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Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses

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Abstract

Loss of synapses and dying back of axons are considered early events in brain degeneration during Alzheimer's disease. This is accompanied by an aberrant behavior of the microtubule-associated protein tau (hyperphosphorylation, aggregation). Since microtubules are the tracks for axonal transport, we are testing the hypothesis that tau plays a role in the malfunctioning of transport. Experiments with various neuronal and non-neuronal cells show that tau is capable of reducing net anterograde transport of vesicles and cell organelles by blocking the microtubule tracks. Thus, a misregulation of tau could cause the starvation of synapses and enhanced oxidative stress, long before tau detaches from microtubules and aggregates into Alzheimer neurofibrillary tangles. In particular, the transport of amyloid precursor protein is retarded when tau is elevated, suggesting a possible link between the two key proteins that show abnormal behavior in Alzheimer's disease.

Keywords: Alzheimer's disease; Tau; Axonal traffic

1. Introduction

The loss of synapses and dying-back of axons is a characteristic feature of neurodegenerative diseases such as Alzheimer's. They appear at early stages and correlate with the incipient loss of memory and brain functions [6,7,32]. A number of triggering events have been implicated, such as oxidative stress, inflammatory cytokines, lack of growth factors, or the toxic Aβ peptide which may lead to the decay of the axon or the neuron as a whole [25,27]. These features may develop gradually and appear to precede the more overt pathological changes in the brain, such as deposition of protein aggregates in the form of amyloid plaques and neurofibrillary tangles.

Since neurons are highly elongated cells they depend on an efficient transport system for delivering proteins, lipids and other cell components from the cell body to the synapse. This system is based on microtubules which serve as tracks, motor proteins which represent the engines, vesicles and organelles which are the cargoes, and microtubule-associated proteins (MAPs) which serve as ties for the stabilization of the microtubule tracks [21]. In axons, tau protein is one of the predominant MAPs. It stabilizes microtubules and promotes neurite outgrowth. This apparently beneficial role of tau contrasts with its anomalous behavior in several neurodegenerative diseases, most prominently Alzheimer's disease, where it occurs in a highly phosphorylated form, detaches from microtubules, and aggregates. It has been hypothesized that the detachment of tau from microtubules is caused by some imbalance in intracellular signaling which favors excessive phosphorylation. This in turn would detach tau from microtubules, prompt their decay, so that axonal transport would be interrupted by the breakdown of the tracks. In addition, the detached, soluble tau would aggregate and thus cause a generalized clogging of cytosolic space. One advantage of this hypothesis is that it relates neuronal degeneration to the abnormalities in tau, in agreement with observations (Braak stages [1,5]). However, the assumptions on the underlying molecular mechanisms are not well supported at present; for example, it is not clear whether detachment of tau and its aggregation and the breakdown of microtubules are early causes of degeneration or later consequences. We, therefore, searched for other cellular roles of tau which might explain a more subtle and early defect in neuronal physiology. This lead us to study the interplay between motor proteins and MAPs, both of which have to interact with the microtubule surface. Unexpectedly, the "ties" are able to inhibit the "engines". This is in contrast to a real railroad where ties and engines are spatially segregated on opposite sides of the tracks. This effect has the potential of blocking diverse transport processes, es-

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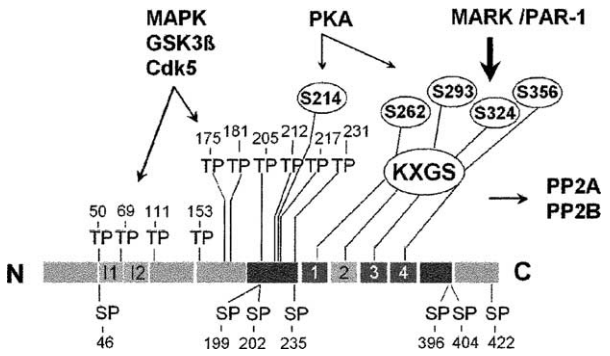

Fig. 1. Diagram of tau protein: isoforms and phosphorylation sites. Human tau in CNS neurons occurs as six isoforms derived from a single gene by alternative splicing (352–441 aminoacid residues). The three or four repeats in the C-terminal half (∼31 residues each) constitute the center of the microtubule-binding domain, as well as the core of Alzheimer paired helical filaments. Tau contains a number of Ser and Thr residues, many of which show abnormally high phosphorylation in Alzheimer's disease and are diagnostic of Alzheimer tau. Phosphorylation sites within the repeats (at KXGS motifs) efficiently detach tau from microtubules.

pecially the anterograde processes from the cell body down the axon towards the synapse. It could be caused, for example, by a local elevation of tau which in its initial stages would not affect microtubule numbers or tau aggregation.

