Staging of Neurofibrillary Degeneration Caused by Human Tau Overexpression in a Unique Cellular Model of Human Tauopathy

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The hyperphosphorylation of human tau and its aggregation into neurofibrillary tangles are central pathogenic events in familial tauopathies and Alzheimer's disease. However, the cellular consequences of neurofibrillary tangle formation *in vivo* have not been directly studied because cellular models of human neurofibrillary degeneration have been unavailable until recently. Incorporation of human tau into filaments *in vivo* and the association of filamentous tau with cytodegeneration were first demonstrated experimentally with the overexpression of human tau in identified neurons (anterior bulbar cells) in the lamprey central nervous system. In this system, filamentous tau deposits are associated with the loss of dendritic microtubules and synapses, plasma membrane degeneration, and eventually the formation of extracellular tau deposits and cell death. Here we show that human tau hyperphosphorylation in anterior bulbar cells is spatiotemporally correlated with a highly stereotyped sequence of degenerative stages closely resembling those seen in human neurofibrillary degeneration. Hyperphosphorylated tau deposits first appear in the distal dendrites and somata, together with degenerative changes that begin in distal dendrites and progress proximally over time. This sequence is independent of the tau isoform used, the presence of epitope tags and the method used to overexpress tau, and thus has important implications for the cytopathogenesis of human neurofibrillary disease. (Am J Pathol 2001, 158:235–246)

The intracellular accumulation of hyperphosphorylated, filamentous tau protein is a hallmark of a large group of neurodegenerative conditions that are collectively responsible for the overwhelming majority of cases of dementing illness among elderly humans. The presence and distribution of neurofibrillary tangles (NFTs) and neu-

ropil threads are tightly correlated with the degree of dementia seen in Alzheimer's disease, suggesting that they play a major functional role in the development of dementia.^{1–3} Furthermore, filamentous tau deposits such as NFTs and Pick bodies have recently been linked to mutations affecting the sequence, splicing, and expression of tau in familial forms of a variety of neurodegenerative conditions,^{4–7} raising considerable interest in the cellular mechanisms underlying NFT formation as a possible point of therapeutic intervention in neurofibrillary degenerative disease (NFD).

Studies of the early stages of NFD in human autopsy material suggest that abnormal tau deposits develop in a stereotyped spatiotemporal sequence, with the earliest changes being seen in the distal dendrites of vulnerable neurons,^{8,9} and the appearance of evenly distributed, granular tau deposits that have been phosphorylated at the AT8/Tau-1 site.^{8,10} Throughout time, large deposits of filamentous, highly phosphorylated tau (NFTs) fill the somata of such neurons, which eventually die, leaving extracellular tombstone NFTs consisting of highly modified tau filaments.^{8,10} Unfortunately, very little is known concerning the time course and the precise sequence of cellular events required to produce these lesions *in vivo*. The total time required for NFT formation and neuronal death can only be determined indirectly from autopsy studies, and such estimates vary from several months to up to 20 years.^{11–13} In particular, it is unclear which of the observed cellular changes play early, causal roles in the cascade of events leading to NFD, and which are merely consequences of more central events. For instance, both axonal¹⁴ and dendritic^{8,15} degeneration are widespread in Alzheimer's disease and other neurofibrillary degenerative conditions, yet it is unknown whether one or the other of these loci is the primary point of attack in NFD or if both dendritic and axonal changes are secondary to pathology affecting the entire cell. Similarly, it is unclear if NFD is primarily a cell-autonomous process, affecting only the cell containing the developing tangle, or if trans-synaptic mechanisms play a critical role. Finally, it is still unclear if the development of argyrophilia and/or tau

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phosphorylation at any one site plays a significant role in NFT formation and neurofibrillary degeneration *in vivo*, and if so, whether these changes are a cause or consequence of the polymerization of tau into filaments. The inability of neuropathologists to address these questions directly and effectively is primarily because of the impracticality of following individual neurons in which NFTs are developing on a prospective basis in humans and/or murine or other mammalian models *in situ*.^{16–18}

Throughout the past few years, we have developed a unique cellular model of tau filament/NFT formation for studying the cytopathological changes that accompany chronic overexpression of human tau that circumvents many of the difficulties outlined above. This cellular model of tau-induced NFD consists of giant neurons [anterior bulbar cells (ABCs)] in the hindbrain of the ammocoete sea lamprey, *Petromyzon marinus*, that have been induced to overexpress human tau by the injection of plasmids containing constructs in which tau expression is driven by the cytomegalovirus (CMV) promoter. With this system, we showed for the first time that overexpression of the shortest human tau isoform (htau23) *in vivo* can cause the incorporation of human tau into filaments, the phosphorylation of the PHF1 and Tau1 epitopes (serines 396 to 404 and 199 to 202, respectively), and gross degenerative changes in the soma and dendrites including the externalization of human tau deposits.¹⁹ We have since characterized the intracellular behavior of human tau filaments in ABC somata and dendrites and their association with dendritic microtubule (MT) and synapse loss.²⁰

