Induction of lysosomal abnormalities and tau hyperphosphorylation in human neuroblastoma cells by colchicine and okadaic acid: Evidence that microtubule disruption contributes to Alzheimer neurodegeneration

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SUMMARY

Two early pathological features of neurons in Alzheimer's disease, the intraneuronal accumulation of lysosomal hydrolases and alterations in tau antigenicity, distribution and electrophoretic mobility, have been suggested to arise in whole or in part from disruption of the microtubule system. To test this hypothesis, we subjected SH-SY-5Y human neuroblastoma cells to treatment with colchicine and the phosphatase inhibitor okadaic acid (OA), both of which have been previously demonstrated to depolymerize microtubules in these cells. We observed that these treatments induced the accumulation of lysosomal hyrolases and AD-like tau immunoreactivity. These findings support the hypothesis that microtubule breakdown may represent a contributing factor to Alzheimer neurodegeneration. Unlike the alteration in tau antigenicity, Bielschowsky staining indicated that neither treatment induced neurofibrillary pathology in these cells. This latter finding underscores the possibility that the interrelated events of microtubule disruption, tau hyperphosphorylation and lysosomal accumulation are antecedent events in AD neuropathology.

Key Words: microtubules, tau, hyperphosphorylation, lysosomes, hydrolases, cathepsin D, Hexosaminidase A, neurodegeneration, Alzheimer's disease

INTRODUCTION

Essential to the development of any treatment to halt or slow the widespread death of neurons that accompanies Alzheimer's disease is to determine the progression of events, and in particular, the earliest such detectable events, that highlight "at-risk" neurons in this disorder. Two such pathological features detected within affected neurons in Alzheimer's disease are alterations in the endosomal-lysosomal system (1-3) and hyperphosphorylation of the microtubule-associated protein tau (for review, see ref. 4).

The endosomal-lysosomal system, hereafter referred to as the lysosomal system for brevity, is comprised of a network of acidic vesicular compartments that contain a variety of acidic hydrolases and represent a major location for intracellular protein processing and degradation (5). Lysosomal hydrolases undergo specific increases in affected neurons in AD, and degenerating neurons are the probable source for the hydrolases routinely detected within extracellular senile plaques in AD brains (1-3). The resultant release of these proteases from their normal sequestered intracellular environment affords them novel access to inappropriate substrates, including membrane proteins of healthy adjacent neurons in addition to other intracellular

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constituents derived from degenerated neurons. This unregulated protease activity is thought to represent a significant component in the development of AD neuropathology (2,3).

Tau proteins are a family of microtubule (MT) -associated proteins which promote the assembly of MTs (6) and stabilize them against subsequent depolymerization (7,8). Site-specific phosphorylation of tau inhibits its ability to associate with MTs (9-14). This phenomenon may be related to phosphorylation-induced lengthening and loss of elasticity of the molecule(15). Dephosphorylation restores both MT-binding and elasticity (15).

Several converging lines of evidence point towards tau having a pivotal role in AD neurofibrillary pathology. A marked increase in total tau content is observed in AD brains, and this increase is apparently due to the accumulation of abnormally phosphorylated forms (16). Paired helical filaments (PHF) that accumulate in affected neurons in Alzheimer's disease are comprised of hyperphosphorylated tau that exhibits electrophoretic and antigenic properties distinct from that of normal adult CNS tau (for reviews, see refs. 17-19). A number of kinases have been reported to induce normal tau to exhibit PHF-like characteristics (e.g., 20-34). PHF-tau exhibits a decreased MT binding ability (35), and dephosphorylation dissociates PHFs and restores MT-assembly promoting properties to tau (36-38).

Both the intraneuronal accumulation of lysosomal hydrolases (2) and of hyperphosphorylated tau (39, 40) in AD have been suggested to arise in whole or in part from microtubule (MT) disruption; this possibility is supported by the observation that MT assembly is defective in AD (39, 40). To test this hypothesis, we subjected SH-SY-5Y human neuroblastoma cells to treatment with colchicine and okadaic acid (OA), both of which previously demonstrated to depolymerize MTs in these cells (e.g., refs. 41-43), and monitored the effect of these agents on levels of lysosomal hydrolases and phosphorylated tau.