Human tau is encoded by a single gene (on chromosome 17) which can be spliced into six main isoforms in the central nervous system containing between 352 and 441 residues; a further higher molecular weight isoform is enriched in the peripheral nervous system (Fig. 1). The domain of the repeats (three or four, each about 31 residues) is essential for microtubule binding, and in addition it forms the core of Alzheimer PHFs [24]. However, domains flanking the repeats have to be present in order to ensure tight binding to microtubules. The N-terminal "projection domain" protrudes from the microtubule surface and presumably interacts with other cell components (e.g. anchor for kinases or phosphatases). Tau has a very hydrophilic composition which makes it highly soluble; the aggregation into insoluble fibers in disease states is therefore counterintuitive. Native tau also has a mostly unfolded structure, contrary to a typical globular protein. It is likely that some of this structure becomes folded when interacting with microtubules. Similarly, the repeat domain adopts a cross-beta structure when tau aggregates into pathological fibers, similar to other amyloids [35]. A notable feature of tau is its many potential phosphorylation sites, targeted by a variety of protein kinases. Most sites outside the repeat domain are of the type Ser–Pro or Thr–Pro and can therefore be phosphorylated by proline-directed kinases (MAP kinase, GSK-3β, cdk5). Other sites within the repeats are targets of non-proline-directed kinases, in particular the KXGS motifs in the repeats which are targets of microtubule-affinity regulating kinases (MARK [8]). Certain phosphorylated motifs are important diagnostic tools because they are among the first signs of neuronal degeneration [2], but their physiological roles are uncertain. Phosphorylation of the KXGS motifs in the repeats has the effect of detaching tau from microtubules and could therefore contribute to microtubule instability.

The results reported here were prompted by long-term observations of CHO cells transfected with GFP-labeled tau. The protein showed apparently normal behavior, i.e. it was attached to microtubules, and the microtubule affinities of different forms of tau were in general agreement with the values measured in vitro. However, there was a very slow rearrangement of cell components such as mitochondria which tended to accumulate in the cell center; the cells rounded up and divided less frequently [11]. Inspection of the movements of mitochondria and exocytotic vesicles by live-cell time-resolved microscopy revealed that movements were inhibited, especially those towards the cell periphery. Thus, the dominance of inward-directed movement lead to the retraction of mitochondria, cells lost their extended shape and rounded up [34]. The effects were clearly related to the binding of tau to the microtubule surface and were explained by an interference between microtubule-dependent motor proteins and tau [26]. Here we report on the consequences of this effect for neuronal cells, especially for their cell processes. We show that traffic inhibition by tau has drastic implications for the distribution of cell components, which leads to energy deprivation, lowered defence against oxidative stress, and dying-back of neurites.

2. Results

2.1. The transport of cell organelles and vesicles is inhibited by tau

Figs. 2–5 show typical experiments which illustrate the effect of tau on intracellular traffic of different cell components and in different cell types. For example, N2a cells can be differentiated by exposure to retinoic acid and develop neurites which grow to typical lengths of 30 μm in 2 days (Fig. 2). These neurites contain the components necessary for sustained growth, such as mitochondria for the generation of chemical energy, peroxisomes for detoxification of H_2O_2, neurofilaments and microtubules for structural stability and intracellular transport, and transport vesicles carrying supplies for the growth cone. The transport and distribution of these components was studied in normal cells (containing only endogenous tau) and cells where the level of tau was increased by stable transfection with human tau. In control cells, organelles such as mitochondria and peroxisomes are normally distributed throughout the cell body and the neurites where they are transported by motor proteins along microtubules [23]. In cells with elevated tau they are virtually absent from the neurites, become clustered in the cell center around the MTOC, and the neurites are inhibited in their growth (Fig. 2). The same is observed for neurofilaments which also rely on microtubules for their transport

