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PROGRESSIVE HYPERPHOSPHORYLATION OF NEUROFILAMENT HEAVY SUBUNITS WITH AGING:
Possible involvement in the mechanism of neurofilament accumulation

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Abnormal accumulations of phosphorylated neurofilaments occur both in normal senescence and in age-associated neurodegenerative diseases. In the present work, we study the physicochemical properties of neurofilaments isolated from rats of controlled ages. Aging induces in vivo hyperphosphorylation of the heavy neurofilament subunit without affecting in vitro neurofilament phosphorylation by the neurofilament-associated protein kinase. Interactions in vitro between neurofilaments from very old rats occur at higher rate and extent than that of neurofilaments from younger animals. These results support the hypothesis that the abnormal accumulation of neurofilaments observed in nervous tissues from aging mammals results from an altered equilibrium in situ between interconnected and independent neurofilaments.

Aging in the nervous tissue is associated with modifications of the neuronal cytoskeleton, including an increase in the phosphorylation state of neurofilaments (NF) (1-5). Similar accumulations of hyperphosphorylated NF occur in several neurodegenerative diseases (4-13). Furthermore, evidence that most neurodegenerative diseases develop with aging (4,5) support the possibility that a specific alteration of NF metabolism in aging nervous tissues favor their abnormal accumulation in pathological situations.

The high phosphorylation level of NF-H inhibits interactions between NF and microtubules (14). In addition, hyperphosphorylated NF subunits are preferentially associated in normal axons with a stationary subpopulation of NF (15), suggesting that phosphorylation of NF sidearms controls the equilibrium between moving and stationary NF (16,17). An interpretation of these observations is that a dynamic equilibrium between cross-linked and independent NF, modulated by the phosphorylation level of NF subunits (18-20), is required for the maintenance and the regulation of their axonal transport (15-17,21-24).

In the present work, we compared the physicochemical characteristics of purified NF from young and aging rats, following an experimental approach previously described

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Abbreviations: NF: neurofilaments; NF-H: neurofilament heavy subunit; NF-M: neurofilament medium subunit; NF-L: neurofilament light subunit. ELISA: enzyme-linked immunosorbent assay; TBS: Tris buffer saline; PBS: Phosphate buffer saline.

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(18,25,26). A correlation was found in vitro between NF-H hyperphosphorylation and a higher bundling capacity of very old rat NF compared to that of young rat NF.

MATERIALS AND METHODS

Chemicals: Mes (morphoethanesulfonic acid), ATP (Vanadate free, Mg salt, from equine muscle), ABTS reagent (2,2'-Azino-bis (3 ethylbenz-thiazoline-6-sulfonic acid, diammonium salt)), bovine serum albumine (BSA), polyvinyl-pyrrolidone and protease inhibitors (see below) were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Adenosine 5'-(γ-^{32}P) triphosphate, triethylammonium salt (3000 Ci/mmole), ^{125}-I labeled anti-mouse sheep immunoglobulins (5-20 μCi/μg protein) and the ECL kit were purchased from Amersham (Les Ulis, France). Ampholines and ultra pure urea were obtained from LKB Pharmacia (Saclay, France). All other reagents were purchased from Merck (Darmstadt, Germany).

Antibodies: Two monoclonal antibodies against NF-H were kindly donated by Dr. V.M. Lee (Dpt. of Pathology and Laboratory Medicine, School of Medicine, Univ. Philadelphia Pennsylvania, U.S.A.): the antibody RMO-217 recognizes the phosphorylated form of the molecule (P++), while the antibody RMDO-9 recognizes non-phosphorylated NF-H epitopes (P-) (27). The monoclonal antibody RS18 (specific of repetitive KSP sequences of the phosphorylated NF-H) was a kind gift from Prof. B.H. Anderton (Institute of Psychiatry, London, U.K.). Its specificity has been previously described (28,29). Antibodies used in ELISA were purchased from Pasteur Diagnostic (Aulnay/Bois, France) (goat anti-rabbit IgG, coupled with peroxidase) and from Biosoft Clonatec (Paris, France) (goat anti-mouse IgG, coupled with peroxidase).

