Hyperphosphorylation and accumulation of neurofilament proteins in Alzheimer disease brain and in okadaic acid-treated SY5Y cells

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Abstract We investigated the role of neurofilament (NF) proteins in Alzheimer disease (AD) neurofibrillary degeneration. The levels and degree of phosphorylation of NF proteins in AD neocortex were determined by Western blots developed with a panel of phosphorylation-dependent NF antibodies. Levels of all three NF subunits and the degree of phosphorylation of NF-H and NF-M were significantly increased in AD as compared to Huntington disease brains used as control tissue. The increase in the levels of NF-H and NF-M was 1.7- and 1.5-fold ($P < 0.01$) as determined by monoclonal antibody SMI33, and was 1.6-fold ($P < 0.01$) in NF-L using antibody NR4. The phosphorylation of NF-H and NF-M in AD was increased respectively at the SMI31 epitope by 1.6- and 1.9-fold ($P < 0.05$) and at the SMI33 epitope by 2.7- and 1.3-fold ($P < 0.01$ and $P < 0.05$). Essentially similar effects were observed in SY5Y human neuroblastoma cells when treated with okadaic acid, an inhibitor of protein phosphatase (PP)-2A and -1. This is the first biochemical evidence which unambiguously demonstrates the hyperphosphorylation and the accumulation of NF subunits in AD brain, and shows that the inhibition of PP-2A/PP-1 activities can lead to the hyperphosphorylation of NF-H and NF-M subunits. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Alzheimer disease; Neurofilament; Phosphorylation; Okadaic acid; SH-SY5Y cell

1. Introduction

The neurofilament (NF) is the intermediate filament of the neuron. NF is composed of three protein subunits with apparent molecular masses of ~200 kDa (NF-H), 140–160 kDa (NF-M), and 68–70 kDa (NF-L) [1,2]. The highly repetitive Lys-Ser-Pro (KSP) motif present in the tail domain/C-terminal region of NF-H and NF-M is highly phosphorylated in vivo [3–5]. The phosphorylated NFs appear to be localized mainly in the axon, whereas the dephosphorylated forms are found in the cell body and dendrites [6–8], suggesting the importance of NF phosphorylation in its transport and interaction with other cytoskeletal proteins.

In Alzheimer disease (AD) brain the neurofibrillary degeneration, a hallmark of the disease, is believed to be the result of a protein phosphorylation/dephosphorylation imbalance [9–11]. The activities of protein phosphatase (PP)-2A/PP-1 which regulate the phosphorylation of tau [12–14], the major protein subunit of paired helical filaments (PHF)/neurofibrillary tangles [15], are compromised in AD brain [16,17] and probably contribute to the protein phosphorylation/dephosphorylation imbalance. Thus, one or more neuronal proteins other than tau might also become hyperphosphorylated in the affected areas of AD brain. Immunocytochemically phosphorylated NF-H and NF-M subunits were reported in tangle-bearing neurons in AD brain previously [18]. However, the cross-reaction of NF antibodies with abnormally phosphorylated tau made these results uncertain [19–21]. In the present study, we resolved this question by using Western blots to distinguish NF proteins (NFPs) and tau by their different apparent molecular weights and show that in AD neocortex as compared with Huntington disease (HD) brain used as a control, the levels of all three neurofilament subunits are increased and that at least NF-H and NF-M are significantly hyperphosphorylated at several sites. The present study also shows that the treatment of human neuroblastoma SY5Y cells with okadaic acid (OA), an inhibitor of PP-2A and -1, also results in the phosphorylation and accumulation of NF-H and NF-M subunits as seen in AD.

2. Materials and methods

2.1. Brain samples and antibodies

For this study neocortex from 20 AD (76 ± 5.8 years) and as controls 20 HD (69 ± 12 years) cases was employed. The brains had been removed 3–6 h post mortem and were stored frozen at −75°C until used. All AD cases had histopathologically confirmed diagnosis. HD diagnosis was based on both family history and neuropathological examination. Autopsied brain specimens were obtained from the Brain Tissue Resource Center (Public Health Service Grant MH/NS 31862), McLean Hospital, Belmont, MA, USA, and from the IBR Brain Bank (Dr. Piotr Kozlowski). The source, specificity, and the dilution of the antibodies used are described in Table 1.