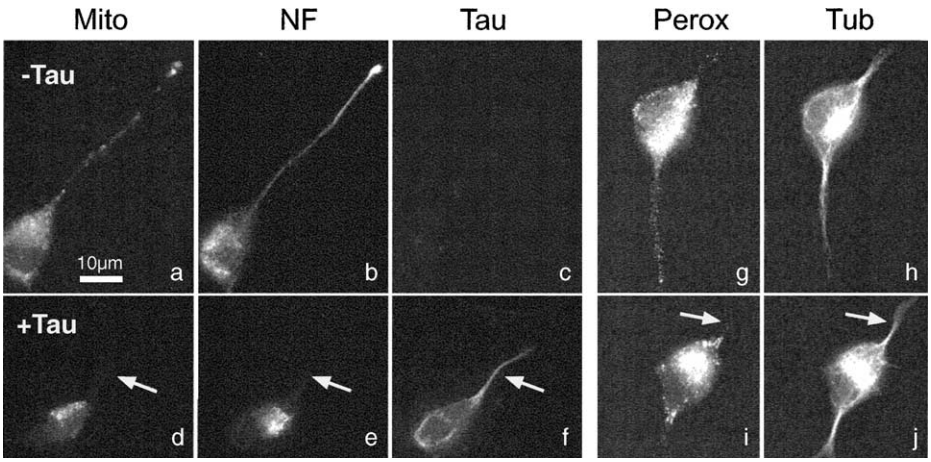

Fig. 2. Inhibition of transport by tau in neurites of N2a cells. Top: control N2a cells, differentiated for 2 days by 1 μM retinoic acid, fixed in methanol, and immunostained; bottom: cells transfected with tau. (a–c) N2a cell stained for mitochondria, neurofilaments, and tau; the cell contains a long neurite since the level of tau is low (c). (d–f) In the tau-transfected cell, mitochondria and neurofilaments are retracted to the cell body (arrows in d, e point to empty neurites), while tau and microtubules still extend into the neurite (f). (g, h) Cell stained for peroxisomes and microtubules which extend throughout neurites. (i, j) When tau is elevated, peroxisomes disappear from neurites (i) whereas tau and microtubules still persist (arrows).

and distribution [28]. By contrast, microtubules are present throughout the neurites, showing that their transport is not perturbed, and the same holds for tau which is associated with microtubules throughout the neurite. The results illustrate that the tracks for axonal transport are present in the neurite, but the transport along them is impaired. This implies that neurites lack the energy of ATP (because of the missing mitochondria) and lack protection against oxidative stress (because of the missing peroxisomes), which explains why tau-expressing cells have shorter neurites and are more sensitive to oxidative stress.

Similar transport phenomena were observed in primary hippocampal neurons from rats which were transfected with tau by adenovirus vectors containing the longest human tau isoform (htau40, Fig. 3). Normal neurons showed mitochondria and peroxisomes throughout the cell body and the cell processes, but after transfection with tau the organelles disappeared from the neurites and accumulated in the cell body.

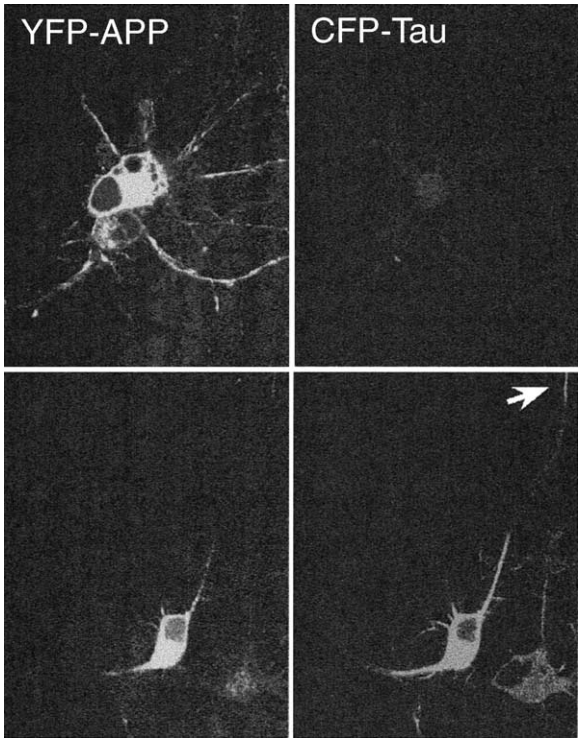

Fig. 3. The transport of APP is inhibited in cultured hippocampal neurons. Two-color imaging of a neuron transfected by adenovirus with APP-YFP alone (top) or cotransfected with APP-YFP and CFP-htau40 (bottom) and analyzed by confocal microscopy. APP alone occurs along the cell body and neurites (top left), but with elevated tau it is largely restricted to the cell body (bottom left), whereas tau is located along the entire axons (bottom right, arrow).

