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Compartmental protein expression of Tau, GSK-3β and TrkA in cholinergic neurons of aged rats

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Summary During aging basal forebrain cholinergic neurons (BFCNs) degenerate, and we hypothesize this to be the result of a degeneration of the cytoskeleton. As a corollary, retrograde transport of the complex of nerve growth factor (NGF) and its activated receptor phospho-TrkA (P-TrkA) is impaired. Using immunocytochemistry, we here compare young and aged rat brains in their subcellular localization of NGF and P-TrkA in relation to the compartmentalization of phosphorylation-dependent tau protein isoforms. Despite lower P-TrkA immunoreactivity in cortex and hippocampus of aged rats, NGF immunoreactivity was not altered in these areas, but was significantly lower in aged basal forebrain. In young animals, expression of tau isoforms and glycogen synthase kinase-3β (GSK-3β) was restricted to neuritic structures in cortex, hippocampus, and basal forebrain. In contrast, tau and GSK-3β labeling was confined to cell bodies in aged rats. Since a somatic localization of phospho-tau is indicative of cytoskeletal breakdown, we suggest this to be the mechanism the breakdown of trophic support in aging BFCNs.

Keywords: Aging, cholinergic neurons, NGF, axonal transport, cytoskeleton, tau, neurodegenerative diseases

Introduction

Nerve growth factor (NGF) is produced in a number of different hippocampal and cortical neurons. Following release, NGF diffuses to presynaptic axons terminals of neurons such as the basal forebrain cholinergic neurons (BFCNs), where it binds to TrkA (a receptor of tyrosine kinase for NGF), including its dimerization and autophosphorylation, and presents the formation of a protein complex involved in its signaling pathways (Huang and Reichardt, 2003). Current models of Trk receptor signaling pathways include complex networks with extensive cross-talk and multiple retrograde signaling mechanisms are likely to coexist and interact. Prominent among these is the signaling endosome model (Campenot and MacInnis, 2004; Delcroix et al., 2004; Saxena et al., 2004). An endosome containing activated Trk may serve as a signaling platform as it is shuttled from the distal axon to the cell body via a microtubule-based transport mechanism (Watson et al., 1999). The retrograde transport of signaling endosomes from axon to the perikaryon appears to rely on dynein-microtubules, in which TrkA is linked directly to the motor through the dynein intermediated chain (Bhattacharyya et al., 2002; Yano et al., 2001; Yano and Chao, 2005). Microtubule depolymerizing agents that block dynein-based transport also markedly disrupt the retrograde movement of both neurotrophin and activated Trk receptors (Watson et al., 1999; Sandow et al., 2000; Yano et al., 2001).

Considerable evidence supports the contention that deficient long-range neurotrophin signaling from distal axons to cell bodies may be causally related to a variety of neurodegenerative disorders (Kruttgen et al., 2003; Delcroix et al., 2004; Schliebs, 2005). However, neither NGF levels nor expression and binding to its receptor were decreased in aged or Alzheimer brains (Boissiere et al., 1997; Dubus et al., 2000; Costantini et al., 2005). Nevertheless, aging is paralleled by reductions in cholinergic markers which critically depend on functional NGF signaling (Niewiadomska et al., 2000). It is therefore suggested that BFCNs may not be lost but due to lack of trophic support down-regulate

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expression of markers used to identify them (Niewiadomska et al., 2002). Taken together, the data invite the hypothesis that impaired transport of NGF and its receptors contribute significantly to the degeneration of BFCNs.

Another element of reduced NGF signaling could arise from the age-related breakdown of the cytoskeleton in neurons (Su et al., 1997; Castejon and Acurero, 2004; DiProspero et al., 2004; Santa-Maria et al., 2005). This includes the principal components of the microtubule system, the transport units involved in active shuttle of organelles and molecules in an antero- and retrograde manner, and the proteins stabilizing the cytoskeleton and providing trophic support. Microtubule-associated proteins, such as tau, modulate the extent and rate of microtubule assembly and play an essential role in morphogenetic processes, such as axonal growth. Tau increases the rate of tubulin polymerization, decreases the rate of transit into the shrinking phase, and inhibits the rate of depolymerization. Tau strongly suppresses the catastrophe rate, and its ability to do so is independent of its ability to increase the elongation rate. Thus, tau generates a partially stable but still dynamic state in microtubules. This state is perturbed by metabolic modification of tau (Drechsel et al., 1992; Mukaetova-Ladinska et al., 2000; Mandelkow and Mandelkow, 1998; Gotz, 2001; Buee and Delacourte, 2001; Avila et al., 2004). Tau pathology is a central neuropathological characteristic of a number of neurodegenerative disorders that are characterized by dementia and are now collectively known as tauopathies (Lai et al., 1995; Wischik et al., 1996). Representative examples of this heterogeneous group of neurodegenerative disorders include AD, Down's syndrome, progressive supranuclear pulsy, Pick's disease, corticobasal degeneration, sporadic frontotemporal dementia, familial frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), postencephalic parkinsonism, and several variants of prion diseases (Avila et al., 2004). In each disease tau is hyperphosphorylated and in a filamentous form. The neurofibrillary tangles in neuronal cell bodies and the neuropil threads in dendrites and axons each consist of fibrillar structures, which have been shown by electron microscopy to be paired helical or straight filaments. These structures are composed largely of hyperphosphorylated tau (Ferrer et al., 2005; Iqbal and Grundke-Iqbal, 2005; Iqbal et al., 2005). Several possibilities exist of how hyperphosphorylated tau could influence neurotrophin signaling. The simplest is that neurofibrillary tangles physically block transport of the neurotrophin signals. Alternatively, hyperphosphorylated tau could compromise both microtubule stability and function, resulting in a decrease of transport (Amos, 2004; Feinstein and Wilson, 2005; Mendieta et al., 2005) and this would include TrkA-NGF complexes.

Several protein kinases are known to act on tau (Ferrer et al., 2005). One that contributes to tau hyperphosphorylation in AD is glycogen synthase kinase-3β (GSK-3β). It is associated with microtubules (Kaytor and Orr, 2002; Kosik and Shimura, 2005) and immunostaining for GSK-3β labels neurofibrillary tangles (Flaherty et al., 2000; Yamaguchi et al., 1996). There is some evidence to suggest that levels of GSK-3β are increased in the AD brains (Baum et al., 1996; Imahori and Uchida, 1997) and that imbalance in GSK-3β activation could compromise neurotrophin signaling. The abnormal tau phosphorylation that results from increased activity of GSK-3β could lead to sequestration of tau, inhibition of microtubule formation, destabilization of existing cytoskeleton, and promotion of assembly of large tau-containing aggregates. These would inhibit both anterograde and retrograde microtubule-based transport.