2.2. Immunocytochemistry

Neighboring sections of formalin-fixed, paraffin-embedded Alzheimer hippocampus (6 μm) were immunostained with monoclonal

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Abbreviations: NF, neurofilament; AD, Alzheimer's disease; PP, protein phosphatase; NFP, neurofilament protein; HD, Huntington disease; OA, okadaic acid; PBS, phosphate-buffered saline; BME, β-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; IR, immunoreactivity

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Table 1
Antibodies employed

Antibody	Type	Dilution	Specificity	Source
SMI31	P[a]	1:5000	NF-H, NF-M	Sternberger Mono Inc.
SMI32	NP[b]	1:5000	NF-H, NF-M	Sternberger Monoclonals Inc.
SMI33	NP	1:5000	NF-H, NF-M	Sternberger Monoclonals Inc.
SMI34	P	1:5000	NF-H	Sternberger Monoclonals Inc.
NR4		1:500	NF-L	Dako
Tau-1	NP	1:50000	Tau Ser 195/198/199/202	[9,44]

[a,b]Reactive with neurofilament proteins H and M only if they are [a]phosphorylated or [b]not phosphorylated at specific site(s).

antibody (mAb) Tau-1 (ascites, 1:50 000) after dephosphorylation of the tissue [9] or with antibody SMI34 (Sternberger Monoclonals, Lutherville, MD, USA; 1:20 000) and developed using the Vector Elite Kit (Vector Laboratories, Burlingame, CA, USA). SY5Y cells were fixed for 10 min in 4% paraformaldehyde in 1×phosphate-buffered saline (PBS) prewarmed at 37°C, washed three times in PBS containing 0.1% Triton X-100 and incubated with PBS containing 10% goat serum, 3% bovine serum albumin, and 2% Tween-20 for 1 h at room temperature to block non-specific binding of antibodies. Primary antibodies were added at the dilutions shown in Table 1 and incubated at 4°C overnight. Bound antibodies were detected by using the Histostain SP kit from Zymed (Beijing, PR China) with diaminobenzidine as a substrate. Levels of antibody bound to cells were analyzed by Kodak Image System IPP Analysis Software (Cold Spring Harbor Inc., Beijing, PR China). At least 500 cells per experiment were analyzed. For statistical analysis of the data Student's *t*-test was used. Each experiment was carried out at least three times.

2.3. Tissue fractionation and quantitative Western blot analysis

Cerebral gray matter was cleaned free of any underlying white matter, washed in ice-cold buffer containing 50 mM HEPES, pH 6.8, 1 mM EGTA, and 1% β-mercaptoethanol (BME), and blood clots and meninges were removed. The tissue was then homogenized in the above buffer (1:0.75 w/v) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, and 10 μg/ml each of leupeptin, pepstatin A and aprotinin. The homogenate was centrifuged at 75 000×*g* for 1 h and separated into a supernatant and a pellet. The pellet was rehomogenized and dissolved by heating for 5 min in Laemmli sample buffer, containing 60 mM Tris–HCl, pH 6.8, 3% sodium dodecylsulfate, 5% BME, 10% glycerol and 0.05% bromophenol blue. Protein concentrations were measured by the modified Lowry method [22].

Proteins were separated by SDS–PAGE. The amount of protein loaded per gel lane was as follows: 16 μg/lane for SMI31, 24 μg for SMI34, 8 μg for SMI33 and 32 μg for SMI32 and for NR4. After transfer of proteins to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA), blots were probed with primary mAbs SMI31, SMI32, SMI33, SMI34 or NR4 (Table 1) and developed with ^{125}I-labeled antibodies to mouse IgG (0.5 μg/ml, Amersham Pharmacia) as secondary antibodies. Images were processed with the aid of a computerized Fuji 1500 Imaging System and expressed as PSL [23]. Student's *t*-test was used for statistical analysis. Dephosphorylation, where required, was carried out on the blot with bovine calf intestine alkaline phosphatase (Sigma, St. Louis, MO, USA) as previously described [9]. For each case the values obtained with the phosphorylation-dependent antibodies were normalized against the corresponding immunoreactivities obtained with mAb SMI33 after dephosphorylation (total NF-H and NF-M). The percent phosphorylation at the SMI33 and SMI32 epitopes was calculated from the immunoreactivities (IR) obtained before and after dephosphorylation by using the following formula: (IR total−IR not dephos)×100/IR total. IR total = immunoreactivity after dephosphorylation.

