–800 nm between the focal planes. **(a-c)** represent the sum of all focal planes (extended focus), while individual focal planes at an interval of 4 µm and 2 µm, respectively, are shown at the bottom of (b) and (c). Bars = 50 µm (a), 25 µm (b,c).

fibers revealed NF and tau protein immunoreactivity. At higher magnification, both cytoskeletal proteins were found in the same cell (Fig. 3b,c). However, these structures were localized in different focal planes (separated by several micrometers) and which is shown as inserts at the bottom of Fig. 3b,c. Neurofilaments were often located near the dendritic shaft (arrowhead), while tau was found in the cell body and mainly disposed around the cell nucleus, suggesting a segregation of both types of aggregates with a different subcellular location of PHF-tau and NF filaments within the same cell. A few yellow spots suggested an occasional co-localization of both filament types in the same cell.

DISCUSSION

Neurofilament triplet proteins and phosphorylation: Over the past years, many reports have shown that in AD the neuronal cytoskeleton is subjected to profound changes [5,17,19,20] and that abnormalities of cytoskeletal proteins, mainly the microtubule-associated tau proteins, are closely related to the pathology of AD [21]. Here, we have investigated a different aspect of the cytoskeleton. Neurofilaments typically provide the cytoskeletal framework of the cell, while tau proteins are intimately linked to microtubules and are involved in a variety of cellular functions, such as regulating microtubule stability, but unfortunately are also involved in the formation of pathological lesions such as PHF in AD [5,11]. In this study we have produced and characterized a panel of monoclonal antibodies that reacted with NF-H and NF-H phospho-forms. In the human entorhinal cortex these antibodies identified a subset of neurons, which also contained tau-PHF and which were characterized by aberrant post-translational modifications. Studies on human brain tissue may be influenced by post mortem delay. In an animal model it was suggested that somatic staining of NF-H and -M may increase with increased delay [22]. Enzymatic control of posttranslational modifications has been recognized to affect the cytoskeleton between death and tissue removal [23]. However, the rather short postmortem delays in our study may not be sufficient to account for the formation of the high number of neurons with an aberrant cytoskeleton and a changed cell morphology.

Twenty years ago, several laboratories described and characterized a variety of monoclonal antibodies and have shown that phosphorylation of neurofilaments may be of importance in the modulation of neurofilament function [24–26]. A series of antibodies described previously [27,28] in part react with neurofilament proteins and identify aberrant cytoskeleton structures, several of these antibodies react also with tau proteins. Here we provide further evidence, using novel antibodies, that aberrant neurofilament phosphorylation may occur in a subset of neurons in the aging human brain, independent of neuropathological changes such as those of AD and without crossreactivity with tau. The antibodies strongly reacted with neurons with aberrantly organized neurofilament cytoskeleton characterized by bundled structures with spike-like extensions into neurites. Using double- anti-tau and anti-NF labelling we provide evidence that NF accumulation is not co-extensive with that of tau containing inclusions typical of AD. About one-third of PHF tau-positive cells contained such NF aggregates. This indicates that NF phosphorylation only occurs in a subset of neurons. On Western blots, several of these antibodies reacted with brain samples from an advanced age-group between 60 and 80 years, suggesting a particular phosphorylation of NF-H in many degenerating neurons. But unlike tau proteins, the NF protein amount on Western blots does not correlate with the severity of AD changes. Therefore, the phosphorylation of NF-H subunits may relate to metabolic- or age-related events. Phosphorylation, especially of the high and medium sized subunits, has been shown to be essential during postnatal maturation, in axonal transport or during aging [8,29].

Conclusion

Initially, we aimed to identify markers, which could be used to characterize incipient changes in neurodegenerative diseases. The presence of NF accumulation in PHF containing neurons raised our hopes. Unfortunately, the extent of NF amount did not correlated to the degree of AD changes particularly to the amount of PHF. Therefore, this particular NF phosphorylation may rather relate to molecular events occurring during normal aging or age-related neurodegeneration.

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