The present study examined this mechanism to link failed neurotrophic factor signaling to age-dependent cytoskeletal degeneration of the basal cholinergic neurons. We propose these dystrophic changes in BFCNs to be due to destabilization of the cytoskeleton and protein transport in these neurons. It seems intuitively plausible that disturbances in tau protein metabolism should be viewed as an initial process of cytoskeletal breakdown. In an attempt to test this assumption we examined expression and cellular compartmentalization of phosphorylated tau isoforms and GSK-3β kinase, as well as βNGF and phospho-TrkA proteins in basal forebrain areas and cholinergic projection targets in cortex and hippocampus. Our results provide evidence that pattern of cellular localization of phospho-tau isoforms and GSK-3β, which regulates tau phosphorylation, is profoundly influenced by age. Furthermore, we report that expression of NGF and the activated isoform of its receptor P-TrkA is different in young and aged rats. These observations may provide insight into mechanisms underlying the relationship between age-dependent deterioration of retrograde transport of NGF-P-TrkA complex and the status of microtubule system in cholinergic neurons.

Materials and methods

Animals

Male Wistar rats (The Animal Farm of the Nofer Institute, Lodz, Poland) 4 ($n = 12$) and 28 ($n = 12$) month of age were used in this study. All animals were housed in an environmentally controlled room (temperature 23 ± 1°C, 12 h light/12 h dark cycle) in groups of four per cage with food and water

ad libitum. This study was conducted with approval of the local Ethics Commission (Polish Law on the Protection of Animals) and was carried out in accordance with the Principles of Laboratory Animals Care (NIH publication No 86-23). All efforts were made to minimize the number of animals used and their suffering.

Perfusion and tissue processing

Animals were deeply anesthetized with Nembutal and the perfusion procedure was begun when all reflex responses to cutaneous stimulation were absent. Generally, tissues were fixed by intraaortic perfusion with cold (4°C) 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and then followed by phosphate-buffered 5% glycerol with 2% DMSO in phosphate buffer. The brains were removed and placed for 1 h in the fixative solution and then immersed for cryoprotection in 10% followed by 20% glycerol + 2% DMSO in phosphate buffer. All perfusion solutions contained 1 mM sodium ortovanadate (Na_3VO_4) to inhibit endogenous phosphatases. Such a procedure is essential for the correct assessment of investigations on phospho-isoforms of proteins because the binding sites of phospho-epitopes are rapidly dephosphorylated postmortem. Functional conclusions on phospho-TrkA and -tau protein require an accurate assessment of phosphorylated sites. For immunohistochemical analysis, brains were positioned in a brain matrix (BAS Inc., Lafayette, USA) and evenly sliced into uniform blocks. Sections were cut coronally through the forebrain at 40 μm thickness with a cryostat (Microm Int., Germany). Consecutive sections were collected throughout BF and hippocampal region (from 1.0 mm anterior to −4.30 mm posterior to bregma; Paxinos and Watson, 1997) of each rat. From eight series of the sections collected, one set was processed for βNGF immunoreactivity (IR), the second set for phospho-TrkA IR, the third and fourth for phospho-tau isoforms IR, the fifth for glycogen synthase kinase-3β IR, the sixth and seventh for choline acetyltransferase (ChAT) and high-affinity NGF receptor (TrkA) IR respectively, and the last one was cresyl violet Nissl staining to evaluate neuroanatomical brain structures and morphology of neurons.

Antibodies and Immunohistochemistry

For immunohistochemical determination of βNGF, a rabbit polyclonal antibody commercially supplied by Chemicon Int. Inc. was used at 1:500 dilution. The free-floating sections were incubated in 0.1 mM phosphate-buffered saline (PBS) pH7.4 with the primary antibody, with continuous agitation at room temperature overnight and subsequently were similarly incubated in a goat anti-rabbit biotin conjugated IgG (diluted 1:1000, Chemicon Int. Inc.) for 1 h followed by peroxidase conjugated streptavidin (diluted 1:1000, The Binding Site Lmt.) for 1 h. Following a brief (5 min) reaction with 0.05% diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide the sections were mounted on glass microscope slides, air-dried, dehydrated in alcohol/xylene serial solutions and coverslipped with permanent mounting media. After each incubation sections were washed 3 times for 5 min. For P-TrkA immunodetection sections were processed with rabbit anti-tyrosine kinase polyclonal antibody (diluted 1:200, Santa Cruz Biotechnology Inc.) raised against an amino acid sequence containing phosphorylated Tyr 496 of TrkA.

Immunohistochemistry for tau was performed using the rabbit polyclonal anti-tau AT180 (diluted 1:50, Innogenetics, Gent) and P-TauS404 (diluted 1:200, Santa Cruz Biotechnology Inc.) antibodies. Both antibodies recognize tau in a phosphorylation-dependent manner. AT180 recognizes tau phosphorylated at Thr 231 and Ser 235, P-TauS404 recognizes tau phosphorylated at Ser 404. The formation of neurofibrillary tangles in Alzheimer's disease is preceded by a pretangle stage of hyperphosphorylated tau. To characterize pretangle tau *in vivo*, we correlated, in our young and aged rats, levels of kinase GSK-3β known to phosphorylate tau in PHFs in Alzheimer's brains with the phosphorylation of tau at specific epitopes and compared localization of staining in axonal and somato-dendritic compartments of neurons. For immunohistochemical detection of GSK-3β, a rabbit polyclonal antibody (diluted 1:200, Santa Cruz Biotechnology Inc.) was used.

For P-TrkA, phospho-tau isoforms, and GSK-3β, bound antigens were visualized using two different methods. One was similar to the procedure applied for βNGF, in which biotin-streptavidin-peroxidase was combined with DAB and H_2O_2. The second one utilized the fluorescent dye fluorescein (FITC) conjugated with streptavidin-peroxidase (diluted 1:500, Vector Staining, Inc.). Sections from all groups were incubated in the same bath to avoid group inter-variability in staining. Controls for the immunohistochemical procedure were obtained running some slides through the entire procedure with the omission of the primary or secondary antibodies as a safeguard against nonspecific staining by the primary antibody. No staining was observed in these control slides. The cellular patterns and intensities of staining reactions obtained with the various antibodies were estimated using bright field and fluorescence microscopy (Nikon Eclipse E400).

Results

Immunohistochemistry of NGF in hippocampus, cortex, and basal forebrain in young and aged rats

In order to determine age-related changes in NGF protein expression we stained brain sections with anti-βNGF antibody (Fig. 1). Sections show no differences in hippocampal and cortical NGF staining in animals of both age-groups (Fig. 1).

However, there was a difference between the NGF staining in young and aged animals in the basal forebrain cholinergic structures, i.e. medial septal nucleus (MS) projecting to hippocampus, and magnocellular basal nucleus (NBM) issuing cholinergic fibers to different cortical sites (Fig. 1). The density of NGF expressing cell bodies in MS and NBM of aged rats was markedly lower relative to the young controls. The intensity of NGF staining in BFCN neurons was also decreased in aged rats. This confirms that NGF synthesis in target areas of cholinergic projections is not altered with aging.