In the present study, we have performed a systematic analysis of the degenerative changes caused by tau overexpression throughout time in ABCs, and have correlated these changes both with the length of time after vector injection and with the appearance of multiple AD-related epitopes on tau, including the PHF1, AT8, AT100, TG3, and ALZ50 sites, in ABCs expressing human tau. We have also compared the cytopathological changes induced by the longest human tau isoform (htau40) to those caused by htau23, and have compared htau23 and htau40 constructs containing Green Fluorescent Protein (GFP) fusions to those that express htau23 and htau40 alone. Finally, we have used a novel method of overexpressing tau in ABCs using self-replicating mRNAs derived from Semliki Forest Virus (SFV) to achieve chronic tau overexpression in ABCs in addition to the plasmid injection method used previously. We show that chronic overexpression of all of these constructs in ABCs induces the same stereotyped sequence of cytopathological changes throughout time, with the earliest and most severe changes occurring in the distal-most dendrites. Moreover, this sequence of degenerative changes is spatiotemporally correlated with the appearance of several AD-related phosphoepitopes. However, only the phosphorylation of the PHF1 epitope accompanies or precedes the earliest morphological changes induced by human tau overexpression in ABCs. These results bear a strong resemblance to sequences of cellular degeneration proposed for human NFD,^{8,10} and their significance

Figure 1. Expression and immunolabeling of human tau from plasmid and SFV-derived mRNA vectors in lamprey ABCs. **A:** Constructs used in this study to express tau in ABCs. In all clones, human tau expression was driven by the CMV promoter. Clone 93 has EGFP fused to the htau23 N-terminal and clone 139 has GFP fused to the C terminal of htau40, whereas n123c and SFV expressed htau23 and htau40 without epitope tags. **B:** A schematic diagram outlining the mechanism of SFV mRNA vector amplification used for htau40 expression in ABCs. The SFV replicase consists of four subunits that combine to synthesize strands complementary (–) to the injected mRNA. Duplicate strands (+) are then synthesized from which both the replicase and the exogenous target protein (ie, htau40) are then translated, amplifying the original mRNA injected into the ABC and permitting the chronic expression of the target protein without the need for either whole virus or nuclear targeting and subsequent transcription of injected plasmids. **C:** Schematic diagram of the tau molecule showing the MT-binding motifs (black) and the approximate locations of the epitopes recognized by mAbs used in this study. AT8, AT100, and TG3 recognize phosphoepitopes on the N-terminal flanking region of the MT binding repeats, whereas PHF1 recognize a phosphoepitope on the C-terminal flanking region. ALZ50 recognizes the N terminal of tau in conjunction with a conformational change that occurs when human tau becomes hyperphosphorylated and dissociates from MTs.

and implications for the cellular mechanisms responsible for human NFD is discussed.

Materials and Methods

Human Tau Constructs Overexpressed in ABCs

All constructs used in this study are shown in Figure 1A. The human tau sequence was either fused with the coding sequence for GFP at the tau N terminal (for htau23—clone 93²⁰) or at the tau C terminal (for htau40—clone 139) as shown in Figure 1A. The plasmid pRC/CMVn123c¹⁹ was used to overexpress htau23 without an epitope tag. Htau40

was expressed without an epitope tag by injecting mRNA purified by standard methods from the SFV-htau40 vector into ABCs as described in Figure 1B. Human tau expression was driven by the CMV promoter as a single transcript in all of the constructs used.

Overexpression of Human Tau in ABCs

Plasmid microinjection was performed as described,¹⁹ and surgery and preparation for fixation and immunohistochemistry was performed as described in Hall and Kosik.²¹ Briefly, the hindbrains of anesthetized ammocoete lampreys 8 to 11 cm in length were exposed and the somata of ABCs identified and injected under visual guidance. The brain was maintained under a constant flow of Ca^{++} -free lamprey Ringers solution^{22,23} at all times, and the membrane potential of injected ABCs was monitored during injection. Microinjection of SFV-derived constructs was performed using a technique similar to that used for plasmids, but with modifications to ensure RNase-free conditions (ie, autoclaving of all stock solutions, electrode holder caps and electrode glass; addition of diethyl pyrocarbonate to stock solutions). In addition, the shanks of all micropipettes used for microinjecting SFV mRNAs were briefly flamed and allowed to cool before use. Otherwise, surgical procedures for all operations were identical to those described above and in Hall and Kosik.²² All procedures were performed under general anesthesia, which was accomplished by immersing the lampreys in a saturated aqueous solution of benzocaine for 10 to 20 minutes. Approximately 120 lampreys were used in this study, yielding 97 expressing cells. Sixty-seven of these cells produced 10- μm sections through their somata and dendritic trees that were immunostained with PHF1, and were thus considered suitable for staging purposes (see Results).