Animals: Two groups of 3 and 20 month-old rats (females, 6 animals for each age, Wistar) and 3 and 30 month-old rats (females, 10 animals for each age, Wistar) were independently raised in the laboratory. Two other groups of 3 and 30 month-old rats and 3, 20 and 30 month-old rats (females, 10 rats of each age, Wistar AG/Rij) were donated by EURAGE (Dr D.L. Knook, Institute for experimental gerontology TNO, Rijswijk, the Netherlands).

Neurofilament purification: Rats were sacrificed under CO_2 asphyxia. NF were isolated from rat spinal cord according to the procedure described previoulsy (25,26). Purified NF resuspended in buffer A (Mes 0.1M, MgCl2 1mM, Ethylene Glycol Tetraacetic Acid 1mM, pH 6.8) containing 0.8M sucrose were dialysed for 24 h at 4°C against the same buffer containing 1mM phenylmethane sulfonylfluoride.

Viscosity measurements were performed by falling ball viscosimetry as previously reported (26), using 75 μl capillaries (Drummond) and 0.7 mm diameter stainless steel ball (Marteau et Lemarié, Paris). Samples were prepared at 4°C in buffer A, containing 0.8 M sucrose, 6 mM $MgCl_2$, and protease inhibitors (final concentrations in assays: 0.1 mg/ml N-p-tosyl-L-Arginine methyl ester, 0.05 U/ml aprotinin, 1 μM pepstatin, 1 μM leupeptin, 1 mM phenylmethane sulfonylfluoride, 0.1 mM chloroquinin and 10 nM soybean trypsin inhibitor). The velocity of the falling ball was measured at 30°C in a water-jacketted chamber over a distance of 5 cm at an angle of 45° or 80° to the horizontal. The viscosity was expressed in Pascals per second by comparison with the velocity of the ball in glycerol solutions of known concentrations.

Autophosphorylation of NF proteins (phosphorylation of endogenous substrates by the associated protein kinase) was measured at 35°C. The incubation medium was buffer A containing protease inhibitors, 6 mM $MgCl_2$, 50 μM γ-^{32}P-ATP (1μCi/μmole) and detection of protein-bound radioactivity was performed as reported earlier (26).

SDS-PAGE and immunochemistry: Proteins were analysed by one or two-dimensional SDS-PAGE according to Laemmli (30) and O'Farrel (31) on mini gel slabs (linear pH gradient: 4.0-6.5). Gels were either stained with Commassie Brilliant blue and dryed for autoradiography (XR films, type 3M, G.E.) or transfered onto nitrocellulose membranes (Immobilon, Millipore) for immunoblotting according to Towbin et al (32). Replicas were saturated with 5% skimmed milk powder in Tris Buffer Saline (TBS) and incubated for 3 h with the primary antibody (polyclonal or monoclonal) in TBS, 3% BSA, 0.5% polyvinyl-pyrrolidone. The washing buffer was TBS containing 0.01% Tween 20. Membranes were incubated for 3 h with secondary antibodies (anti-mouse IgG labelled with ^{125}I) in 5% skimmed milk powder in TBS. The bound radioactivity was detected by autoradiography. The intensity of autoradiograms was quantified using an image analyzer (Sapphire, Quantel Instruments, Cambridge, U.K.). ELISA were

performed according to Coleman and Anderton (29): 100 µl/well samples diluted in coating buffer (Na_2CO_3 15 mM, $NaHCO_3$ 35 mM, pH 9.6) were incubated overnight at 24°C in Costar-96-wells vinyl assay plates and washed with phosphate buffer saline containing 0.05% Tween 20 (PBS-Tween). Incubations with the monoclonal antibody RS18 (1/1000) and anti-mouse IgG coupled with peroxidase (1/2000) were made for 2 hours at 37°C in PBS-Tween 3% BSA, with extensive washing with PBS-Tween. The bound peroxidase was detected using 1 mg/ml ABTS reagent in the presence of H_2O_2 (0.03%) in 28 mM citric acid-44mM Na_2HPO_4 buffer. The reaction was detected at 540 nm (Multiskan II, EFLAB, Finland) after 1 h incubation at 37°C.

Protein measurements were conducted according to Lowry et al (33), using bovine serum albumin as a standard.

Statistical tests of significance were carried out on unpaired groups of values and compared with bilateral t tests, using the Statview Student test, version 1.03 (Abacus Concepts Inc., Berkeley, USA).