2.4. Cell culture and treatment with okadaic acid

SH-SY5Y cells were obtained from Dr. J.L. Biedler (Sloan Kettering Institute, New York, NY, USA) and were propagated in Dulbecco's modified Eagle's medium (Gibco BRL, Beijing, PR China) with 5% fetal bovine serum and penicillin/streptomycin (5% CO_2 and 95% air). Cells were plated at a density of 1.0×10^5 cells/cm^2 on glass coverslips for immunocytochemistry or at a density of 5×10^6 cells/35 mm dish for immunoblotting. For OA treatment, cells were cultured in the presence of 0, 15 or 30 nM of the drug for 24 h, and then either washed, lysed in sample buffer containing phosphatase and protease inhibitors and employed for Western blots, or fixed in 4% paraformaldehyde and used for immunocytochemical staining with antibodies SMI31, SMI32, SMI33 or SMI34.

3. Results

3.1. Subcellular distribution of NFPs in AD brain

The distribution of phosphorylated NFPs in AD brain was examined immunocytochemically by staining sections of hippocampus with mAb SMI34 at a 1:20 000 dilution. On Western blots at this dilution the antibody only reacted with NF-H/-M but not with Alzheimer hyperphosphorylated tau, whereas in AD hippocampus tissue sections it strongly labeled a number of tangles and neuropil threads. However, considerably fewer structures were labeled by the neurofilament antibody when compared to immunolabeling of the alkaline phosphatase-treated sections with mAb Tau-1 (Fig. 1).

The distribution of NFPs between the 75 000×*g* pellet and supernatant of AD brain homogenate was studied by Western blots (Fig. 2). In the particulate fraction all antibodies tested strongly labeled protein bands with apparent molecular mass ∼200 kDa, 160–150 kDa or 68 kDa corresponding to NF-H, NF-M and NF-L, respectively. Antibodies SMI31 and SMI33 detected both NF-H and NF-M, whereas SMI34 detected only NF-H and SMI32 detected preferentially NF-M. In the same fraction antibody NR4 to NF-L labeled a protein of a molecular mass of 68 kDa. In contrast, in the supernatant fraction, a <150 kDa doublet and a ∼230 kDa protein were detected by antibody SMI33. When the blot was overexposed the same proteins were also labeled by SMI31 (data not shown). Preliminary data indicate that these proteins were not NFPs but spectrins (Wang et al., manuscript in preparation). These findings show that, in contrast to fresh animal brain where NFPs were observed both in supernatant and in pellet fractions [24,25], in human autopsy tissue the neurofilament polypeptides separate in the pellet fraction.

3.2. Protein levels and degree of phosphorylation of NFPs in AD and HD brains

Since NFPs were only detected in the particulate brain fractions, we studied the total protein levels and the phosphorylation states of NFPs in this fraction from AD and as neurological control age-matched HD cases (Fig. 3). Total NF-H and NF-M levels were determined by quantitative Western blots using antibodies SMI32 and SMI33. We found that antibodies SMI33 and SMI32 reacted maximally with NF-H and NF-M, respectively, after treatment of the blots with alkaline phosphatase prior to antibody application. The blots showed that the total immunoreactivity levels of NF-H