Age-dependent changes in protein expression of P-TrkA in hippocampus, cortex, and basal forebrain

Since protein expression of NGF in hippocampal and cortical neurons is not altered during aging, but NGF immunoreactivity was reduced in BFCN, we explored the possibility that axonal transport involved in retrograde NGF signaling may be impaired in aged neurons. Towards this end, we labeled the activated isoform of TrkA receptor, phospho-TrkA.

We found that aged rats differed from the young animals in terms of cellular P-TrkA staining (Fig. 2) in brain regions analyzed. The intensity of P-TrkA immunostaining was much stronger in hippocampus and cortex from young rats compared to that in brains from aged animals (Fig. 2).

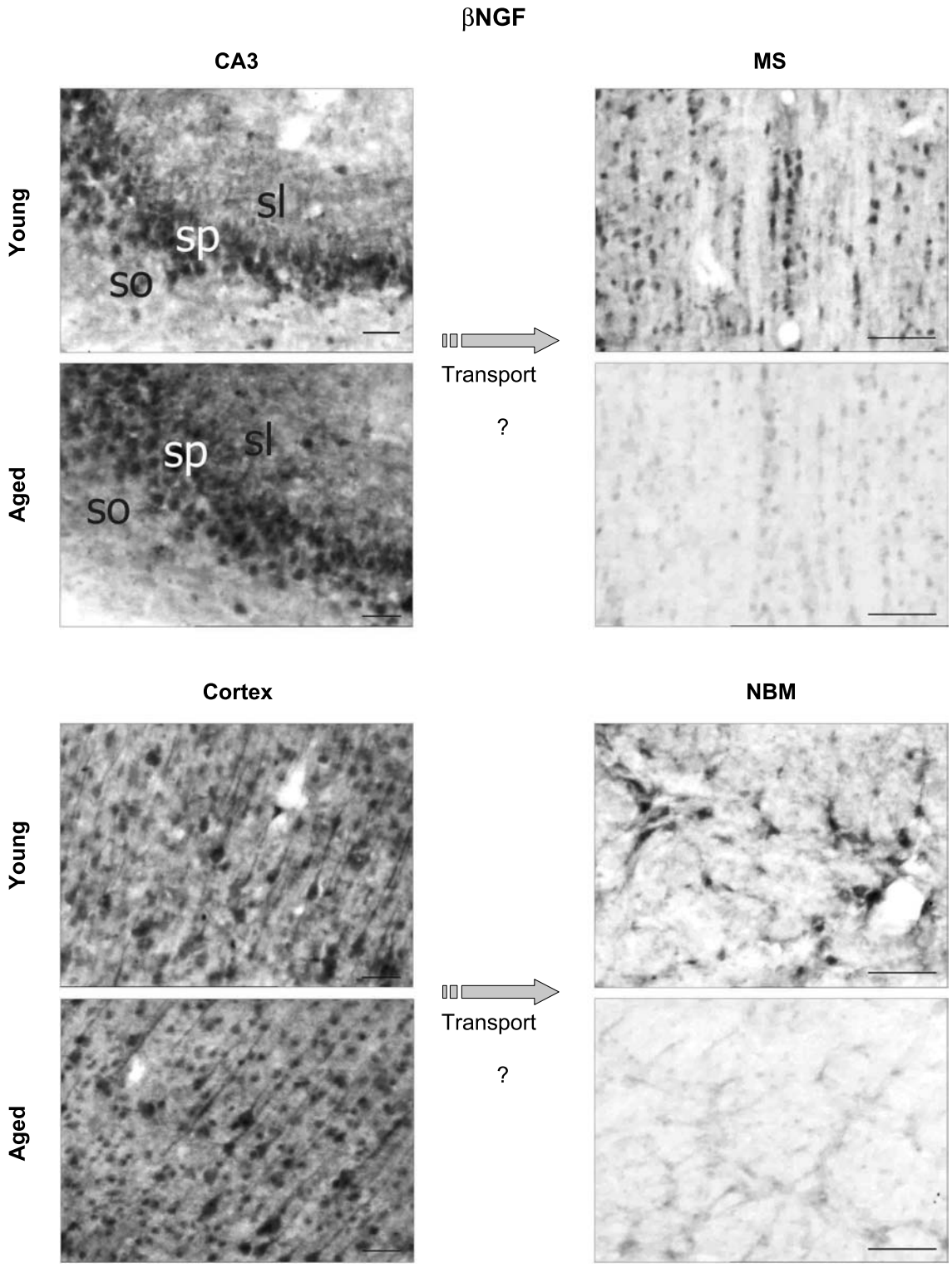

Fig. 1. Protein expression of βNGF in hippocampus, cortex and basal forebrain in young and aged rats. Immunoreactivity of NGF in hippocampal CA3 (top) and in parietal cortex (bottom) of aged rats was not altered when compared to the young controls. However, NGF expression in medial septal nucleus (MS, top) and in magnocellular basal nucleus (NBM, bottom) was significantly lower in aged brain. so *stratum oriens*, sp *stratum pyramidale*, sl *stratum lucidum*. Bars = 50 μm in cortex and hippocampus and 100 μm in MS and NBM