Electron Microscopy

Fixation, embedding, and sectioning was done as described in Hall et al.¹⁹ Targeting of expressing ABCs was done by using the features of the brain visible in the block provided by photographs similar to that shown in Figure 2 as visual cues. ABC dendrites in mildly degenerated cases were identified by established morphological criteria for identifying distal dendrites of axotomized ABCs in transverse thin sections through the lamprey hindbrain.^{22,23} In sections processed for immunoelectron microscopy, lamprey brains were fixed for 2 hours in 4% paraformaldehyde/0.05% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, and then transferred to phosphate-buffered saline containing 2 mol/L sucrose/0.2 mol/L glycine for 30 minutes followed by immersion in liquid nitrogen. Frozen ultrathin sections were then cut at -120°C with a cryo-diamond knife and transferred to Formvar-coated grids. Immunolabeling was performed on the grids by standard methods with 0.5% fish skin gelatin (Sigma Chemical Co., St. Louis, MO) as a blocking agent. A 1:10 dilution of anti-GFP antiserum (Clontech, Palo Alto, CA) was then used to identify tau fila-

ments. Protein A-gold/anti-rabbit secondary (10 nm, Sigma) was then used to label filaments for the electron microscopy. Grids were then stained in 0.3% uranyl acetate for 10 minutes at 0°C and examined on a JEOL 1200EX electron microscope.

Immunocytochemistry and Antibodies

Lamprey brains were fixed and sectioned as described.²² Immunocytochemistry was performed on 10- μm transverse sections of paraffin-embedded lamprey heads that had been fixed by immersion in FAA (10% formalin, 10% glacial acetic acid, and 80% ethanol). The mAbs PHF1 (1:100), ALZ50 (1:25), TG3 (1:25), AT8 (1:100), and AT100 (1:100) were used to identify phosphoepitopes on htau23 and htau40. PHF1, ALZ50, and TG3 were kindly provided by Dr. Peter Davies (Department of Pathology, Albert Einstein College of Medicine, Bronx, NY), and AT8 and AT100 were purchased from Innogenetics Corp. (Leuven, Belgium). None of these mAbs cross-reacted with endogenous epitopes in FAA-fixed lamprey brain. Sections were deparaffinized in Histoclear and then photographed if they contained fluorescent profiles of ABC somata and dendrites. They were then rehydrated, quenched in excess H_2O_2 , washed, and incubated with primary overnight at 4°C . Biotinylated secondary antibodies were then applied and antibody staining revealed with an Biotin/Extravidin/HRP kit (Sigma Chemical Co.), with diaminobenzidine as the chromagen.

Results

In this study, we have characterized the development of neurofibrillary pathology and assess its correlation with changes in the phosphorylation state of human tau in ABCs using a panel of mAbs directed at phosphoepitopes in regions of tau that flank the MT-binding repeats. We found that all of these mAbs recognize subsets of the human tau expressed in ABCs, but that none of them label as heavily or as extensively as PHF1. Moreover, we found no significant differences in the patterns of human tau immunostaining and/or cytodegeneration that were attributable to the effects of using different splice variants of tau (ie, htau23 and htau40) and different expression techniques on the response of ABCs to human tau overexpression. All of the constructs used resulted in the same stereotyped pattern of degenerative changes, which were in turn spatiotemporally correlated with the immunolabeling patterns of the anti-tau mAbs listed above and in Figure 1C. These patterns are described in detail below.

The Earliest Changes Because of Human Tau Overexpression Appear in the Distal Dendrites of ABCs and Occur before Tau Hyperphosphorylation

Lightly expressing ABCs (Figure 2C) and cells examined within a few days of plasmid injection often showed no

Figure 2. Effects of human tau overexpression on lamprey ABCs. **A:** Dorsal view of the hindbrain of a living lamprey showing an ABC that is expressing htau23 viewed in a combination of fluorescence and bright-field illumination under a dissecting microscope. **B:** A transverse section through the soma and dendritic field of an ABC (at the point indicated by the **arrows** in **A**) injected with Lucifer Yellow, showing normal ABC dendritic gross morphology in the absence of tau expression, with ABC dendrites tapering smoothly from base to tip (**arrow**). **C:** An ABC 9 days after injection with clone 93 exhibiting typical early (stage 1) dendritic changes, including a slight expansion of distal dendrites (**caret**) relative to proximal dendrites (**arrow**). PHF1 labeling is even and granular, except in some distal dendrites, where it is concentrated near the plasma membrane. **D:** GFP fluorescence image from two ABCs 79 days after injection with clone 93. The **circle** delineates the normal outline of the cell body; it is likely that this neuron is at a relatively severe stage of degeneration involving the entire somatodendritic region (ie, stage 3 or 4); note that despite this the cell retains a grossly normal axon (**arrow**). **E:** A cross-sectional view of a section from an ABC 21 days after injection with clone n123c, showing relatively severe (stage 3) degeneration. Note the marked contrast between the cells shown in **C** and **E**, in particular the roughened and distorted outline of most dendrites in **E**. Much of the tau in **E** is localized to membranous structures, as well as to filamentous deposits, and that some is outside the cell (**E**, **large asterisk**). Nuclei of ABCs showing stage 3 degeneration are typically tau-positive (**E**, **small asterisk**) unlike earlier stages (**C**, **asterisk**). **F** and **G:** Examples of dendritic ultrastructure in normal (**F**) and severely degenerated (**G**) ABCs. **G** is from a cell 70 days after injection of clone 93. ABC dendrites normally contain well-organized arrays of MTs (**F**, **carets**) that are disrupted by chronic tau overexpression and eventually replaced by straight filaments containing human tau (immunolabeled with anti-GFP in **G**). Scale bars: 50 μ m (**A–E**), 200 nm (**F**), and 100 nm (**G**).