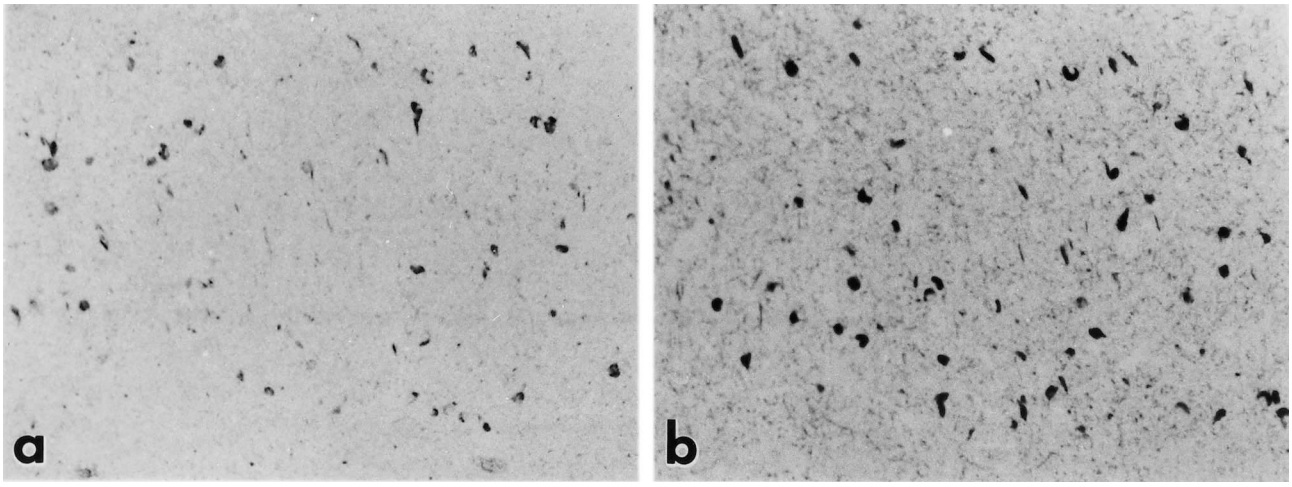

Fig. 1. Immunohistochemical staining of neighboring sections of an AD hippocampus with (a) phosphorylation-dependent neurofilament antibody SMI34 (1:20 000) and (b) an alkaline phosphatase-treated section with Tau-1 (1:50 000). SMI34 at 1:20 000 dilution does not cross-react with hyperphosphorylated tau on Western blots.

and NF-M determined by SMI33 after dephosphorylation were respectively 1.7-fold ($P < 0.01$) and 1.5-fold ($P < 0.01$) higher in AD than in HD brains ($n = 20$ each), whereas without dephosphorylation no significant difference was detected in the NFP levels between AD and controls (Fig. 3A,B). Similarly, with mAb SMI32 after dephosphorylation the levels of NF-M were 1.6-fold ($P > 0.05$; $n = 11$ each) higher in AD than in HD brains (Fig. 3A,B). The levels of NF-H could not be determined in AD brain with this antibody since even after extensive dephosphorylation this band was not labeled in most of the samples studied ($n = 11$). This was probably due to the blockage of the SMI32 epitope by a posttranslational modification other than phosphorylation in AD. As

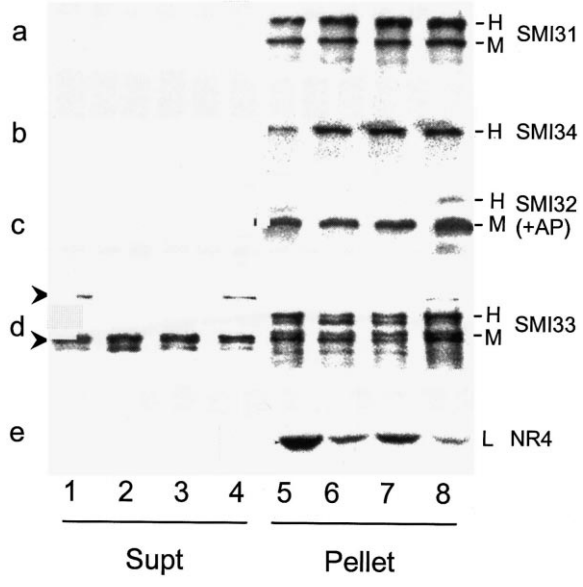

Fig. 2. Distribution of NFPs in supernatant (lanes 1–4) and particulate fractions (lanes 5–8) of the neocortex from four AD cases. Western blots of NF-H and NF-M immunostained with antibodies (a) SMI31; (b) SMI34; (c) SMI32, after treatment of the blot with alkaline phosphatase (+AP); (d) SMI33; (e) Western blot of NF-L with NR4. The position of ~230 kDa and <150 kDa mass proteins on the blot in panel d is marked by arrow heads.

in the case of NF-H and NF-M a 1.6-fold ($P < 0.01$; $n = 7$ each) increase in the NF-L levels in AD as compared to HD brains was detected by mAb NR4 (Fig. 3A,B).