MATERIALS AND METHODS

Cell culture and treatment SH-SY-5Y cells (originally obtained from the stocks of Dr. June L. Biedler, Memorial Sloan-Kettering Cancer Center, Rye, New York) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 10% CO_2. Twenty-four hours later, the medium was replaced with medium containing 5nM-1μM okadaic acid for 1-24hr (OA) or 3μM colchicine for 1hr. All reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Immunocytochemistry Treated cultures and untreated controls were rinsed in Tris-buffered saline (TBS; pH7.4), fixed with 4% paraformaldehyde in 0.1M phosphate buffer for 15 min at room temperature, and rinsed 3x in TBS. Immunocytochemistry was carried out on fixed cultures using 1:100 dilutions of monoclonal antibody 6-11B-1 (directed against acetylated α-tubulin; obtained from Sigma) (44), a monoclonal antibody directed against all forms of α-tubulin (Sigma), monoclonal antibody ALZ-50 (raised against a neurofibrillary tangle preparation from AD brains (45,46), monoclonal antibody TAU-1 (which reacts only with tau that has not been phosphorylated at a site that is hyperphosphorylated in AD; obtained from Boehringer-Mannheim, Indianapolis, IN) (47), or 1:1000 dilutions of polyclonal antisera directed against cathepsin D (Dako, Inc.) and Hexosaminidase A (generous gift of Dr. Sirinivasa Raghavan, New York University Medical Center, Manhattan, NY). This was followed by incubation with the appropriate peroxidase- (Sigma) or alkaline phosphatase-conjugated (Boehringer-Mannheim) secondary antibody and visualization by standard

methodologies as described previously (48). Additional untreated and OA-treated cultures were processed for perikaryal accumulation of argyrophilic material by the Bielschowsky method as described (49).

Microdensitometry Microscopic images of cells obtained at 60X on an Olympus inverted microscope were captured via CCD, then digitized and subjected to microdensitometry using the NIH Image analysis software (version 1.57) on a Power PC 7100AV Macintosh equipped with video import capabilities as described previously (24,33). Identical magnification, illumination, capture and record settings were maintained for all cells in all cultures in these analyses. For each antibody, a total of 100-200 cells from duplicate cultures from duplicate experiments were identified by phase-contrast illumination, outlined on-screen with the NIH Image program's freehand selection tool and subjected to densitometric quantitation under bright-field illumination. Tubulin and tau-specific immunoreactivity was typically dispersed throughout the perikaryon and putative neurites, although acetylated α-tubulin immunreactivity was often concentrated at the base of and within putative neurites (e.g., Fig. 1); the entire cell body and any putative neurites were therefore selected and measured as one unit in densitometric analyses. Cathepsin D and hexosaminidase A immunoreactivity was entirely confined to lysosomes, with only minor measurable extra-lysosomal density that was unaltered by either colchicine or OA treatment as ascertained by microdensitometric analyses (not shown). Accordingly, the entire perikaryon of cells was selected and measured for densitometric analyses for hydrolase-specific as well as tau-specific antibodies. For each microscopic field, 5 representative areas that were devoid of cells were selected with the freehand selection tool, and the average densitometric value of these areas designated as "background"; only microscopic fields in which all background areas were of similar density (both visually and following quantitation) were utilized for further analysis. All digitization and quantitation operations were carried out manually without invoking any of the program's automatic densitometric functions. Resultant raw densitometric values from individual fields were accumulated into Excel spreadsheet software. The average background density was individually subtracted from all recorded cells from each respective field, and statistical analyses were carried out on the resultant "corrected" cell density values using Student's *t* test. Values are presented as the mean ± standard error of the mean. Micrographs present representative digitized images, annotated and printed using Power Point software and a Fargo dye-sublimation printer, that were obtained directly from representative fields from the above analyses.

Numbers of lysosomes per cell were also quantified from representative digitized images under optimized contrast using equivalent magnification and illumination. At least 25 cells with clearly discernible boundaries in each of 3-6 microscopic fields obtained from duplicate cultures were scored for total numbers of cathepsin D-positive lysosomes and the resultant values statistically compared using Student's *t* test.