RESULTS

No significant modification of the yield in purified NF was observed between 3 and 30 month-old rats (table I). NF analyzed on two dimensional gels revealed nearly identical polypeptide patterns between the two preparations (figure 1). However, a shift of NF-H molecules from aged rat NF toward a more acidic isoelectric point than that of NF-H from young rat NF was regularly observed (figure 1). No age-induced changes of the electrophoretic properties of NF-M and NF-L molecules were observed.

The eventuality of an age-dependent hyperphosphorylation of NF-H subunits, reflected by their change in isoelectric migration, was examined by immunoblotting with phosphorylation-dependent antibodies (figure 2). Identical amounts of NF from several preparations of 3, 20 and 30 month-old rats were tested by immunoblotting with monoclonal antibodies directed against either the dephosphorylated (figure 2A) or the phosphorylated NF-H subunits (figure 2B), followed by incubation with ^{125}I-coupled second antibodies. Quantitative measurements of the corresponding spots on autoradiograms of immunoblots revealed that non-phosphorylated NF-H epitopes decreased in 20 month-old rat NF to a level which remained identical in 30 month-old rat NF preparations (figure 2A). Conversely, an age-dependent increase in the phosphate-dependent epitopes was recorded (figure 2B). However, an increase in

TABLE I

Neurofilament yield from 3 and 30 month-old rat spinal cord

Age of rats	3 months	30 months
Number of preparations (10 rats each)	3	3
mg NF/g wet weight (+/- S.D.)	0.42 +/- 0.17	0.36 +/- 0.07 (N.S.)

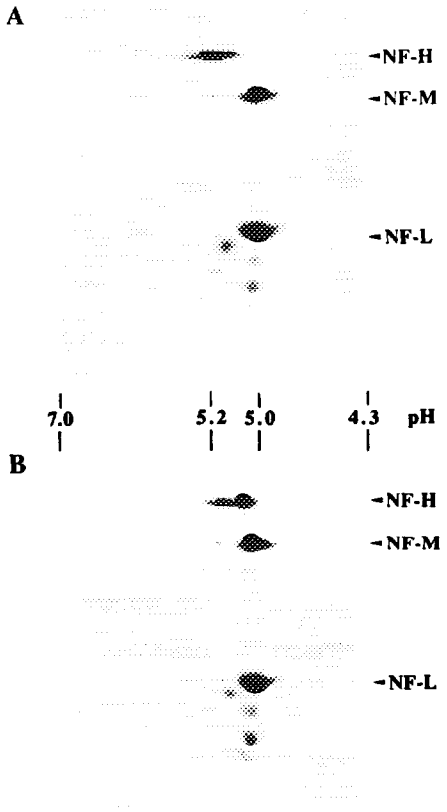

Figure 1. Two-dimension gels of purified NF from 3 (A) and 30 (B) month-old rat spinal cord. 40 µg NF from both preparations was solubilized in 9.5 M urea, 2% (vol./vol.) NP40, 5% 2β-mercaptoethanol and 2% ampholines,pH 3.5-10. Isofocalisation was made with a prerun for 2h (400V) followed by focusing for 12 h (800V). Separation in the second dimension was on 7.5% acrylamide SDS-PAGE. Proteins were detected by Commassie brilliant blue staining.

phosphorylated NF-H epitopes was measured between 3 and 20, but also between 20 and 30 month-old rat NF, respectively (figure 2B). The higher phosphorylation level of KSP repetitive sequences of NF-H in 30 month-old rat NF, relative to that of 3 and 20 month-old rat NF, was further demonstrated by ELISA with the NF-H phosphorylation-dependent monoclonal antibody RS18 (figure 2C).

The age-dependent increase of the phosphorylation level of NF-H subunits might be the consequence of a higher activity of the NF-associated protein kinase which phosphorylates the three NF subunits in vitro (34,35). However, no significant modification of the autophosphorylation of the NF suspensions was detected between NF preparations from 3 and 30 month-old rats (table II). SDS-PAGE and autoradiography of the proteins from 3 and 30 month-old rats NF phosphorylated in vitro by the NF-associated kinase revealed a similar incorporation of radioactivity in the three NF subunits (NF-M>NF-L>NF-H) of both age-groups (figure 3).