The degree of phosphorylation at the SMI33 epitope was deduced from the immunoreactivity levels calculated for NF-H and NF-M before and after dephosphorylation (Fig. 3C). Despite high individual variations, we found that both NF-H and NF-M were generally phosphorylated to a higher degree in AD than in HD brains. In NF-H ~40% of the SMI33 epitope was phosphorylated in AD versus ~15% in HD brains ($P < 0.001$; $n = 20$ each). For NF-M the degree of phosphorylation was ~43% in AD and ~32% in HD but the difference was not statistically significant (Fig. 3C). The degree of phosphorylation at the SMI32 epitope was not determined because in most of the samples no staining was detected without prior dephosphorylation (Fig. 3A). The degree of phosphorylation at the SMI31 epitope was 1.9-fold ($P < 0.05$; $n = 11$ each) higher in both NF-H and NF-M in AD than in HD brains, and at the SMI34 epitope of NF-H it was 1.4-fold ($P < 0.05$; $n = 11$ each) increased in AD (Fig. 3D). Because of the increased levels of the NFPs in AD, phosphorylation at the SMI31 and SMI34 epitopes was further calculated by normalizing the values obtained with these antibodies in the individual samples against the respective total levels of NF subunits determined with SMI33 after dephosphorylation (Fig. 3E). Under these conditions we found that the degree of phosphorylation at the SMI31 epitope was 1.6-fold ($P < 0.05$) and 1.9-fold ($P < 0.05$) respectively higher in the NF-H and NF-M subunits of AD as compared to the HD controls. However, no significant difference in phosphorylation between AD and HD was detected with SMI34 after normalization.

3.3. Dose-dependent induction of hyperphosphorylation and accumulation of NF-H/-M in SY5Y neuroblastoma cells by treatment with OA

To investigate the possibility that the abnormal hyperphosphorylation and elevation of NFPs observed in AD brain might be caused by a decrease in the activities of protein phosphatases, we induced an AD-like decrease in PP-2A/PP-1 activities by treating SY5Y cells with 15 or 30 nM OA.

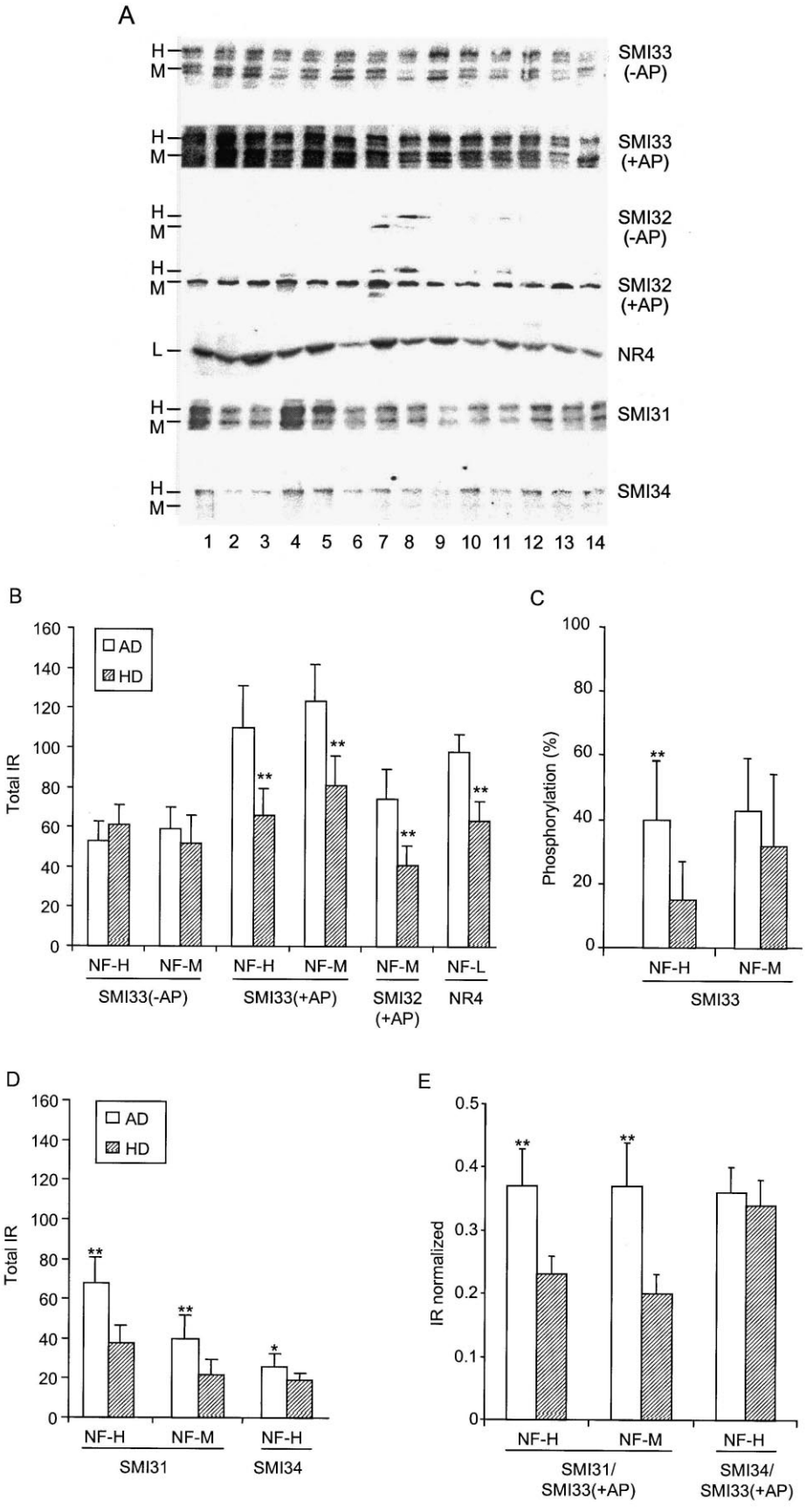
A
H
M
L
SMI33 (-AP)
SMI33 (+AP)
SMI32 (-AP)
SMI32 (+AP)
NR4
SMI31
SMI34
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
AD
HD
Total IR
NF-H
NF-M
NF-L
SMI33(-AP)
SMI33(+AP)
SMI32 (+AP)
NR4
C
Phosphorylation (%)
SMI33
D
Total IR
SMI31
SMI34
E
IR normalized
SMI31/ SMI33(+AP)
SMI34/ SMI33(+AP)