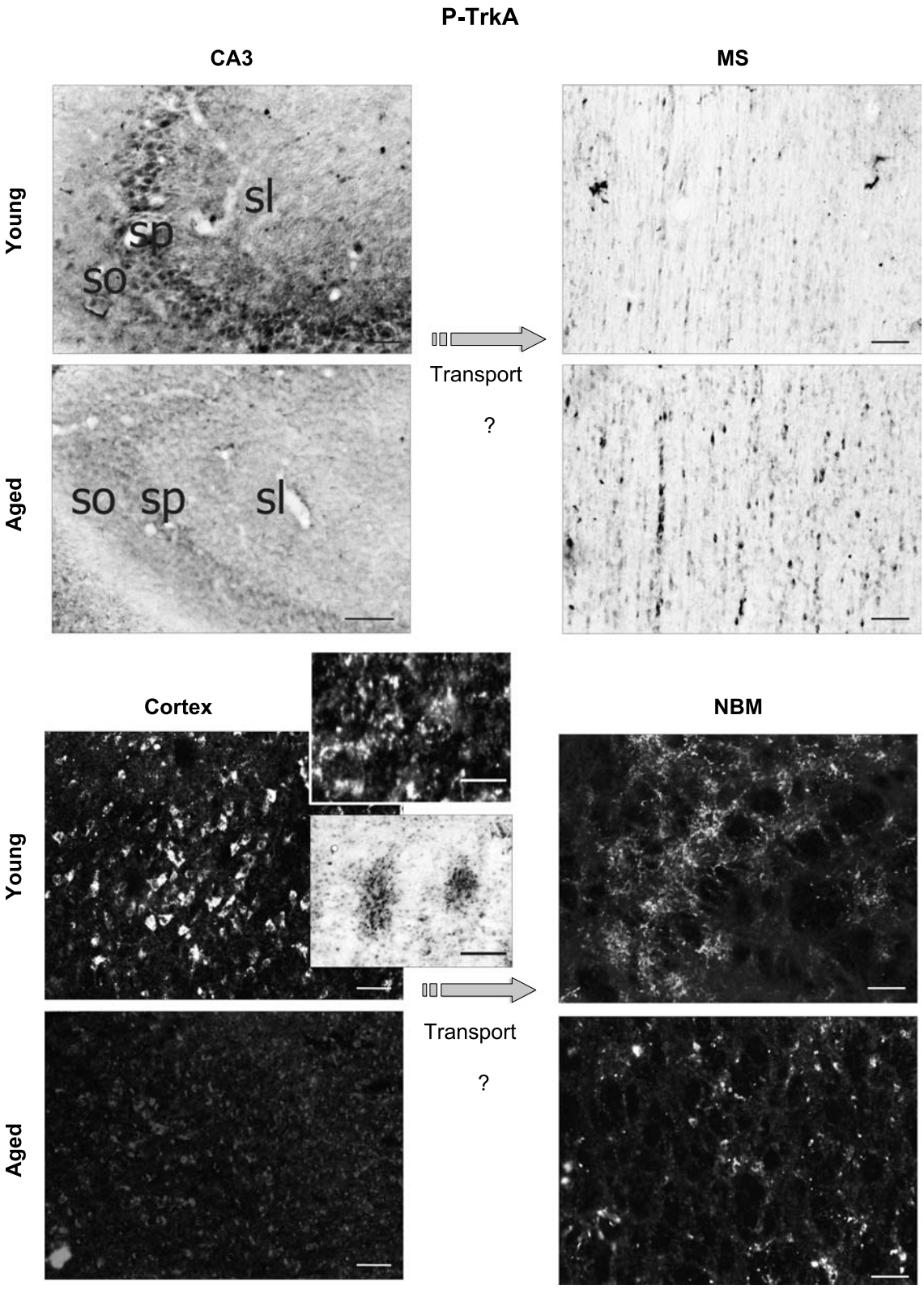

Fig. 2. Protein expression of P-TrkA receptor in hippocampus, cortex and basal forebrain in young and aged rats. P-TrkA immunoreactivity was revealed with diaminobenzidine – DAB (top) and with fluorescein – FITC (bottom). In young but not in aged animals, intensive P-TrkA staining was present in the neuropil of *stratum pyramidale* (sp) and *stratum oriens* (so) in hippocampal CA3 area (top left) and in the pyramidal cell bodies and their apical dendrites in cortex (bottom left and insertions). In medial septal nucleus (MS, top right) and in magnocellular basal nucleus (NBM, bottom right) of young rats cell bodies were free of P-TrkA staining, whereas in aged animals some cellular staining was observed. Inserts present higher magnification of FITC and DAB staining. sl *stratum lucidum*. Bars = 50 μm in cortex and hippocampus, 100 μm in MS and NBM, and 10 μm in insertions

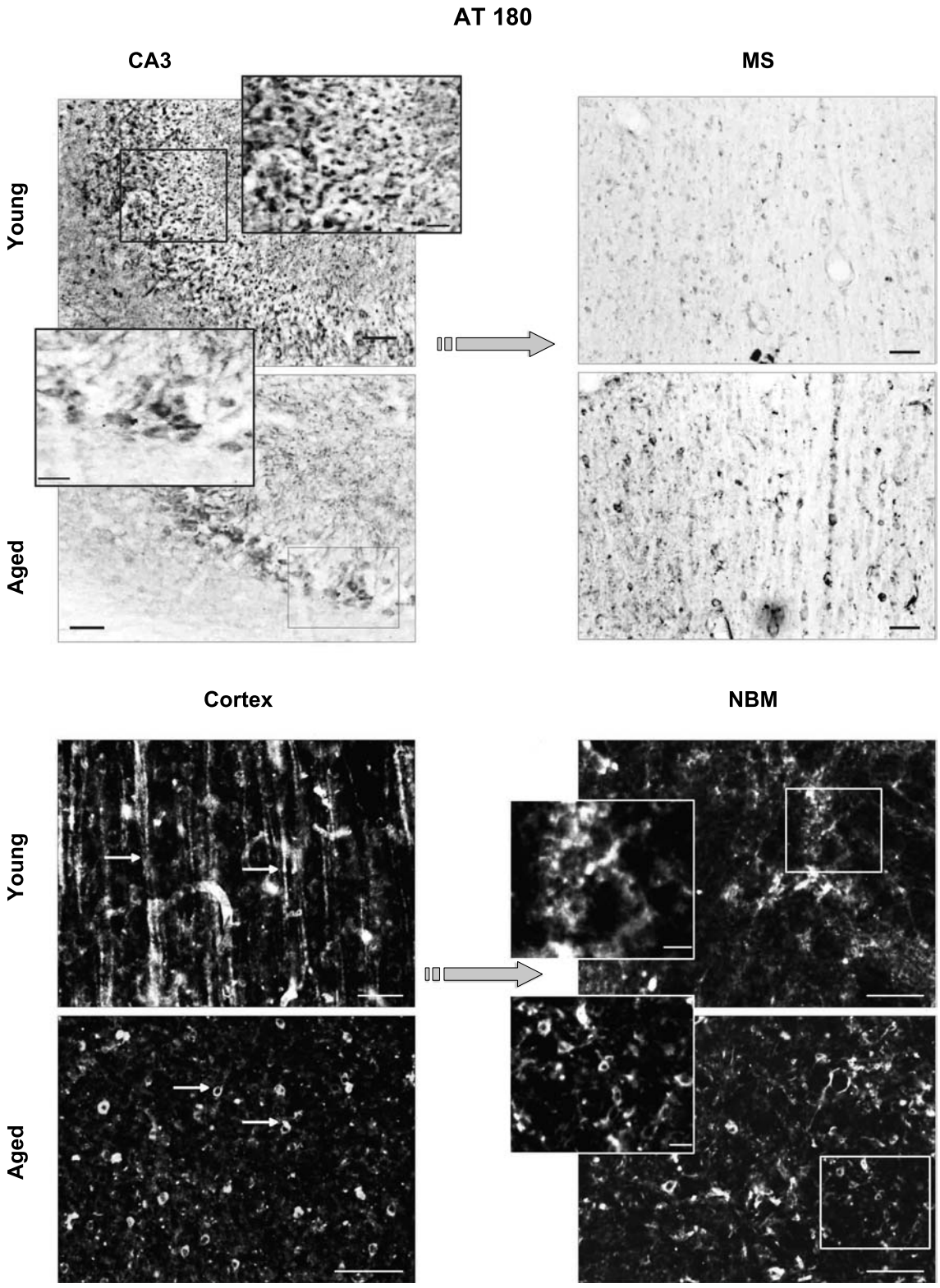

Fig. 3. Protein expression of AT180 phospho-tau isoform in hippocampus, cortex and basal forebrain in young and aged rats. AT180 immunoreactivity was revealed with diaminobenzidine – DAB (top) and with fluorescein – FITC (bottom). In young animals, AT180 staining was present in neuropil of hippocampal CA3 area, while in aged rats AT180 immunolabeling appeared exclusively in neuronal cell bodies (top left). Immunostaining for AT180 in cortex revealed similar changes of tau localization (bottom left, arrows). In medial septal nucleus (MS, top right) and in magnocellular basal nucleus (NBM, bottom right) of young rats cell bodies were free of AT180 staining, whereas in aged animals cellular staining was observed. Inserts present higher magnification of the area framed. Bars = 50 μm in cortex and hippocampus, 100 μm in MS and NBM, and 10 μm in insertions