10cytopathological changes at all.¹⁹ These cells showed PHF1/GFP staining in a granular, evenly distributed pattern throughout their somata and dendrites, and did not

label with any of the other mAbs directed at tau phosphoepitopes (ie, AT8, TG3, AT100) or with ALZ50. Most of these cells exhibited slight swelling of some distal den-

Figure 3. Swollen dendritic tips produced by localized aggregations of membrane-bound organelles are the first morphological changes seen as the result of human tau overexpression in ABCs. **A:** A swollen dendritic tip from a lightly expressing (stage 1) ABC immunostained with PHF-1. **B:** Electron micrograph of a similar swollen dendritic tip showing pronounced aggregations of membranous organelles. **Inset** region is shown at high magnification at **right C:** These aggregations contain MTs (**carets**), tau filaments (**arrows**), and mitochondria (**m**), small vesicles (**v**), and smooth endoplasmic reticulum (**sER**) oriented in an apparently random arrangement, with some clumping of both vesicles and mitochondria. Scale bars: 10 μm (**A**), 5 μm (**B**), and 500 nm (**C**).

drites and many of them tended to have stronger PHF1 staining in their distal-most dendrites than elsewhere.¹⁹ Cellular profiles were otherwise normal, and nuclei were tau-negative. No lightly expressing ABCs exhibited clearly fibrillar deposits, but such cells frequently showed heavier immunolabel localized to the plasma membrane, especially in distal dendrites (Figure 2C). We observed a total of 30 cells fitting this description, which were examined between 4 and 56 days after vector injection.

Human Tau Hyperphosphorylation First Occurs in ABC Distal Dendrites and Somata, and Is Correlated with Dendritic Swelling and Beading

Sixty-seven ABCs examined between 8 and 79 days after mRNA or plasmid injection were found to have expressed human tau heavily enough so that clear alterations to their normal somatodendritic morphology were evident. These alterations usually took the form of a pronounced swelling and/or beading of most or all of their distal dendrites. When examined in the electron microscope, swollen dendritic tips were found to contain aggregations of membrane-bound organelles mixed with tau filaments and some MTs (Figure 3; also see Hall et al.²⁰). Of the 63 cells immunostained with mAbs directed against phosphoepitopes other than PHF1, 45 exhibited immunolabeling of one or more of the following mAbs: ALZ50 (25 of 57 cells sampled) TG3 (13 of 36 cells sampled), AT100 (4 of

10 cells sampled), or AT8 (24 of 57 cells sampled). These hyperphosphorylated deposits were highly localized, occurring in either the soma and/or the distal dendrites (Figure 4), except in cells exhibiting severe degeneration, where they were distributed throughout the cell (Figure 5). A typical example of a somatic deposit of hyperphosphorylated human tau at a relatively early stage of degeneration is shown in Figure 4; examples of distal dendritic deposits are shown in Figures 3 and 4. Although somatic hyperphosphorylated tau deposits tended to have a fibrillar appearance (Figure 4), much of the distal dendritic staining seemed to be localized to membranous structures. This was particularly true of ALZ50 staining (see Figures 4 and 5B), although examples of ALZ50-positive fibrillar deposits were also found (Figure 4, asterisk). All of the mAbs used labeled both fibrillar and membranous structures. Immunostaining for ALZ50, TG3, AT8, or AT100 appeared in the distal dendrites of 10 cells that did not exhibit somatic hyperphosphorylated tau deposits, whereas only two cells were found that exhibited the reverse pattern, suggesting that distal dendritic changes involving tau hyperphosphorylation usually precede the onset of somatic tau hyperphosphorylation. Proximal dendrites were hardly ever labeled with any phosphoepitope-specific mAb other than PHF1 unless cytodegenerative changes were present throughout the cell as well (Figure 5). There was no clear temporal sequence of immunolabeling of human tau deposits with

Figure 4. Swelling and beading of dendritic tips is characteristically accompanied by tau hyperphosphorylation in ABCs showing a moderate degree of tau-induced degeneration (stage 2). Each micrograph shows immunolabel with a different mAb: PHF1 (**A**), ALZ50 (**B**), AT8 (**C**), TG3 (**D**). **A** and **C**: Adjacent sections from the same ABC (28 days after injection of pRc123c). **B** and **D** are from different cells 28 and 34 days, respectively, after the injection of this plasmid. Note that although some sections show somatic tau deposits (asterisks), and all cells show immunolabeling in distal dendrites (arrows), there is no labeling of dendritic shafts (small carets) in the proximal dendritic field by any mAb except PHF1. Note the parallel nature in the development of morphological degenerative changes (ie, distal dendritic beading, **A**, and the appearance of AT8, ALZ50, and TG3 immunolabel in these areas) Also note that although many of the distal dendritic deposits of hyperphosphorylated tau appear to be membranous, some are fibrillar in appearance (**C**, arrow). Scale bar: 25 μ m.

the four mAbs that were used to identify hyperphosphorylated tau deposits. Although ALZ50 was the only PHF-tau marker other than PHF1 recognizing tau in some ABCs, TG3 and/or AT8 staining appeared to precede ALZ50 in others. Moreover, there was no significant difference between the proportions of cells labeling with each mAb (other than PHF1), suggesting that the events triggering the onset of hyperphosphorylation may make each of these epitopes equally likely to appear.