Fig. 3.

Fig. 3. ^{125}I Western blot profiles and levels of NFPs in the particulate fraction of AD and HD brains. A: Western blots were developed with antibodies to non-phosphorylated (SMI33, SMI32) and phosphorylated (SMI31, SMI34) epitopes on NF-H and NF-M (AD, lanes 1–7; HD, lanes 8–14) and the NF-L (NR4) subunits (AD odd and HD even numbers). (−AP) and (+AP) indicates whether the blots had been treated with alkaline phosphatase prior to application of the antibodies to unmask the SMI33 and SMI32 epitopes. B–E: Bar graphs represent means ± S.D. of (B) the relative levels of total ^{125}I immunoreactivities (IR) from scans of Western blots that were developed with SMI33 ($n = 20$ AD and HD cases each) or SMI32 ($n = 11$ each) for NF-H and NF-M without or with prior treatment of the blot with alkaline phosphatase (+AP; −AP) and with NR4 for NF-L ($n = 7$ each); (C) the percentage of phosphorylation at the SMI33 epitope; (D) the degree of phosphorylation at the SMI31 and SMI34 ($n = 11$ each) epitopes of NF-H and NF-M subunits; (E) the degree of phosphorylation at the SMI31 and SMI34 epitopes after normalization against the total NF-H or NF-M levels (SMI33 after dephosphorylation). For SMI33 the percentage of phosphorylation was calculated from the IR obtained in B as $\{(SMI33(+AP)-SMI33(-AP))/SMI33(+AP)\} \times 100$ where IR SMI33(+AP) is assumed to represent the total IR. Phosphorylation at the SMI31 and SMI34 epitopes in AD and HD cases is expressed in IR that had been normalized in pair against the total NF subunit levels (IR SMI33+AP). $^{*}P < 0.05$, $^{**}P < 0.01$.
←

Previously, we have shown that the treatment of SY5Y cells with 10 nM OA for 24 h inhibits almost 100% of PP-2A and ~75% of PP-1 activities [23]. Since tau in OA-treated SY5Y cells is hyperphosphorylated at several sites [23] we first studied the degree of its cross-reactivity with the phosphorylation-dependent antibodies SMI31 and SMI33 to NF on Western blots. We found negligible immunoreactivity with the phosphorylated tau in OA-treated cells (data not shown)