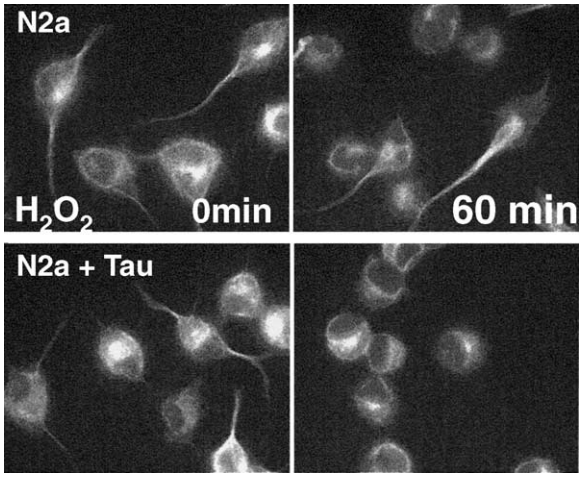

Fig. 4. Neurites of N2a cells expressing tau become vulnerable to oxidative stress. Top, control N2a cells differentiated for 2 days and exposed to 250 μM H_2O_2 and immunostained for microtubules. After 1 h long neurites are still clearly visible. Bottom, tau-stable N2a cells treated with H_2O_2. Neurites disappear quickly and are no longer visible at 60 min.

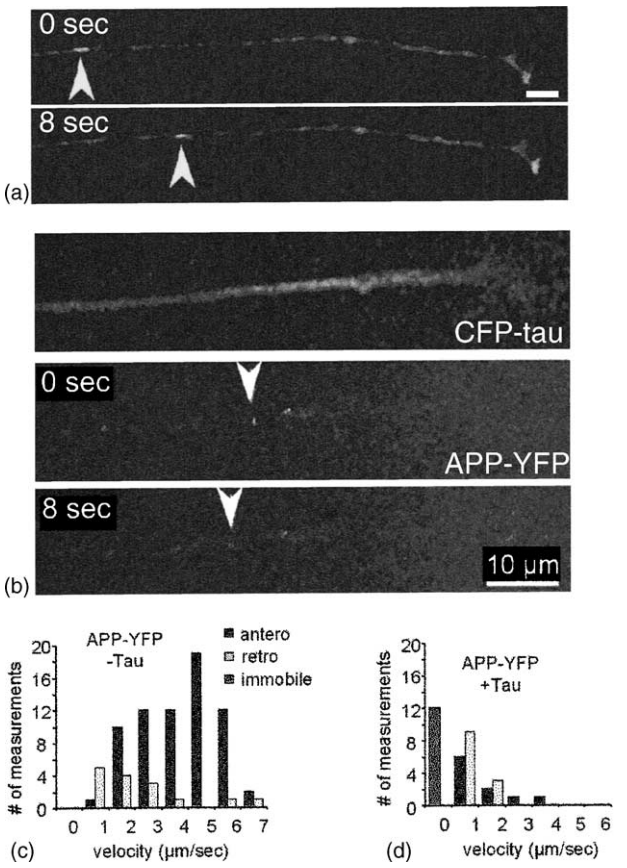

Fig. 5. Transport of APP-YFP vesicles in retinal ganglion neurons and inhibition by tau visualized by time-lapse microscopy. (a) RGCs transfected with APP-YFP adenovirus, two-image frames separated by 8 s. There are numerous APP-vesicles moving rapidly, mostly in the anterograde direction. (b) RGCs doubly transfected with CFP-htau40 (upper panel) and APP-YFP (middle and lower panels), two frames separated by 8 s as in (a). There are only few particles moving slowly or immobile. (c and d) Histograms of particle velocities without and with tau. Note that without tau most APP-vesicles (~80%) move rapidly forward, in the presence of tau they slow down, many are immobile, and net transport is now reversed.