Gel elecrophoresis and immunoblot analyses Nitrocellulose replicas of heat-stable Triton-soluble fractions from untreated and colchicine-treated cells were probed with ALZ-50, and monoclonal antibodies directed against total tau (5E2; generous gift of Dr. K. Kosik) and PHF-tau (PHF-1; generous gift of Dr. S. Greenburg) as described previously (24).

RESULTS

OA and colchicine rapidly deplete MTs MT assembly states under various conditions were readily monitored by observation of immunoreactive levels of acetylated α-tubulin; acetylation of α-tubulin is a posttranslational modification carried out exclusively on subunits that have been incorporated into MTs, while these subunits are deacetylated prior to MT disassembly (e.g., see refs. 48, 50 and refs. therein). Colchicine treatment induced large-scale disruption of MTs within 1hr as evidenced by the depletion of acetylated a-tubulin immunoreactivity; that these treatments induced disruption rather than tubulin degradation was confirmed by the observation of identical levels of total (including assembled and unassembled) tubulin using an antibody that reacts with all α-tubulin isoforms (Fig. 1, 2).

Colchicine induces tau hyperphosphorylation and lysosomal accumulation Colchicine treatment (1hr) induced an increase in ALZ-50 immunoreactivity and a decrease in TAU-1 immunoreactivity (Fig. 1, 2). Colchicine treatment for this length of time also induced an increase in lysosomes as judged by cathepsin D and hexosaminidase A immunoreactivity (Fig. 1, 2); lysosomal hydrolase immunoreactivity was exclusively vesicular under all conditions. A markedly heterogeneous range of lysosomal diameters was observed following immunostaining for both hydrolases (e.g., Fig. 1), with no significant overall difference was observed among the ranges obtained in the absence or presence of colchicine. However, occasional lysosomes within colchicine-treated cells were of significantly larger diameter than the majority (Fig. 1). While these results are in accord with previous immunocytochemical analyses (50), other studies utilizing immunoblot analyses have indicated a decrease in AD-like tau phosphorylation following colchicine treatment (51). To address these apparently dicordant findings, we subjected colchicine-treated cultures to immunoblot analyses. Colchicine-treated cells demonstrated an overall increase in ALZ-50 immunoreactivity (Fig. 3). Total tau immunoreactivity demonstrated an overall increase, while PHF-1 immunoreactivity, by contrast, was decreased following colchicine treatment, while (Fig. 3). We therefore interpret these findings to indicate that these cells undergo a complex series of responses to colchicine-induced MT disruption which includes a degree of site-specific dephosphorylation coupled with an overall increase in certain phospho-isoforms, apparently as a reflection of an increase in total tau.

OA induces tau hyperphosphorylation and lysosomal hydrolase accumulation As previously demonstrated (52, 53), acetylated α-tubulin immunoreactivity was eliminated with 1hr of OA treatment (21.5 ± 1.2 in untreated controls vs. 12.1 ± 2.5 in OA-treated cultures; mean ± standard error of the mean in arbitrary densitometric units; $p < 0.0007$, Students *t* test). As was observed following colchicine treatment, however, total tubulin levels remained unchanged, indicating that OA treatment induced MT disassembly rather than tubulin depletion (28.8 ± 2.2 in untreated controls vs. 25.3 ± 2.0 in OA-treated cultures; mean ± standard error of the mean in arbitrary densitometric units; $p < 0.0007$, Students *t* test).

A progressive increase in the numbers of cells displaying prominent ALZ-50 immunoreactivity, and a progressive decrease in numbers of cells displaying TAU-1 immunoreactivity accompanied incubation with OA from 1-24hr (Fig. 4, 5). Variation in staining intensity was observed among individual cells throughout cultures in multiple experiments and with multiple antibody dilutions and processing times; however, densitometric analyses confirmed an overall net increase in ALZ-50 and net decrease in TAU-1 immunoreactivity (Fig. 5).