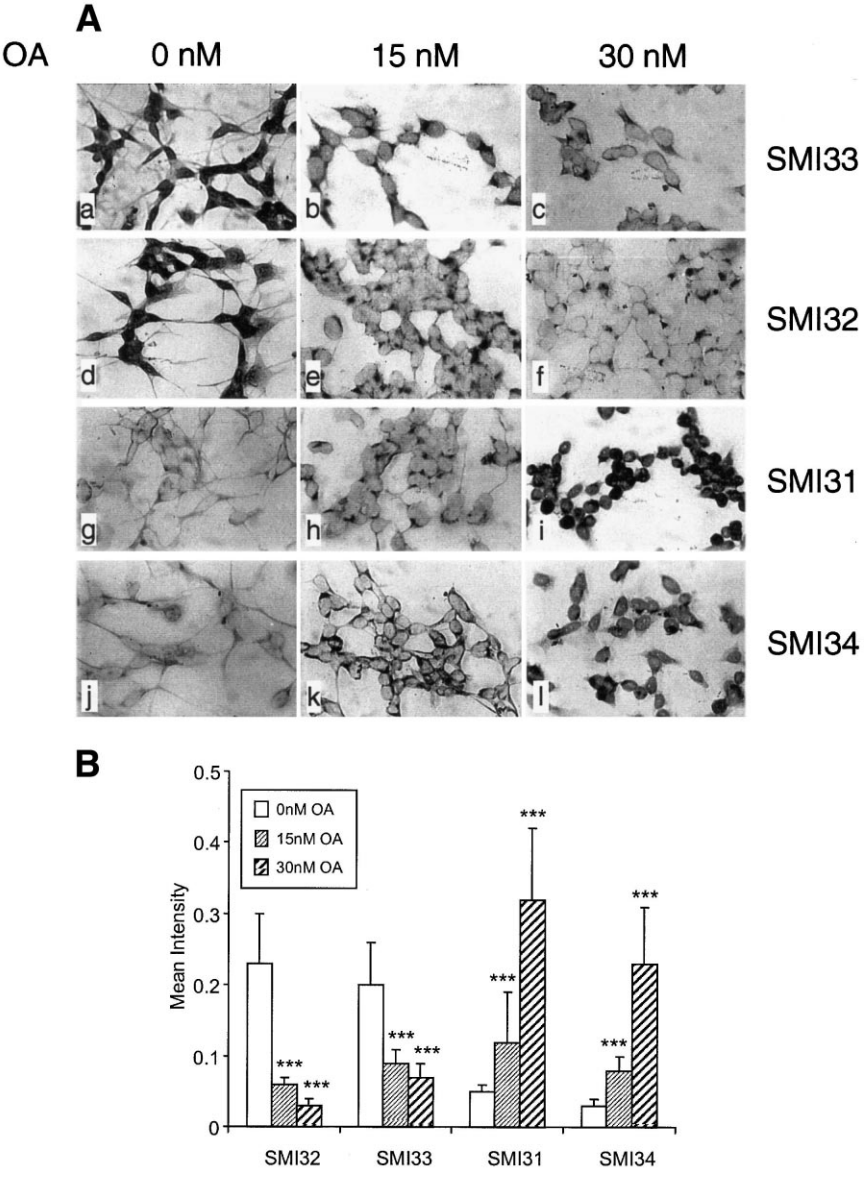

Fig. 4. Hyperphosphorylation of NFP in OA-treated SY5Y cells. A: SY5Y cells were treated with 0, 15 or 30 nM OA and immunocytochemically stained with NF antibodies. Representative micrographs of cells that were treated without OA (a,d,g,j); 15 nM OA (b,e,h,k); and 20 nM OA (c,f,i,l) for 24 h, and immunostained with SMI33 (a–c); SMI32 (d–f); SMI31 (g–i) and SMI34 (j–l). B: Bar graphs represent the intensity (in arbitrary units) of immunostaining per cell. The values are the averages obtained from at least 500 cells and three separate experiments. $^{***}P < 0.001$.

indicating the NF specificity of the immunocytochemical reaction. Antibodies SMI33 (Fig. 4A, a–c) and SMI32 (Fig. 4A, d–f) to non-phosphorylated epitopes of NF-H and NF-M stained most strongly the untreated SY5Y cells. The immunolabel was mostly in the cell bodies and to a lesser degree in the processes. In the OA-treated cells the staining dramatically decreased and was mostly concentrated around the nuclei and in the proximal ends of the processes. The reverse picture was observed with SMI31 (Fig. 4A, g–i) and SMI34 (Fig. 4A, j–l) to phosphorylated NFP epitopes; namely, only weak labeling of cell bodies and processes of the untreated cells and increased staining densities of the cells exposed to OA. Very similar results were obtained with image analysis of the immunolabeled cells, i.e. decreased levels of labeling of the OA-treated cells with the antibodies to dephosphorylated NFPs and increased levels of staining with the antibodies recognizing phosphorylated NFPs (Fig. 4B).