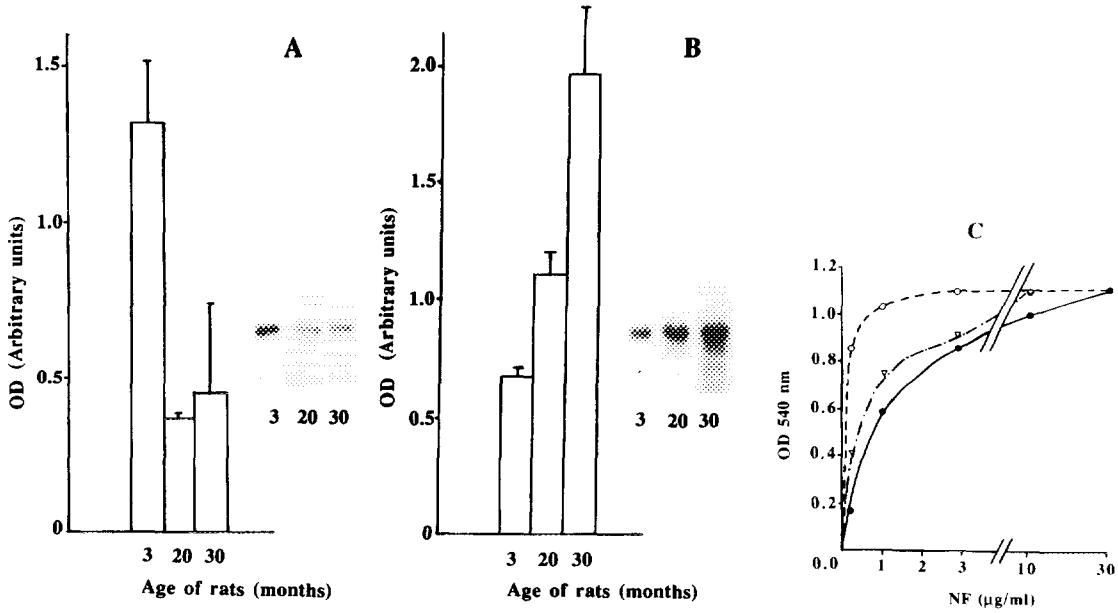

Figure 2. Age-dependent changes in dephosphorylated and phosphorylated epitopes of NF-H subunits. Identical amounts of purified NF (2.5 µg) from 3 (three preparations) (**1**), 20 (two preparations) (**2**) and 30 (three preparations) (**3**) month-old rat spinal cords were analyzed by 7.5% acrylamide SDS-PAGE and Western blotting. Bound ^{125}I-labelled anti-mouse antibodies were revealed by autoradiography, followed by quantification of the spots on autoradiograms. Histograms show means +/- S.D.
A: quantification of intensity of spots on autoradiograms of non-phosphorylated dependent NF-H epitopes detected with the monoclonal antibody RMD0-9 (P-). Amounts of P- epitopes decreased significantly between 3 and either 20 or 30 month-old rat NF ($p=0.008$ and 0.01, respectively). No significant difference was measured for P- between 20 and 30 month-old rat NF. Representative autoradiograms are shown in inset.
B: Quantification of intensity of spots on autoradiograms of phosphorylated NF-H epitopes detected by the monoclonal antibody RMO-217 (P++). Amounts of P++ epitopes differ significantly between 3 and either 20 or 30 month-old rat NF ($p=0.004$ and 0.016, respectively). Amounts of P++ epitopes differ significantly between 20 and 30 month-old rat NF ($p=0.03$). Representative autoradiograms are shown in inset.
C: ELISA quantification of phosphorylated NF-H epitopes from 3 (●), 20 (▽) and 30 (○) month-old rat NF with the monoclonal antibody RS18.

A physicochemical property of native purified NF is their association in viscous gels at a slightly acidic pH, resulting from lateral interactions between filaments through NF-H and NF-M sidearms (25). Gelation kinetics of NF from 3, 20 and 30 month-old rats after extensive dialysis against buffer A containing 0.8M sucrose are shown in figure 4A. At the low NF concentration adopted, close to the critical gelation concentration of 1.2 mg/ml (25), the viscosity reached a plateau value significantly higher in the sample from 30 month-old rat NF than that of the 3 and 20 month-old rat NF (figure 4A). No significant difference was found between the gelation kinetics of NF from 20 month-old rats and 3 month-old rats (figure 4A). Measurements of the viscosity changes of increasing NF concentrations from 3 and 30 month-old rats after a fixed incubation time showed a six to eight-fold increase in gelation velocity in 30 month-old rat NF samples, compared with 3 month-old rat NF (figure 4B).