In contrast to the dramatic alterations in tau immunoreactivity, only a slight trend towards increased numbers of Bielschowsky-positive perikarya was observed (Fig. 6). Quantitative analysis of perikarya in Bielschowsky-stained cultures were arbitrarily designated as Bielschowsky-positive or negative based on

staining intensity, no significant difference could be detected between the percentage of Bielschowsky-positive cells in untreated cultures (12.5 ± 2.3% positive) and OA-treated cultures (15.9 ± 2.6% positive; mean ± standard deviation, $p < 0.189$).

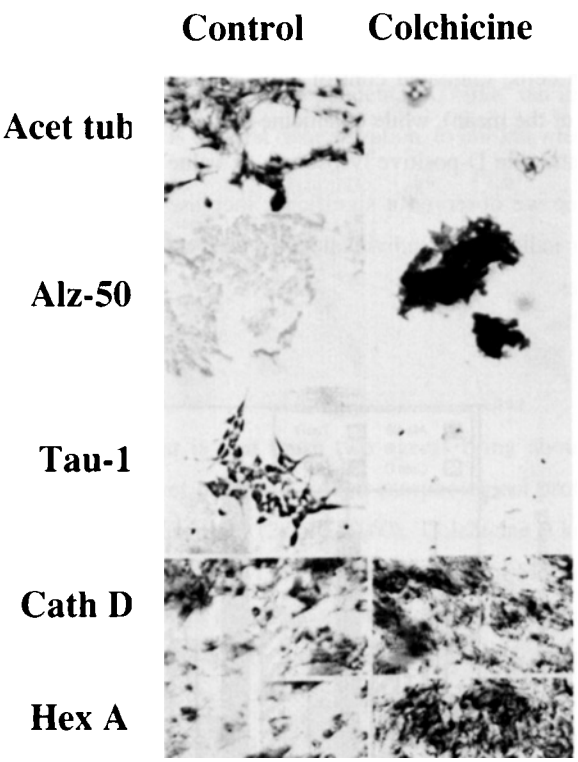

Fig. 1: Colchicine treatment alters microtubule, tau and lysosomal hydrolase immunoreactivity

SH-SY-5Y neuroblastoma cells were treated with colchicine for 1hr and processed along with untreated controls for immunocytochemical distribution of acetylated α-tubulin (Acet tub), Alz-50, Tau-1, cathepsin D (Cath D) and hexosaminidase A (Hex A) immunoreactivity as indicated. Representative images are presented. Note that colchicine treatment eliminates acetylated a-tubulin and Tau immunoreactivity, and increases Alz-50, cathepsin D and hexosaminidase A immunoreactivity. Cell borders are difficult to discern in panels immunostained for lysosomal hydrolases since this immunoreactivity is confined to lysosomes; however, all micrographs are the identical magnification for reference.

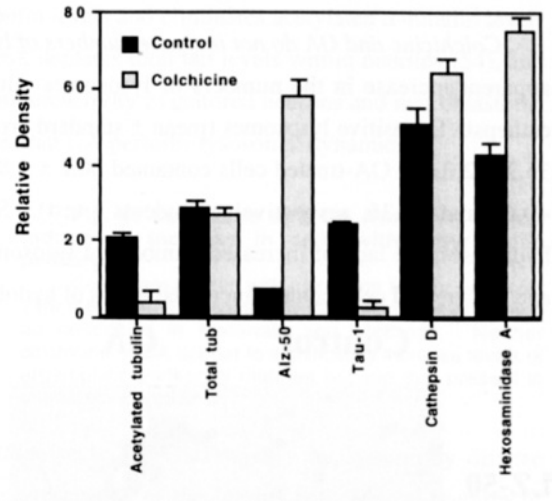

Fig. 2: Densitometric analyses of the effect of colchicine treatment alters microtubule, tau and lysosomal hydrolase immunoreactivity

Cells were treated with colchicine for 1hr and processed along with untreated controls for immunocytochemical distribution of acetylated α-tubulin, total tubulin, Alz-50, Tau-1, cathepsin D and hexosaminidase A immunoreactivity and subjected to densitometric analyses as described in Materials and Methods. Note that colchicine treatment increases Alz-50, cathepsin D and hexosaminidase A immunoreactivity and eliminates Tau-1 immunoreactivity.