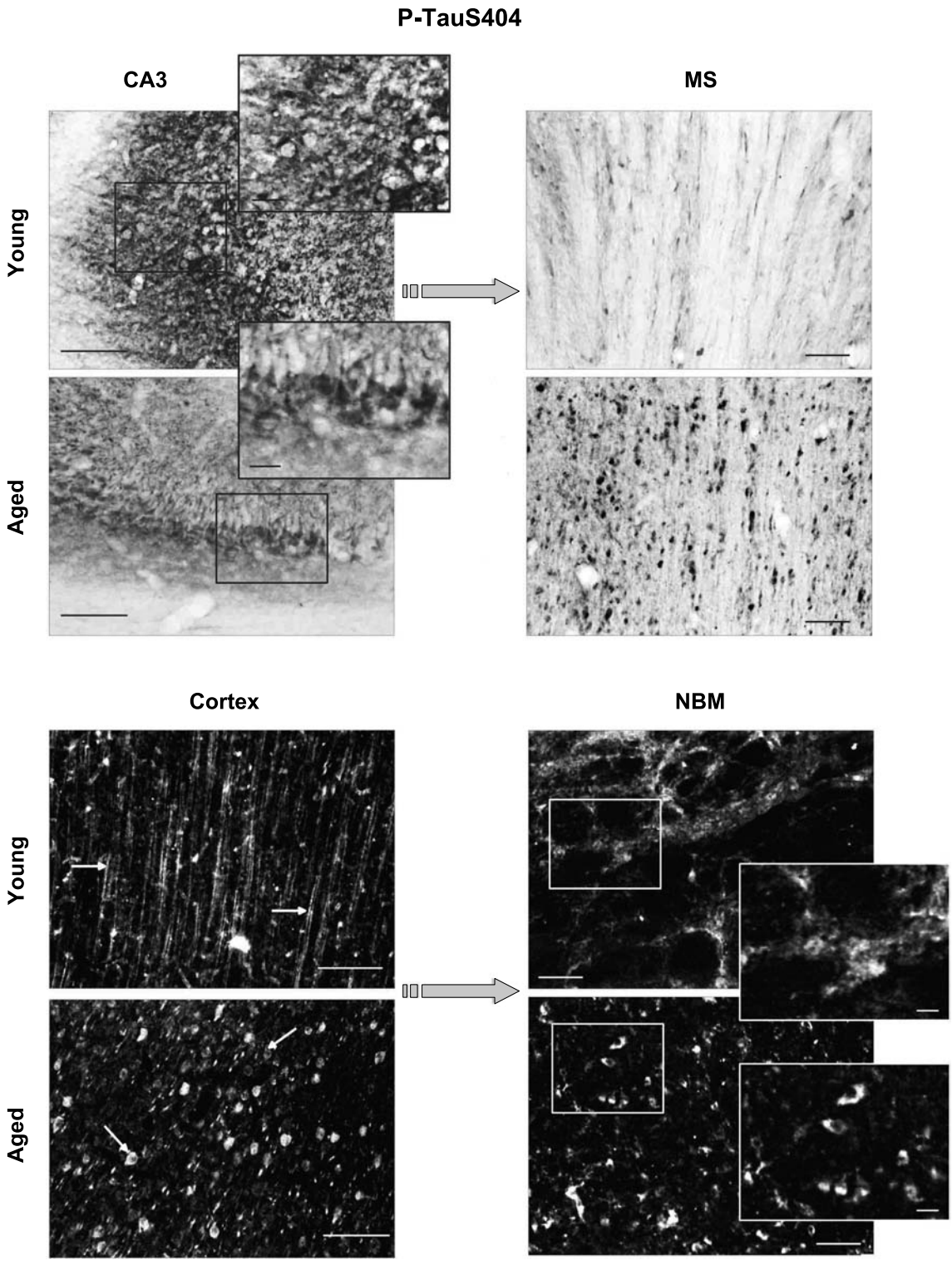

Fig. 4. Protein expression of P-TauS404 phospho-tau isoform in hippocampus, cortex and basal forebrain in young and aged rats. P-TauS404 immunoreactivity was revealed with diaminobenzidine – DAB (top) and with fluorescein – FITC (bottom). In young animals, intense P-TauS404 staining was present in the neuropil of hippocampal CA3 area, whereas in aged rats P-TauS404 immunolabeling appeared exclusively in neuronal cell bodies (top left). Immunostaining for P-TauS404 in cortex revealed similar changes of tau localization (bottom left, arrows). In medial septal nucleus (MS, top right) and in magnocellular basal nucleus (NBM, bottom right) of young rats cell bodies were free of P-TauS404 staining, whereas in aged animals cellular staining was observed. Inserts present higher magnification of the area framed. Bars = 50 μm in cortex and hippocampus, 100 μm in MS and NBM, and 10 μm in insertions

Pyramidal neurons which are present in the hippocampal CA3 area and in the layer V of parietal cortex of young rats exhibited perinuclear and cytoplasmic diffuse P-TrkA immunoreactivity. High-power magnification in both light and fluorescence microscopy revealed P-TrkA staining as punctate immunoreactivity distributed along the cytoplasmic surface of the cells (Fig. 2, insertions). This pattern is reminiscent of somatic nerve endings of projecting axons. These P-TrkA IR deposits were, in general, not detected within hippocampus and cortex of aged rats. Instead we observed some intracellular P-TrkA staining in MS and NBM of aged rats, but no P-TrkA IR was observed in cell bodies of basal forebrain neurons in young animals (Fig. 2).

Distribution of phosphorylated tau in the cellular compartments of neurons in hippocampus, cortex, and basal forebrain in young and aged rats

Since it is conceivable that the age-related degeneration of cytoskeleton in BF cholinergic neurons renders the NGF-TrkA signaling system non-functional we compared the cellular compartmentalization of phosphorylated tau protein in hippocampal and cortical neurons as well as in basal forebrain neurons of both young and aged rats. Phosphorylation of tau protein can be detected by the AT180 and P-TauS404 antibody and this is associated with tau pathology (Spillantini and Goedert, 1998). Both antibodies recognize PHF-tau and tangles, the major fibrous components of neurofibrillary lesions associated with Alzheimer's disease (Mahmoud et al., 1996; Steiner et al., 1990).

Figures 3 and 4 show that the pattern of AT180 and P-TauS404 immunoreactivity was different in young and aged rats. These differences refer to all brain regions analyzed. The AT180 and P-TauS404 in the hippocampus of young adult rats was confined mainly to the neuropil of the *stratum oriens* in the area CA3. By contrast, there was no detectable IR in the CA3 pyramidal cell layer (Figs. 3 and 4).