TABLE II

Relative activities of NF-associated protein kinase from 3 and 30 month-old rat purified NF preparations

Age of rats	3 months	30 months
Number of preparations (10 rats each)	3	3
NF-associated protein-kinase (pmoles Pi incorporated/min./mg NF) (+/- S.D.)	104.31 +/- 13.22	104.14 +/- 8.25 (N.S.)

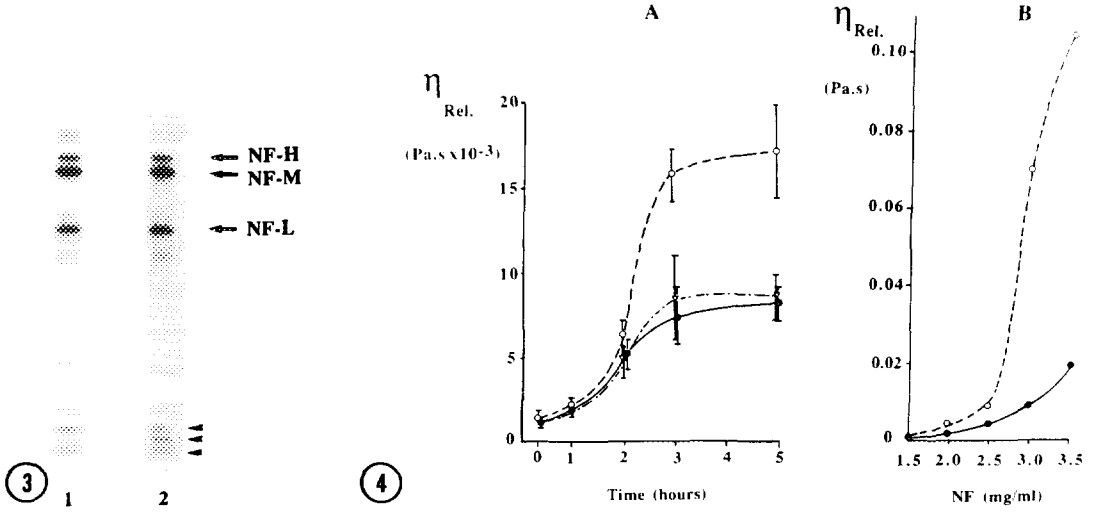

Figure 3. Phosphorylation in vitro of the three NF subunits by the NF-associated kinase is unmodified by aging: Autoradiogram of ^{32}P-labelled NF preparations from 3 (**1**) and 30 (**2**) month-old rat spinal cord analyzed on 12% SDS-PAGE after phosphorylation by the NF-associated protein kinase (0.2 mg/ml NF) for 20 min at 35°C in the presence of protease inhibitors and γ-^{32}P-ATP (see materials and methods). 5 µg proteins were loaded on gels. Small NF-associated phosphorylated polypeptides (apparent Mr: 2.4, 2.8 and 3.5 kD) are associated with both preparations. One of these labelled polypeptides is exclusively present in 30 month-old rat NF (**2**, middle arrowhead).

Figure 4. Effect of aging on viscosity kinetics of purified NF.
A: Viscosity kinetics of purified NF (1.35 mg/ml) from 3 (●), 20 (▽) and 30 (○) month-old rat spinal cords prepared simultaneously. Measurements were made in triplicates at 30°C in buffer A, 0.8M sucrose, containing 6mM $MgCl_2$ and protease inhibitors. At the low NF concentration used, no gel was obtained: the viscosity reached a plateau after 3 h of incubation. Bars indicate standard deviation of viscosity values. Both the velocity and the plateau of viscosity kinetics were significantly higher in 30 month-old rat samples ($p < 0.001$) as compared to the 20 and 3 month-old rat samples, nearly identical.
B: Measurement of the viscosity of increasing concentrations of purified NF from 3 month (●) and 30 month (○) old rats. NF viscosity was recorded after 6 h at 30°C (samples were in buffer A, 0.8M sucrose, 6 mM $MgCl_2$, protease inhibitors) after extensive dialysis (24 h at 4°C) against buffer A, 0.8M sucrose and 1mM phenylmethane sulfonylfluoride.