The onset of human tau hyperphosphorylation in distal dendrites was invariably either preceded or accompanied by at least one of two characteristic dystrophic changes seen with heavy chronic human tau overexpression in ABCs. These were the localized distension of the distal-most dendrites, and the localized collapse of these dendrites at multiple sites, resulting in a series of swellings interspersed with constrictions causing the affected dendrites to resemble beads on a string (Figures 4 and 5A). Of the 34 ABCs that showed only distal dendritic swelling and beading and no other morphological abnormalities, 19 did not exhibit any hyperphosphorylation-related epitope (ie, AT8, TG3, ALZ50, AT100) at all.

Those ABCs at this stage of degeneration that did exhibit tau hyperphosphorylation invariably had it limited to distal dendritic and somatic regions (Figure 4). Proximal dendrites and somata were grossly unaffected by the morphological changes in the distal dendrites of these cells, exhibiting smooth, unbroken profiles (see Figure 4 for examples).

Progressive Degeneration of the Proximal Dendrites and Soma Is Spatiotemporally Correlated with Human Tau Hyperphosphorylation

Thirty ABCs showed more extensive and severe degeneration than that described above, exhibiting degenerative changes involving proximal as well as distal dendrites. In these cases, tau hyperphosphorylation was also extended to these regions (Figure 5). In most of these cells, extracellular human tau deposits that immunolabeled with PHF1 were also visible (see Hall et al¹⁹ and

Figure 5. Severe degeneration of ABCs overexpressing human tau is accompanied by widespread tau hyperphosphorylation in both membrane-associated and fibrillar deposits. **A**, **C**, and **E** were immunolabeled with PHF1 (**A** and **E**) or anti-GFP (**C**) to show the distribution of total human tau within severely degenerated ABCs, whereas adjacent sections were labeled with a mAb against a hyperphosphorylation marker epitope [ALZ50 (**A**), TG3 (**C**), AT8 (**E**)]. **A** and **B** are from an ABC expressing htau40 28 days after injection with SFV-tau mRNA, **C** and **D** are from a cell 49 days after injection of clone 93 (EGFP-htau23) plasmid, and **E** and **F** are from a cell 34 days after injection of clone 93. Note that the somatodendritic profiles of the htau-expressing cells are highly distorted and swollen compared to normal, especially in distal dendrites (**arrows**). We classified these cells into stage 3 degeneration (if the nucleus was present and the somatic profile was still clearly defined as shown in **A–D**), or stage 4 degeneration (**E–F**), which represents the unequivocal breakup and death of the cell. Stage 4 degeneration was also accompanied by the loss of most dendritic immunostaining and the presence of human tau both extracellularly (**asterisk**) and within adjacent glial or ependymal cells. This tau was presumably scavenged from the dead ABC or the extracellular space via endocytosis, and was frequently hyperphosphorylated. Note that precisely the same pattern of pathology can be seen in ABCs expressing htau40 via the SFV mRNA vector (**A** and **B**) as by either EGFP tagged htau23 (**C** and **D**) or untagged htau23 expressed from pRc123c (Figure 2E). Scale bar: 50 μ m.

Figures 2E and 5C). These deposits were clearly located outside of the ABC plasma membrane, but some immunostaining appeared to be in the extracellular space

(Figure 5A) whereas other deposits appeared to be inside of ependymal and/or glial cells (Figure 5, E and F). Some extracellular tau deposits also labeled with TG3,

ALZ50, or AT8, but usually less extensively than with PHF1. Such cells exhibited beading, distortion, and loss of dendrites throughout the dendritic field. As with the milder cases of degeneration described above, human tau deposits in severely degenerated ABCs appeared to be either membranous or fibrillar, with the former predominating in dendritic tips and the latter most prominent in the soma and proximal dendrites. There was no sign that immunolabeling of any epitope (other than PHF1) was more widespread or intense than the others.

We found that these severely degenerated ABCs (in which the entire soma and dendritic field was involved) could be divided into relatively mild cases, in which the nucleus was still present and the somatodendritic profile was still clearly defined, and the most severe cases, where the expressing ABCs no longer exhibited a recognizable nucleus or a continuous plasma membrane. Axonal profiles were still present in most cases and often appeared grossly normal, although extracellular PHF1 staining was occasionally found around axonal profiles (not shown). Nuclei, when present, were invariably tau immunopositive, unlike their appearance in milder cases of tau-induced degeneration (Figures 2E, 5A, and 5C, asterisks).