In aged rats AT180 and P-TauS404 IR was distributed preferentially in the cell bodies of hippocampal CA3 pyramidal neurons, whereas the neuropil in the stratum oriens did not display strong tau immunostaining (Figs. 3 and 4, top left). In agreement with these findings, AT180 and P-TauS404 labeling in the MS of young animals was absent (Figs. 3 and 4, top right), whereas in aged rats both antibodies AT180 and P-TauS404 preferentially stained somata of large MS neurons with the characteristic morphology of cholinergic neurons. A similar distribution of AT180 and P-TauS404 immunoreactivity was observed in cortex (Figs. 3 and 4, bottom left) and NBM (Figs. 3 and 4, bottom right) of young and aged rats.

In general, in young rats two tau protein isoforms were found predominantly in dendrites and axonal processes, whereas in aged rats tau protein was almost exclusively localized to the neuronal cell bodies. The greater intensity of cytoplasmic immunoreactivity in aged rats suggest that tau protein is sequestered from the axo-dendritic compartment (in young tissue) to cell soma during aging. This implies that the microtubule binding capacity of tau protein may be reduced in aged brain tissue.

Immunohistochemical analysis of kinase GSK-3β protein expression in hippocampus, cortex, and basal forebrain in young and aged rats

The biological activity of the phosphoprotein tau is regulated by its degree of phosphorylation. GSK-3β is serine/threonine, proline directed kinase involved in a diverse array of signaling pathways and is implicated in Alzheimer's disease (Hernandez et al., 2003). Six tau protein isoforms have been identified, all of which are phosphorylated by GSK-3β. Phosphorylation of tau protein by GSK-3β at serine 404 is primarily responsible for the functional loss of tau-mediated tubulin polymerization and tau dissociation from the microtubules. In trying to elucidate GSK-3β effect on phosphorylation of tau we examined the relationship between tau isoforms and GSK-3β distribution in neuronal compartments of young and aged rats. Fig. 5 summarizes differences in GSK-3β IR in cortex, hippocampus and basal forebrain of both age groups.

Similar to the age-related redistribution of TrkA and tau, GSK-3β IR was concentrated in the cell bodies in aged, and in soma and processes in young animals. Although there was a week staining of GSK-3β-positive cell bodies in cortical neurons of young rats, their dendrites showed much stronger immunolabeling forming a characteristic striping pattern (Fig. 5, bottom, left). In contrast, GSK-3β staining in aged rats was greatly enriched in the soma and devoid of labeling in neuronal fibers. The age-dependent redistribution of GSK-3β in hippocampal neurons was also similar to that observed in neocortex (Fig. 5, top left). By contrast, GSK-3β immunostaining in basal forebrain structures was week in neuronal processes in young and old subjects, but strong GSK-3β IR was obtained in the perikarya of aged rats (Fig. 5, right). These changes in compartmentalization of GSK-3β kinase are in accord with the translocation of tau from the axo-dendritic compartment in young rats to the perikaryal cytoplasm in aged rats.

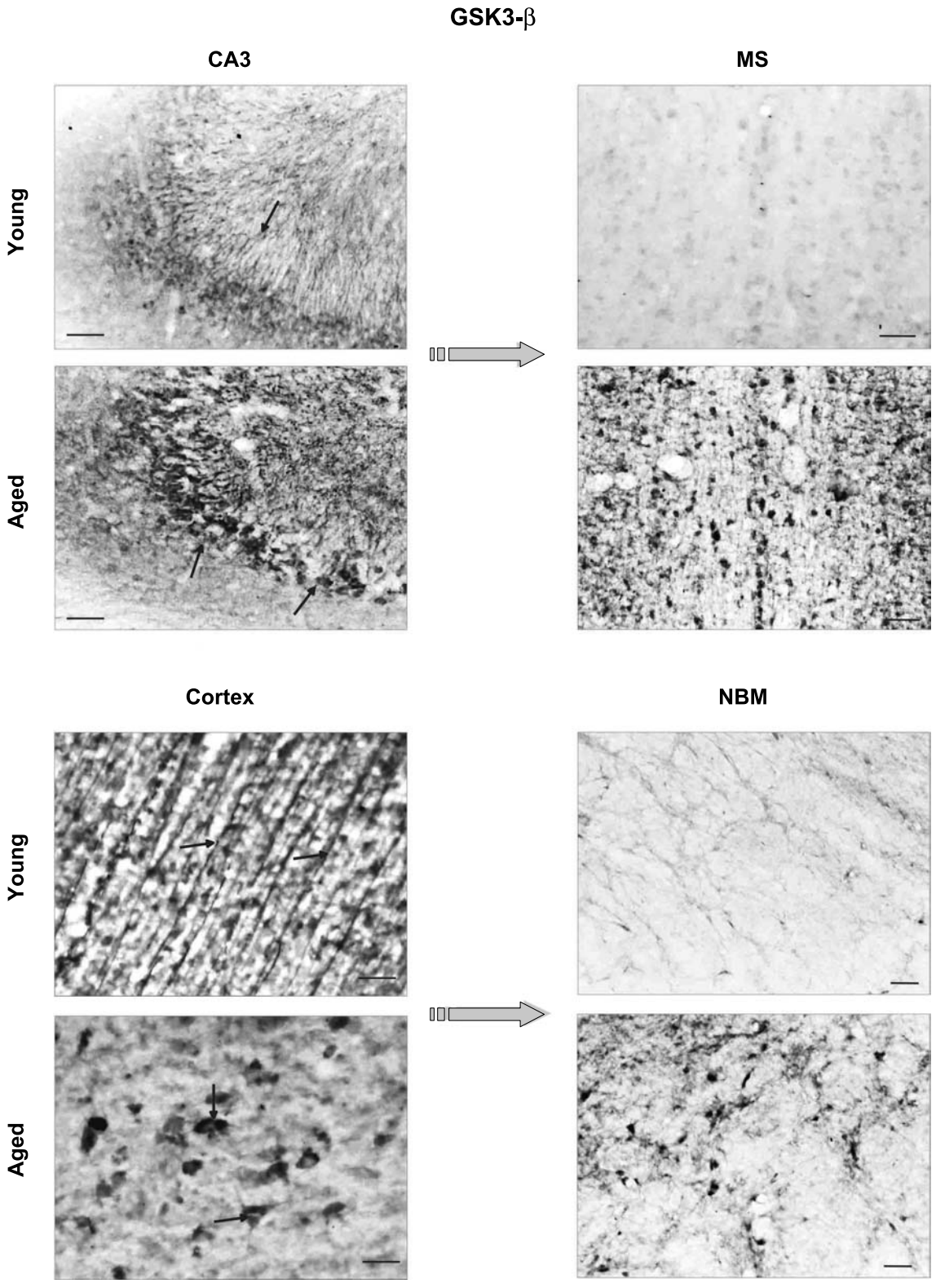

Fig. 5. Protein expression of GSK-3β kinase in hippocampus, cortex and basal forebrain in young and aged rats. In young rats, GSK-3β immunoreactivity was localized mainly in fibers with some light staining of cell bodies both in hippocampal CA3 area (top left, arrows) and in cortex (bottom left, arrows). In aged animals, GSK-3β was restricted to the neuronal somata both in hippocampus and cortex. In medial septal nucleus (MS, top right) and in magnocellular basal nucleus (NBM, bottom right) of young rats no GSK-3β immunoreactivity was expressed, whereas in aged animals cellular staining was clearly evident (right vertical panel). Bar = 20 μm in cortex of young rats, 10 μm in cortex of aged rats, 50 μm in hippocampus, and 100 μm in MS and NBM