DISCUSSION

The comparison of physicochemical properties of purified NF from young and old rat spinal cords revealed discrete age-dependent modifications of these polymers. No significant decrease was found with aging in the relative amount of NF proteins per g of wet tissue (table I), and neither was the polypeptide pattern of purified NF modified in preparations from aged rats (figure 1). Among the three NF subunits, the pI of NF-H was specifically affected by aging, in contrast with that of NF-M and NF-L (figure 1). This finding suggests an age-dependent hyperphosphorylation of the molecule resulting in a shift in pI toward acidic values (27,36). This possibility was further demonstrated by quantitative immunoblotting. Non phosphorylated NF-H epitopes decreased strongly in 20 month-old rat NF and remained at the same low level in 30 month-old rat NF (figure 2A). In parallel, a significant increase in the phosphorylated epitopes of the same NF-H subunits occured between 3 and 20, and also between 20 and 30 months of age (figure 2B,C). The hyperphosphorylated sites which appear in NF-H between 3 and 20 and between 20 and 30 months of age include phosphorylated KSP epitopes detected by the monoclonal antibody RS18 (figure 2C). These findings suggest that a constant addition of phosphates with time on the NF-H molecules occurs from the early adulthood, resulting in the NF-H hyperphosphorylation level observed in very old animals. The present results in vitro confirm and extend earlier findings that NF are hyperphosphorylated in aging tissues (1-5).

The distribution of the sites on NF-H sidearm which become phosphorylated between 20 and 30 months of age is unknown. Nevertheless, in vitro phosphorylation sites for the NF-associated kinase are identical in all subunits of both NF from 3 and 30 month-old rats (figure 3 and Table II). Thus, the phosphorylation level in situ of the sites in NF-H which are substrates of the NF-associated kinase in vitro does not increase with aging, possibly as a consequence of a high turnover of their phosphate groups (15). From the present results, it is likely that the high NF-H phosphorylation level of aged rat NF (figure 2) results from the activity of kinases other than the NF-associated enzyme. Recent reports point at a direct role for a NF-H associated kinase (37) and cdc2-like kinases (14,38) in phosphorylating efficiently NF-H. A cdk5 kinase isolated from the nervous system was characterized, which phosphorylates a KSPXKX sequence in NF-H (39,40). In addition, other phosphorylated regions of the NF-H sidearm containing the sequence KSPXXX might be phosphorylated by another still unknow kinase (41). A hypothesis which remains to be investigated is either the possible increasing activity of such enzymes in neurons from aging animals or the lower efficiency of phosphatases affecting in situ the turnover of phosphates in specific sites of NF-H.

NF from 30 month-old rats form gels in vitro more efficiently than NF from 3 and 20 month-old rats (figure 4). This could be the consequence of the high phosphorylation level of NF-H subunits in 30 month-old rat NF (figures 1 and 2). The rate of NF gelation in samples from 3, 20 and 30 mont-old rats (figure 4A) parallels the relative amount of phosphorylated KSP epitopes of NF-H detected by the monoclonal antibody RS18 (figure 2C). Opposit effects on NF gelation kinetics were shown to result from the removal of distinct sets of phosphate groups on NF subunits, which differ in their accessibility to phosphatases in vitro (18). These

indications in vitro favor a model of the control of NF interactions by distinct phosphorylation sites which activate or inhibit the interactions between NF, depending on their location on NF subunits (18). Accordingly, a possible interpretation of the present results might be that the large increase in gelation kinetics of 30 month-old rat NF compared with that of 3 and 20 month-old rat NF (figure 4A) is the direct consequence of the incorporation of phosphates in NF-H which occurs exclusively between ages of 20 and 30 months (figure 2B,C).

In conclusion, the present study demonstrates the increase of NF-H hyperphosphorylated isoforms with aging. NF-H hyperphosphorylation in situ occurs in parallel with a higher efficiency in NF gelation in vitro. This mechanism might contribute to the abnormal accumulation of hyperphosphorylated NF in aging nervous tissues (1,5). Whether this process could also participate in similar NF accumulations in neurodegenerative situations remains to be investigated.

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