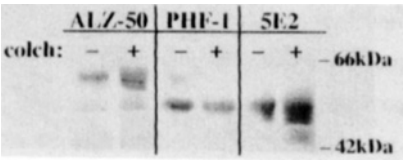

Fig. 3: Colchicine treatment alters tau phosphorylation and steady-state levels

Heat-stable, Triton-soluble fractions from colchicine-treated and untreated cells were immunostained with ALZ-50, PHF-1 and 5E2 as indicated. Note the overall increase in ALZ-50 immunoreactivity despite a degree of depletion of the slowest migrating isoform, a decrease in PHF-1 immunoreactivity, and an increase in 5E2 immunoreactivity.

OA treatment induced a progressive increase in cathepsin D and hexosaminidase A immunoreactivity (Fig. 4, 5). Lysosomal hydrolase immunoreactivity was exclusively vesicular under all conditions (Fig. 4). As was observed following colchicine treatment, a somewhat heterogeneous range of lysosomal diameter was

observed following immunostaining for both hydrolases under all conditions (e.g., Fig. 4), but no significant difference was observed among the ranges obtained for each condition.

Colchicine and OA do not increase numbers of lysosomes per cell Neither colchicine nor OA induced an apparent increase in the numbers of lysosomes within cells; untreated control cells contained 34.0 ± 2.5 cathepsin D-positive lysosomes (mean ± standard error of the mean), while colchicine-treated cells contained 35.2 ± 2.0 and OA-treated cells contained 30.2 ± 1.8 cathepsin D-positive lysosomes (p value vs. control of <0.62 and <0.36, respectively, Students t test). Since we observed a significant increase in lysosomal hydrolases, the lack of increased numbers of lysosomes indicate that individual lysosomes within colchicine and OA-treated cells contain increased levels of hydolases.

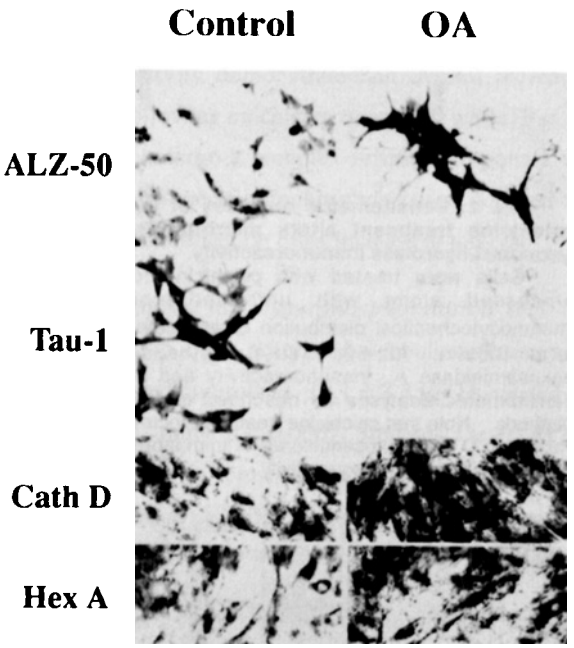

Fig. 4: OA treatment alters tau and lysosomal hydrolase immunoreactivity

Cells were treated with OA for 0-24hr and processed along with untreated controls for immunocytochemical distribution of Alz-50, Tau-1, cathepsin D (Cath D) and hexosaminidase A (Hex A) immunoreactivity as indicated. Representative images obtained from cells treated for 0 (Control) and 24 (OA) hr are presented. Note that OA treatment eliminates Tau-1 immunoreactivity, and increases Alz-50, cathepsin D and hexosaminidase A immunoreactivity.