Proposed Staging of Tau-Induced Neurodegeneration in ABCs

To determine whether the differences between mildly and severely degenerated ABCs described above were because of a progressive sequence of degenerative changes over time, we re-examined sections from all of the ABCs used in this study and assigned each to a stage of neurofibrillary degeneration based entirely on morphological criteria. We used only cells that had either a PHF1- or GFP-immunolabeled slide through their somata, proximal dendrites, and distal dendrites for staging purposes. Sixty-seven of the 97 cells examined in this study met these criteria and were staged as described below by a person who was blind to the age and identity of each cell examined. We thus specifically excluded the length of time that human tau was expressed in ABCs and the presence of hyperphosphorylated tau as staging criteria so as to test directly the relationship between the degree of morphological degeneration of htau-expressing ABCs, their phosphorylation state and time after injection. We broke down the sequence of htau-induced neurofibrillary degeneration in ABCs into the following intervals between major events that were characteristic of all of the tau constructs used, which we then used as the basis of assigning stages of degeneration: 1) the first detectable presence of tau immunolabel up to the first noticeable changes from normal morphology (usually mild swelling of some but not all distal dendrites); 2) clear beading or swelling of all distal dendrites up to the distortion and roughening of some proximal as well as distal dendrites (cells showing extracellular tau label were excluded); 3) clear involvement of the entire somatodendritic region, including nuclear staining, pinching off or beading of entire dendrites, the formation of fibrillar tau in proximal

Figure 6. Stages of neurofibrillary degeneration caused by chronic human tau overexpression in ABCs. The most prominent and/or typical changes seen in degenerating ABCs that are overexpressing human tau. Stages were assigned to 67 ABCs according to morphological changes visible in immunostained sections on the basis of PHF1 and anti-GFP immunolabeling only, without reference to either the presence of tau hyperphosphorylation, the clone or technique being used to induce tau overexpression, or the length of time after injection. Although the presence and/or pattern of tau hyperphosphorylation was not an *a priori* criterion for assigning cells to a particular stage, the close correlation between the patterns of neurodegeneration (shown by PHF1 label at **left**) and hyperphosphorylated tau label (defined as immunolabeling with AT8, TG3, AT100, or ALZ50) is shown for each stage (**right**).

dendrites, and extracellular tau deposits up to the visible breakdown of the plasma membrane; 4) clear indications of cell death, including nuclear loss, fragmentation of the plasma membrane and the cellular profile as a whole, and endocytosis of human tau deposits by adjacent glial cells. These stages of htau-induced degeneration, plus associated changes in tau phosphorylation are summarized schematically in Figure 6.

There was a clear progression through stages 1 to 4 over time after injection (Figure 7A), with the average ABC spending ~10 days each in stages 1 to 2, and somewhat longer in stage 3. A clear positive correlation ($P < 0.05$ or better, chi-square test) between the time of human tau expression and the stage of degeneration reached in ABCs was retained when the cells were sorted according to: 1) tau isoform used, 2) the presence or absence of an epitope tag, and 3) whether expression was achieved with a plasmid or with the SFV mRNA vector (Figure 7B). Thus the presence of a GFP epitope tag, the method used for overexpression, and the isoform used (three *versus* four MT-binding repeats) did not significantly affect either the pattern or overall time course of degeneration. Moreover, both htau23 and htau40 produced virtually identical patterns of phosphotau labeling as well, with PHF1 present at highest concentrations in dendrites in stage 1 cells, and throughout the cells in all later stages. Immunostaining for hyperphosphorylated tau was first seen by late stage 2 in distal dendrites and

Figure 7. Steady progression from early to late stages of NFD occurs with all human tau constructs in ABCs. **A:** A total of 67 ABCs examined at progressively later times after injection showed a steady progression through stages 1 to 4. Cells expressing htau40 via clone 139 and the SFV vector and htau23 via pRCMV123c and clone 93 were combined. Each bar shows the percentage of the total number of cells examined in each time period; these were: 1 to 9 days, 9 cells; 10 to 19 days, 21 cells; 20 to 29 days, 17 cells; and 30+ days, 22 cells. There were a total of 19 stage 1 cells, 22 stage 2 cells, 19 stage 3 cells, and 8 stage 4 cells among the cells sampled. **B:** The stages of degeneration reached by cells sampled at early (E, 20 days or less after injection) and late (L, more than 20 days after injection) times are compared on a per clone basis. The presence of EGFP/GFP as an epitope tag, the use of the SFV expression system, and the presence of the fourth MT-binding repeat did not seem to interfere with the progression of cytopathology from mild (stages 1 to 2) to severe (stages 3 to 4) stages of degeneration ($P < 0.05$). Progression of degeneration within the sample as a whole was highly significant ($P < 0.001$). Statistical comparisons between stage distributions in early and late samples were performed using the chi-square test.

somata, and in stage 3 cells throughout the cell, suggesting that tau-induced degeneration is clearly correlated with tau hyperphosphorylation, and relatively insensitive to the htau isoform used in ABCs. This pattern is shown schematically in Figure 6.

Discussion

In this study, we show that a single, stereotyped sequence of events emerges with the staging of tau-induced NFD in ABCs. This sequence is unaffected by the presence of epitope tags, independent of the isoform

used, and is tightly correlated with the immunolabeling pattern of several epitopes (ALZ50, TG3, AT8, AT100) that are associated with tau hyperphosphorylation and neurodegeneration in human neurofibrillary disease. This pattern of neurodegeneration seems to be specific to human tau, as overexpression of lamprey neurofilament protein from a construct derived from the same parent plasmid causes accumulation of neurofilaments in ABCs, but no cytodegeneration.²⁰ There are key features of this sequence that may have important implications for the cellular mechanisms responsible for the development of human NFD including the similarity that tau-induced neurofibrillary degeneration in ABCs bears to NFD cytopathology in human neurons, and the fact that it can be produced simply by overexpressing human tau. These features are particularly noteworthy as they suggests that simple accumulation of human tau can instruct a neuron to degenerate in a highly stereotyped way that is conserved among vertebrates and is specific to human tau. It is thus likely that a detailed investigation of the interactions between human tau and the cytophysiology of ABCs will provide critical insights into the mechanism of tau-induced neurofibrillary degeneration in humans that are unavailable from more complex and less accessible systems.