Immunohistochemistry for cholinergic neurons in the basal forebrain

To determine whether cholinergic parameters were changed with age, we evaluated staining density of the acetylcholine synthesis enzyme ChAT and non-phosphorylated TrkA receptor (specific cholinergic neuronal markers) in the basal forebrain of young and aged rats. Data (not shown) of ChAT and TrkA immunoreactivity were consistent with that of our previous study (Niewiadomska et al., 2002) and revealed an overall lower ChAT and TrkA staining intensity in cell bodies as well as in fibers of aged rats. Compared to the young control brains, a substantial depletion of cholinergic neurons was observed within the basal forebrain of all aged brains. These results suggest a general down regulation of cholinergic activity as well as diminished TrkA expression and are indicative of a loss of cholinergic markers in the aged BFCN neurons.

Discussion

Collectively, the data suggest that aging coincides with a progressive reduction in cytoskeletal function as revealed by a redistribution of tau protein and related enzymes. Such cytoskeleton breakdown impacts, especially in cholinergic pathways, on the anterograde and retrograde signaling of neurotrophin receptors, such as TrkA, and fails to maintain NGF-mediated support. We thus hypothesize that reduction in cholinergic transmission in aging is a corollary of destabilization of the cytoskeleton. The evidence for this notion in terms of results presented here is:

1. Tau is hyper-phosphorylated in the aging brain

Since neurons are highly elongated cells they depend on efficient transport systems for delivery of proteins, lipids, and other components generated in the soma and transported to the synapse. Transport is based on microtubules which serve as tracks, on motor proteins which represent the engines, on vesicles and organelles which are the cargoes, and on microtubule-associated proteins (MAPs) which serve as ties for the stabilization of the microtubule tracks (Mandelkow and Mandelkow, 2002).

In axons, tau protein is one of the prominent MAPs (Binder et al., 1985). It stabilizes microtubules and promotes neurite outgrowth. This apparently beneficial role of tau contrasts with its anomalous behavior in several neurodegenerative diseases, mostly evident in Alzheimer's disease, where it occurs in highly phosphorylated form and detached from microtubules and self-assembled into aggregates. Tau binding per se promotes microtubule assembly, the degree of tau phosphorylation determines the binding capacity to microtubules. Correlating with higher phosphorylation of tau its binding capacity is diminished. Therefore, regulating tau phosphorylation is of utmost importance for the dynamic instability of microtubules (Brandt and Lee, 1993).

In agreement with previous work, we here report that tau protein is almost exclusively expressed in neuronal processes (Brandt and Lee, 1993). Both isoforms identified based on specific phosphorylation-sensitive antibodies revealed a similar compartmental distribution of tau in young rats. By contrast, tau protein expression was restricted to the neuronal cell bodies in aged rats. This could be viewed as a novel mechanism of axonal transport retardation, whereby abnormal redistribution of tau from axons to neuronal perikarya destabilizes the existing microtubule and thus impairs axonal transport. Such a redistribution of tau protein may also take place in AD and other neurodegenerative tauopathies.

2. Tau redistributes to the soma in all aging neurons

There are relatively a few studies describing cellular compartmentalization of tau protein isoforms in aging brain or in neuropathological situations. In accordance with our study, similar accumulation of neuronal filamentous inclusions identified as the longest human tau isoform were detected in two previous studies over-expressing this isoform in transgenic mice (Lim et al., 2001; Tatebayashi et al., 2002), but the mechanisms underlying this were not addressed. A more recent report (Zhang et al., 2004) examined the effect of tau gene mutation on its cellular organization in transgenic mice over-expressing this long isoform. Immunohistochemical analysis of tau proteins confirmed an age-dependent accumulation of insoluble filamentous tau aggregates in neuronal perikarya of cerebral cortex, hippocampus, cerebellum and spinal cord. The somato-dendritic compartment of neurons in transgenic mice carrying the tau mutation showed the most intense tau immunostaining relative to a predominantly axonal localization in non-mutated tau transgenic controls. Moreover, there was a progressive age-dependent increase in perikaryal accumulation of tau pathology, and NT-like tau pathology appeared in the hippocampus of 12-month-old mutation group that resembled NTs identified in hippocampus of human frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) patients with the similar *tau* gene mutation. Mutant tau presented with lower binding affinity to microtubules, increased accumulation

with insoluble pathological tau and retarded axonal transport. In line with our observations, alterations in tau protein affinity lead to its accumulation in the form of filamentous perikaryal aggregates in an age-dependent manner.

3. GSK-3β as a modulator of tau follows this redistribution

There are 79 putative serine or threonine phosphorylation sites on the longest tau isoform. These sites have been divided into proline-directed kinases-sensitive phosphorylation sites, like protein kinase GSK-3, protein kinase Cdk5, MAP kinase, JNK kinase, and other stress kinases or cdc2, and sites sensitive to non-proline-directed kinases like protein kinase A (PKA), protein kinase C (PKC), calmodulin kinase II (CaMKII), and MAPK kinases (Goedert et al., 1997; Lucas et al., 2001; Avila et al., 2004). Phosphorylation regulates the binding of tau to microtubules or to the membrane (Brandt et al., 1995; Canudas et al., 2005) suggesting that tau phosphorylation can determine tau function (Lee et al., 2001). The two kinases that significantly contribute to the hyperphosphorylation of tau are GSK-3β and Cdk5. Both associate with microtubules (Flaherty et al., 2000), and immunostaining for both kinases labels neurofibrillary tangles (Ymaguchi et al., 1996). Importantly, there is some evidence to suggest that levels of GSK-3β is increased in AD brain (Pei et al., 1997), and studies *in vitro* (Loveston et al., 1994) and *in vivo* (Lucas et al., 2001) argue that dysregulation of GSK-3β activity could be linked to the hyperphosphorylation of tau. This is further implicated by the finding that GSK-3β kinase is co-localized with abnormal tau in selected structures and cells, including neurons with tangles (Ferrer et al., 2002).