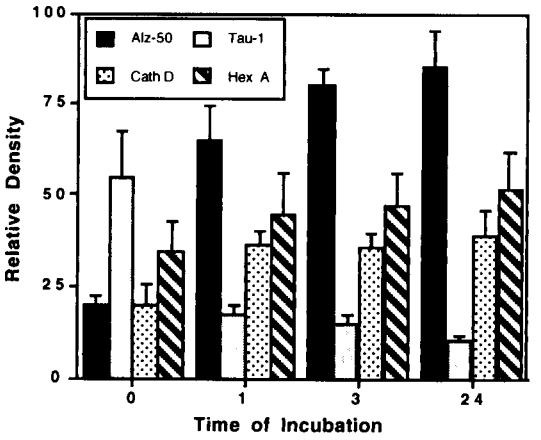

Fig. 5: Densitometric analyses of the effect of OA on tau and lysosomal hydrolase immunoreactivity

Cells were treated with OA for 0-24hr and processed along with untreated controls for immunocytochemical distribution of Alz-50, Tau-1, cathepsin D (Cath D) and hexosaminidase A (Hex A) immunoreactivity and subjected to densitometric analyses as described in Materials and Methods. Note that OA treatment progressively eliminates Tau-1 immunoreactivity, and increases Alz-50, cathepsin D and hexosaminidase A immunoreactivity.

DISCUSSION

In the present study we observed that treatment of cultured human neuroblastoma cells with colchicine and OA induces the composite effects of MT disassembly, tau hyperphosphorylation, and lysosomal hydrolase

accumulation. Colchicine is routinely utilized to invoke MT disassembly, and a previous study has demonstrated a colchicine-mediated increase in ALZ-50 immunoreactivity (50). Previous studies from this and other laboratories have demonstrated that the phosphatase inhibitor OA alters MT organization. OA prevents MT-mediated neuritogenesis, decreases total tubulin levels and eliminates acetylated α-tubulin levels within neurites (51, 54), inhibits spindle organization (56), depletes total tau levels within neurites (54), and increases phosphorylation-dependent, "AD-like" tau immunoreactivity in cultured neurons and neuroblastoma (56-58). This is the first demonstration, to our knowledge, that OA perturbs lysosomal dynamics.

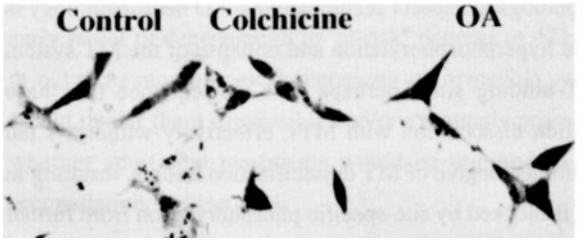

Fig. 6: Colchicine and OA treatment do not induce an increase in cells with argyrophilic perikarya

Cells were treated with colchicine for 1hr or OA for 24hr then fixed and stained by the Bielschowsky method as described in Materials and Methods. Neither colchicine or OA appear to significantly increase levels of argyrophilic perikaryal material beyond that present in untreated control cells.

Of interest is that these two agents bring about large-scale MT disassembly by apparently diverse mechanisms, yet present a similar morphological profile reminiscent of the "early" pathological features of affected neurons in AD (2,3,40,59,60). Colchicine is known to prevent MT polymerization by binding directly to free tubulin subunits (58). The mechanism of MT destabilization by OA is less clear, and may by mediated by interfering with dephosphorylation of both tubulin and tau, since both the ability of α-tubulin to assemble into MTs and the ability of tau to promote MT assembly and stabilize MTs are inhibited by site-specific phosphorylation (6-15, 61). Whether or not OA-induced phosphorylation events actually dissociate tau from MTs, leading to global MT disassembly, or whether tau can undergo assembly-restrictive OA-induced phosphorylation only following dissociation from MTs during normal association-dissociation cycles is not clear. That increased tau phosphorylation accompanies colchicine-induced MT disassembly as seen herein and in a previous study (50) is consistent with the latter possibility. Continued "scavenging" of tau by OA-induced phosphorylation may eventually be expected to destabilize MTs to the point of collapse of the MT network. Moreover, the relative contribution, if any, of OA-induced tubulin hyperphosphorylation to inhibition of MT assembly remains unclear. While the present analyses cannot differentiate among these possibilities, however, the additional observation of lysosomal accumulation following MT destabilization by colchicine and by OA supports the previous hypothesis (39,40), that disruptions in the MT system may represent a critical early event leading to AD neuropathology. Importantly, the effects of OA are not confined to neuronal culture; OA also increases ALZ-50 levels in brain *in situ* (62,63) and in brain slices (64).