Comparison of Neurofibrillary Degeneration in ABCs and Human NFD

The stages of neurofibrillary degeneration in ABCs exhibit striking points of resemblance to the staging of neurofibrillary changes in human hippocampal pyramidal cells proposed by Braak et al,⁸ and Braak and Braak.⁹ These investigators based their studies on autopsy material from persons who did not show widespread neurofibrillary pathology, allowing them to assign stages of cellular degeneration without reference to extracellular or trans-synaptic influences. They found that the mildest degree of degeneration featured the appearance of tau immunostaining in an even, granular distribution throughout the soma and dendrites, followed by the expansion and distortion of the distal-most apical and basolateral dendrites. Stages 2 and 3 of Braak et al,⁸ in which distal dendritic tufts show swelling, distortion, and beading at the same time as fibrillar tau deposits (ie, early NFTs) are forming in the somata of hippocampal pyramidal cells, closely resemble stage 2 in ABCs (Figure 4). More severe stages of degeneration in the hippocampus, as in ABCs, feature the breakdown of somatic morphology, nuclear loss, and the appearance of extracellular tau deposits. On the other hand, there are features of the NFD modeled in ABCs that are not identical to human neurofibrillary disease. For instance, the order of appearance of specific phosphoepitopes appears to be somewhat different in ABCs and in incipient human NFD, with PHF1 rather than AT8 appearing throughout the cell at pretangle stages (stages 1 and 2) in ABCs. AT8 appeared in tau-expressing ABCs only at a time when morphological degeneration was widespread in the distal dendrites, and did not precede overt morphological changes as they did in the

Braak study.⁸ Furthermore, Gallyas silver impregnation and Thioflavin-S labeling were negative in all tau-expressing ABCs examined (not shown), even when heavy filamentous deposits were present, whereas most fibrillar tau deposits appeared to be argyrophilic in the Braak study.⁸ This may have something to do with the very different time scales involved in NFD in humans (up to 20 years)¹² versus ABCs (weeks or months at most)—possibly the argyrophilia of NFTs *in vivo* is acquired gradually by the slow accumulation of one of the many NFT-associated substances found in human pathology. One possible candidate for this role is intracellular A β , which may serve to link NFT formation to APP metabolism in Alzheimer's disease,^{24–27} and that has been put forward as a source of Thioflavin-S birefringence in NFTs.²⁷ It is interesting to note in this context that the spatiotemporal pattern of human tau hyperphosphorylation in ABCs presented in this study closely resembles that of the development of argyrophilia in human hippocampal pyramidal cells, with foci in the distal dendrites and somata spreading eventually to most of the cell.⁸ It is thus tempting to speculate that hyperphosphorylation might be a preliminary to the development of argyrophilia, and that the latter fails to develop in ABCs because of the accelerated time course of tau accumulation and degeneration relative to that seen in human NFD.

A key advantage of being able to directly correlate the progression of degenerative stages with time in an *in vivo* cellular model system is that it became possible for the first time to compare the times of onset of several degenerative changes (tau hyperphosphorylation, dendritic swelling, dendritic degeneration, axonal loss) that are characteristic of neurofibrillary degeneration. The implications of our results for the interrelationships between these events in ABCs and in human NFD are discussed below.

Hyperphosphorylation and Neurofibrillary Degeneration in ABCs

Human tau has been induced to form filaments under a variety of conditions *in vitro*^{28–33} and these studies have suggested several possible mechanisms by which deposits of filamentous tau might form in NFD. However, although there is abundant *in vivo* and *in vitro* evidence that the phosphorylation of human tau protein regulates its ability to bind to and stabilize MTs,^{34–36} and good reason to suppose that tau filament formation plays a critical role in tau-induced cytodegeneration,^{19–20,37} there is no clear evidence that the hyperphosphorylation of tau is required for either tau filament formation *in vivo* or the cytodegeneration caused by NFD. We showed in a previous study of tau-induced NFD in ABCs²⁰ that human tau forms large numbers of filaments throughout ABC somata and dendrites by 10 days after plasmid injection, at a time when all of the cells expressing that construct (clone 93—htau23 with an N terminal fusion of EGFP) are in either stages 1 or 2 (see Figure 7A). Because there is little if any tau hyperphosphorylation outside of the distal-most dendrites before stage 3, it seems unlikely that tau

hyperphosphorylation is prerequisite for tau filament formation, at least in ABCs. Immunolabeling for PHF1 alone, by contrast, occurs very early after the onset of tau expression in ABCs, and precedes the first morphological signs of degeneration. Thus, it is possible that the phosphorylation of the PHF1 site (but not other AD-related phosphoepitopes) is a necessary preliminary for tau filament formation and consequent degenerative changes in ABCs. Constitutive phosphorylation of the other AD-related sites might occur as a consequence of filament formation, especially if the incorporation of tau into filaments were to block access to tau phosphoepitopes by phosphatases that normally dephosphorylate tau at these sites.^{38,39} However, the tight association of tau hyperphosphorylation with the progress of degenerative changes in ABCs, murine, and ovine model systems,^{16–18} and human tauopathies suggests that it plays an important but as yet obscure role in NFD cytopathogenesis.