To confirm the type of neurofibrillary lesions that are immunoreactive for GSK-3β, Ishizawa et al. (2003) double stained brain sections with an antibody against GSK-3β and Ab39; this is a NT-specific monoclonal antibody, which recognizes conformational epitopes in NT. Ab39 detects late stages of neurofibrillary degeneration, and Ab39 immunoreactivity is absent from pretangles. GSK-3β was not detected in neuronal cell bodies that were not labeled by Ab39. Similar results were obtained with confocal immunofluorescence microscopy, where both GSK-3β and Ab39 immunoreactivities were located in NTs but not in pretangles. These results indicate that NT-bearing neurons have abnormal accumulation of tyrosine-phosphorylated GSK-3β and raise the possibility that formation of pathological tau epitopes could be connected with the accumulation of GSK-3β in the cell bodies.

Although we did not find neurofibrillary tangles, our findings suggest that sequestration of GSK-3β from neuronal processes to soma in aged rats might occur in neurons at the same time when phosphorylated tau is aggregated into the cytoplasm. However, our results could also indicate that redistribution rather than increased protein expression of GSK-3β is the most likely basis for this observation (Ishizawa et al., 2003, Planel et al., 2001). It is of note, that the GSK-3β localization pattern found by us in aged rats is comparable to that detected in AD (Augustinack et al., 2002) or in mutant transgenic animal models of neurodegenerative diseases (Ishizawa et al., 2003).

4. Origin of age-dependent differences in P-TrkA staining

Similar to tau protein, P-TrkA receptors redistribute in aged cholinergic cells to the soma. In general, P-TrkA immunoreactive deposits were not detected within hippocampus and cortex of aged rats. Instead we observed some intracellular P-TrkA staining in MS and NBM, whereas no traces of P-TrkA immunoreactivity were detected within cell bodies of basal forebrain neurons in young animals. Decrease in the number of TrkA receptors in the membrane of axonal terminals is an obvious reason for the lack of substrate (i.e. TrkA) which could be phosphorylated. It is clear however, that reduction in trophic support is not due to lower levels of NGF expression in cortex/hippocampus since levels of the neurotrophin remain unchanged in both age groups. This leaves the possibility that anterograde transport of TrkA, retrograde transport of P-TrkA, or both are disturbed. In light of cytoskeletal breakdown observed in the same tissue, we favor the latter interpretation. This is further supported by the observation that anterograde transport of TrkA as determined with specific antibodies is missing in aged animals (Niewiadomska et al., 2002). Overall, the reduced capacity of neurotrophin receptor transport is associated with neuronal atrophy and may contribute to the pronounced vulnerability of cholinergic neurons for degeneration in aging (Cooper et al., 1994).

The expression of P-TrkA in MS and NBM of old rats seems difficult to reconcile in relation to young animals, in which we did not detect any cellular immunoreactivity against this receptor. It is unlikely that this pool of P-TrkA is due to an accumulation of retrogradely transported receptors. Rather, a process unrelated to axonal transport might be involved in cholinergic neurons as a compensatory means to maintain cellular activity during phases when trophic NGF signaling from projecting areas is prevented. It is conceivable that GABA-ergic (Lauterborn ey al., 1985)

neurons and glial cells (Müller et al., 1995) synthesize and release NGF within the basal forebrain and somatically expressed TrkA receptors of BFCNs cell bodies may detect this signal. These receptors could bind NGF, undergo phosphorylation and become internalized, and this would explain the intracellular P-TrkA staining in BFCNs obtained in aged rats. Once internalized, the somatic NGF-P-TrkA can stimulate signaling pathways different from the ones activated by NGF derived from cortex and/or hippocampus (Williams et al., 2005).

The failure of continuous shuttle of TrkA to and from synapses could be explained in terms of cytoskeletal breakdown. Towards this end, hyperphosphorylated tau may be a crucial link as its expression level is known to influence the rate of attachment and detachment of motor proteins from microtubules (Trinczek et al., 1999). Moreover, tau overexpression led to a marked decrease in both anterograde and retrograde transport (Stamer et al., 2002) and this could include transport of neurotrophins. Such an interpretation is in line with a redistribution of P-TrkA and tau to the soma in cholinergic neurons (see Figs. 2–4). In addition, recent studies have shown that GSK-3β is also a kinase for the kinesin light chain (Morfini et al., 2002), a component of the protein motor for anterograde transport. Phosphorylation by GSK-3β leads to dissociation of the motor from its cargo. Thus, even in the absence of changes in the level of phosphorylated tau, increased GSK-3β activity could alter microtubule-based trafficking of structures important for neurotrophin signaling.

5. Basal forebrain cholinergic, but not hippocampal or cortical neurons, lack NGF immunoreactivity in aging brain

Failing neurotrophic support could explain neurodegeneration. This may, on one hand, be due to reduced synthesis of neurotrophin and/or impairments in neurotrophin signaling, for instance in the release from the target structures, binding of neurotrophin to its receptor(s) and resulting activation of signaling pathway, and the cellular response to the signal.

Our findings provide strong evidence for age-related abnormal NGF signaling. NGF levels were unchanged or increased in hippocampus and cortex but are decreased in the basal forebrain. In addition, decreased P-TrkA receptor expression is consistent with the apparent disruption of retrograde transport of NGF. Other studies (Scott et al., 1995; Mufson et al., 1995) have shown that there are decreased levels of NGF in the cell bodies of BFCNs suggesting a failure of NGF retrograde transport and signaling in BFCNs in aging. The retrograde transport of ^{125}I-NGF is also inhibited in the presence of colchicine, an antagonist that interferes with the polymerization of microtubules, suggesting that the transport process is highly dependent upon the microtubule system (Tatebayashi et al., 2004; Yano, 2001; Kopec, 1997; Watson et al., 1999). The tau protein and its hyperphosphorylation may exert a critical role in this as it facilitates breakdown of the axonal cytoskeleton in aging. This may reflect aberrant cell trafficking and could include failure of NGF retrograde transport in cholinergic neurons.

It is interesting to add that certain neurotrophins induce phosphorylation of kinases on Ser9 position, which may inactivate GSK-3β (Grimes and Jope, 2001). The phosphatidylinositol 3-kinase (PI-3) and anti-apoptic kinase (Akt) pathway express their activity prominently through the ability of Akt kinase to phosphorylate Ser9. NGF acts through TrkA to activate the PI-3-kinase-Akt pathway (Sofroniew et al., 2001) and this may be a viable pathway to suppress the activity of GSK-3β through Akt-mediated phosphorylation of Ser9 (Crowder and Freeman, 2000). It raises the possibility that in aged cholinergic neurons, the lack of trophic function supports pathological activity of GSK-3β.

In summary, our data reveal that during aging expression of GSK-3β kinase and its two tau protein substrates are reduced in axons and this may severely compromise the efficiency of retrograde cytoskeletal transport. As this is obvious in cholinergic projection pathways, it is reasonable to assume that abnormal tau phosphorylation is coincident with or functions as a precursor of the collapse of trophic signaling in aged cholinergic neurons. Age-dependent tau redistribution between cellular compartments may be a general phenomenon in many neuronal populations as we observed tau immunostaining in somata of hippocampal, cortical and basal forebrain neurons.

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