It is noteworthy that the identical paradox applies equally well to consideration of the order of pathological events in AD. That is, it remains impossible to confirm at present whether in AD tau is first hyperphosphorylated, which induces its dissociation from MTs, leading in turn to their collapse, or whether

MT collapse represents an initiating phenomenon, and newly-dissociated tau subsequently becomes hyperphosphorylated. Consistent with the former possibility is that phosphorylation of tau at critical MT-binding site(s) prevents proper association of tau with MTs (9-14), and may therefore subject tau to AD-like hyperphosphorylation. That tubulin from AD brains remains assembly-competent is also consistent with the former possibility (40). By contrast, however, the observation of rapid tau hyperphosphorylation following colchicine-induced MT collapse in the present and in a previous study (50) suggests that the latter possibility (i.e., initial MT collapse) could at least partially underlie tau hyperphosphorylation in AD. An additional, plausible hypothesis for the development of these morphological aspects accompanying AD neuropathology is that both phenomena contribute to the full extent of tau hyperphosphorylation and collapse of the MT system as follows: *(1)* some phosphorylation of tau at MT-binding sites, perhaps as a consequence of kinase hyperactivation during ongoing cycles of tau association-dissociation with MTs, effectively withdraws tau molecules from the binding-competent tau pool; *(2)* a limited degree of MT destabilization ensues, resulting in additional tau liberation; *(3)* this newly-dissociated tau is blocked by site-specific phosphorylation from further MT association; *(4)* additional rounds of these phenomena lead to collapse of the MT system; *(5)* binding-incompetent tau would be subjected to progressive AD-like hyperphosphorylation. In this regard, a recent examination of MT-promoting activities of tau from AD and control brains suggested that hyperphosphorylated, binding-incompetent tau may further exacerbate MT breakdown by scavenging normal tau (39). Our demonstration of PHF-1 depletion coupled with and overall increase in ALZ-50 immunoreactivity, although on faster-migrating (and therefore presumably containing fewer overall associated phosphate groups) further underscore the importance of considering site-specific and/or heirarchical tau phosphorylation in neuronal homeostasis. Also consistent with this proposed series of events is the progressive increase in lysosomes, although the minimal extent of MT depletion required to foster a significant increase remains unclear and was not addressed in the present study. Our observation of an increase in total tau immunoreactivity following colchicine treatment may be interpreted as an attempt to restore the collapsing MT system; such possibilities are currently under investigation.

OA activates and potentiates the activity of certain kinases that are themselves regulated by phosphorylation (65-68), including candidate kinases, e.g., MAP kinase, shown to hyperphosphorylate tau under certain conditions (69-72). It therefore remains possible that, in addition to direct inhibition of tau and/or tubulin dephosphorylation, OA-induced potentiation of kinase activity may also contribute to increases in tau and, potentially tubulin, hyperphosphorylation. It furthermore remains possible that the increase in lysosomal hydrolases following OA treatment is also mediated in whole or in part by undisclosed altered phosphorylation of MT receptor/binding sites on lysosomal membrane proteins that normally mediate lysosomal association with and transport along MTs. This likelihood of this possibility as a major contributor

towards the observed increase in lysosomal hydrolases is, however, not supported by the observation of a similar increase following colchicine treatment.

The failure to observe an increase in Bielschowsky-positive perikarya indicates that, while tau hyperphosphorylation has occurred, PHF formation following OA treatment remains at best minimal. Since Bielschowsky-positive perikarya can readily be induced in neuroblastoma by other neurotoxic treatments (50), these latter findings in the present study support the hypothesis (59) that significant accumulation of hyperphosphorylated tau precedes PHF development, and highlight the relationship of these phenomena to the early stages of degeneration in "at-risk" neurons in AD (2,3,59,60). Of interest would be to determine whether or not these morphological alterations are reversible, and if so, whether protracted colchicine or OA treatment would render them irreversible and/or eventually promote significant PHF accumulation within these cells, or whether additional treatments would be required to induce, or any would be capable of inducing, PHF accumulation in these cells.

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