The Dendrites as an Initial Focus for Neurofibrillary Pathology

The other major implication of these data for the pathological mechanisms underlying NFD at the cellular level is the localization of the first pathological changes to the dendritic tips. This is particularly interesting in light of the membranous nature of most of the distal dendritic tau deposits and the involvement of membranous organelles in the swelling of dendritic tips that initiate the degeneration process in ABCs, and suggests a number of possible routes by which human tau overexpression might initiate a degenerative cascade. For instance, recent studies have implicated the N-terminal domain of human tau as a potential point of interaction between tau and the plasma membrane,⁴⁰ and tau may also compete with kinesin in its interactions between membranous organelles and MTs under conditions where tau is overexpressed,^{41,42} leading to the disruption of kinesin-mediated transport of membranous organelles. Either of these interactions might plausibly lead to accumulations of membranous organelles both in the cell body and at dendritic tips, especially because dendritic MTs have mixed polarity.⁴³

The contrast that we found between the time of onset of gross morphological changes to dendritic tips and axons may also have important implications for the issue of where the initial changes leading to NFT formation and neurofibrillary degeneration occur in human NFD. Our results lend some support to the proposal that degenerative changes in the dendrites precede axonal degeneration in NFD. Although the dendritic tips began to show PHF1 immunolabel and signs of swelling during stage 1, overt changes to ABC axonal morphology suggestive of degeneration were not seen until stage 3. Normal looking axons were seen in a number of stage 2 to 3 cases that were processed for electron microscopy, where dendritic degeneration was later shown to be severe, with extensive MT and synapse loss.²⁰ This is in agreement with earlier work in this system where the phosphorylation of

both the PHF1 and Tau1 sites appeared to occur in the sequence: distal dendrites → proximal dendrites/soma → axon at early stages of degeneration.¹⁹ This sequence is later recapitulated in the pattern of both hyperphosphorylation and cytodegeneration in stages 2 and 3. Thus a direct interference with functions such as MT-mediated dendritic transport by tau seems to be more plausible than an initial effect on axonal function, which might be expected to cause early changes in axonal morphology as well as dendritic changes.

It remains possible, however, that the observed dendritic changes were induced by an initial interference with normal axon function (such as an inhibition of axonal fast transport via an interaction of tau with kinesin) without obvious early effects on axonal morphology. Such an interaction (if strong enough) might cause the cell to react as if it had been axotomized. Because axotomy at a point very close to the soma can cause loss of normal neuronal polarity, with axonal sprouting from dendritic tips,^{44,45} this might account for the initial dendritic swelling in tau-expressing ABCs, especially as swollen dendritic tips are the first morphological change visible in ABCs after both close axotomy and tau overexpression. Polarity loss is a characteristic event in the cytopathology of Alzheimer's disease as well as in axotomized ABCs,^{46,47} where massive dendritic sprouting gives rise to axon-like processes such as neuropil threads.^{48,49} No direct connection between polarity loss and axonal loss has yet been established in human NFD, although it should be noted that htau40 overexpression in mice has produced axonopathy in the absence of clear filamentous pathology, suggesting a possible early role for axonal dysfunction in tau-induced NFD in at least some cell types.¹⁸

Implication of These Findings for Interpreting the Role of Tau Mutations in Producing Human NFD

Finally, the observation that htau23 and htau40 produces virtually identical effects when overexpressed in ABCs has possible implications for understanding the roles played by the characteristic tau isoform differences between neurofibrillary lesions of clinically defined tauopathies^{50,51} and the mechanism of action of some tau mutations that induce NFD in humans. One of the most puzzling issues raised by the recent identification of mutations affecting the tau coding sequence and tau splicing has been the difficulty in accounting for the widely different neuropathology resulting from these mutations, some of which appear to merely change the relative frequency of tau isoforms with three *versus* four MT binding repeats.^{4,6,7,51} For instance, although we found that htau23 and htau40 produce patterns of degeneration that are indistinguishable from one another when overexpressed in ABCs, two recent reports of the effects of overexpressing htau23¹⁷ and htau40¹⁸ in mice showed quite different patterns of pathology. These differences emphasize the following points: 1) the likely importance of cell-type specificity in determining how the presence or absence of a particular tau isoform produces cell-type-specific susceptibility and patterns of neurofibrillary pa-

thology in familial tauopathies, and 2) that the underlying cellular mechanisms responsible for tau-induced neurofibrillary degeneration are not isoform-dependent and depend on interactions between human tau and highly conserved mechanisms of neuronal physiology. Thus although the development of murine models that faithfully reproduce the characteristic pathological features associated with the various familial tauopathies will be essential for understanding their pathogenesis, there is also clearly a complementary need for further intensive study of the fundamental cell biological mechanisms responsible for tau-induced neurofibrillary degeneration at the level of a single, identified neuron. The large cell size, unique accessibility, and stereotyped morphology and physiology of ABCs should make them ideal for this purpose.

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