4. Discussion

Based on immunocytochemical studies at light and electron microscopic level with polyclonal and monoclonal antibodies to neurofilaments it had been believed for a number of years that the Alzheimer neurofibrillary tangles, the hallmark lesion of this disease, were composed of NF [18,26–29]. However, subsequently it was shown that some of these antibodies immunologically cross-reacted with abnormally hyperphosphorylated tau [19–21] which is the major protein subunit of paired helical filaments/neurofibrillary tangles [9,15]. Whether and to what extent neurofilament proteins are actually hyperphosphorylated in the AD brain has remained an enigma. In the present study we demonstrate that NFPs are indeed accumulated in hyperphosphorylated form in the AD brain. Furthermore, we show that the treatment of SY5Y cells in culture with OA, a PP-2A/PP-1 inhibitor, induces the accumulation of phosphorylated NFPs in the cell bodies.

In the normal neuron all three axonal NFPs are phosphorylated. However, there are differences in degree of phosphorylation between the subunits. Unlike NF-H and NF-M in which the numbers of phosphorylation sites are very large and which are mainly located in the C-terminal long tail region, NF-L is less phosphorylated and the phosphorylation sites are in the N-terminus of the protein [30]. NFPs in highly phosphorylated form are normally major components of the axonal cytoskeleton. They are synthesized and assembled for axonal transport in the cell body and their phosphorylation occurs principally upon entry into the axon [31]. We found that both NF-H and NF-M were significantly hyperphosphorylated at SMI31 and SMI33 epitopes in AD brain. It is believed that the abnormal phosphorylation of the microtubule-associated protein tau in AD brain is due to an imbalanced regulation of protein kinases and protein phosphatases. The reduced activities of the tau protein phosphatases PP-2A and PP-1 in AD brain [16,17] would induce increased phosphorylation of tau either by diminished dephosphorylation of this protein directly or by tau kinases that are activated by phosphorylation, such as ERK and CaMKII (see [32]). That there might indeed be a general activation of kinases by phosphorylation in the AD brain is indicated by their immunohistochemical association in activated forms with the neurofibrillary tangles in the AD hippocampus (see [32]). NF are a component of the cytoskeleton and are located in the same cellular compartment as microtubules and tau and, like tau, would also be exposed to the abnormal regulation of phosphorylation in AD. In vitro studies have shown that NF subunits can be further phosphorylated over and above their normal phosphorylation by various protein kinases [3,33–35]. In situ such hyperphosphorylation might then lead to disassembly of NF [36] as was shown in vitro by phosphorylation with protein kinase A [37].

In addition to hyperphosphorylation, we also found that the levels of all three NF subunits were significantly increased in AD brain. A previous study reported a similar finding but based on investigation of only two AD and two control cases [38]. To date no indications for increased synthesis of the NFPs but rather, in the case of NF-M and NF-L, a decrease in its message in AD have been found [39]. It is, therefore, more likely that the NFPs accumulate in AD because the access of the catabolic enzymes to these proteins is inhibited by their abnormal hyperphosphorylation as has been shown in vitro [40]. Similar mechanisms have been implied for the abnormally hyperphosphorylated tau in AD [13,41]. Whether the accumulation of hyperphosphorylated NFPs might lead to their association with the neurofibrillary tangles in AD brain is not clear yet, although it is somewhat indicated by the immunohistochemical staining (see Fig. 1). However, these experiments are not fully conclusive since it cannot be excluded that the antibodies, although not reactive with tau on Western blots, might still be reactive with tau polymerized into PHF in tissue due to conformational changes.

Since SH-SY5Y, a human neuroblastoma cell line, only contains negligible amounts of phosphorylated tau proteins that did not cross-react with the phosphorylation-dependent NF antibodies at the concentrations employed here, we used this cell line as a model for the study of NFPs. To explore the mechanism of NF phosphorylation and elevation in AD brain, we treated the SY5Y cells with OA, a potent inhibitor of PP-2A and PP-1. A dose-dependent hyperphosphorylation and accumulation of NFPs in the cell body were observed after inhibition of phosphatases. It has been shown previously that phosphorylation regulates the intracellular transport of NFPs from cytoplasm to the processes [42]. According to the data obtained from the present study, we speculate that in AD the deficiency in PP-2A/PP-1 activities induces hyperphosphorylation of NFPs which thereafter leads to an accumulation of these proteins in the cytoplasm. The effect of accumulated hyperphosphorylated NFPs on the biology of the neuron is as yet not understood. A recent report has raised the possibility that NFPs might chaperone the assembly of tau into filaments [43].

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