This means that the elevation of tau has similar effects on intracellular transport in primary neurons and neuron-like cell models. As in non-neuronal cells [11] there is a preferential inhibition of plus-end directed transport by kinesin motors along microtubules so that minus-end directed transport by a dynein-like motor becomes dominant.

2.2. Depletion of organelles makes neurites sensitive to stress

If organelles disappear from neurites of cells containing elevated tau one would expect major defects in their local metabolism because diffusion from the cell body would not be able to compensate the loss of local production. One consequence would be the reduction of ATP levels in the absence of mitochondria. A second consequence would be a higher sensitivity to oxidative damage, caused by the absence of catalase in the peroxisomes. This can be tested by exposing differentiated N2a cells to H_2O_2 (e.g. 250 mM, Fig. 4). In control cells one observes a progressive loss of neurites over the period of several hours, and eventually to cell death. By contrast, in tau-transfected cells the degradation of neurites is much more rapid. The decay depends on the length of neurites; long ones are more vulnerable than short ones. Notably, the higher vulnerability was restricted to the neurites. In other words, peroxisomes were relocated to the cell body, but they were not incapacitated where present. To check that the degeneration effect was due to the extracellular added hydrogen peroxide, catalase was added to the medium. This protected the neurites efficiently against oxidation-induced stress. On the other hand, the function of peroxisomes could be inhibited by 3-aminotriazol which exacerbated the sensitivity of the neurites, independently of the expression of tau. We conclude that tau has no negative effect on the overall biochemical pathways of neurons, and that the decrease in the viability of neurites can be explained by its effect on transport and the resulting redistribution of organelles.

2.3. Trafficking of amyloid precursor protein is inhibited by tau

Amyloid precursor protein (APP) is a membrane protein implicated in Alzheimer's disease due to its inappropriate proteolysis and accumulation of its Aβ peptide fragment. APP is initially transported to the axon by Golgi-derived vesicles [29]. Because tau is inhibitory to the transport down the axon we asked whether tau would also affect the anterograde transport of APP. We used retinal ganglion cells stably transfected with the human isoform APP_{695} labeled with YFP [33]. The advantage of these cells is that the polarities of the axons are well defined so that transport directions are readily analyzed. Without tau, APP-vesicles are seen to move rapidly (several µm/s) in the cell body and neurites, and the anterograde direction predominates (Fig. 5a). When the cells are co-transfected with tau it is transported into the neurites, but now the APP-vesicles become strongly depleted, and the remaining vesicles move mostly in the retrograde direction or are immobile. The results emphasize the connection between the elevation of tau and the inhibition of anterograde APP trafficking. The same effect of tau on the transport of APP was seen in cultured hippocampal neurons transfected with APP-YFP and CFP-htau40 using recombinant adenoviral vectors (data not shown). Transfection of APP alone showed APP-vesicles in the cell body and neurites, whereas cells transfected with APP and tau showed that APP was restricted mostly to the cell body. By contrast, dendrites and axons were almost devoid of YFP fluorescence (Fig. 3).

3. Discussion

The established physiological functions of tau include the stabilization of microtubules and the promotion of neurite

outgrowth [12,20]. More recently it was recognized that tau can regulate the transport of cell components by molecular motors along microtubules [11]. Tau influences the rates of attachment and detachment of motors from microtubules [26,34]. The result is that movements towards the cell center become predominant. This leads to the gradual retraction of cell components such as the ER, mitochondria or peroxisomes. Starting from these observations we asked what the effect of tau would be on highly asymmetric cells such as neurons. Tau is the predominant MAP in axons [4]; if the plus-end directed transport were retarded by tau this would hinder the supply of material into the axon and towards synapses. This may be significant in the context of Alzheimer's disease where the affected neurons contains higher levels of tau and tau aggregates [18]. We tested three types of cells, differentiated neuroblastoma cells, primary hippocampal neurons from rat or mouse brain, and chick retinal ganglion cells. Tau was elevated either by stable or transient transfection, by transfection with viral vectors, or by the calcium phosphate method. We analyzed the distribution of cytoskeletal components (microtubules, tau, neurofilaments), organelles (mitochondria, peroxisomes), and Golgi-derived vesicles, in particular vesicles carrying APP. We also studied the growth of neurites and their response to oxidative stress. The observations support the view that tau inhibits transport along microtubules, preferentially in the anterograde direction. As a result organelles are mostly excluded from cell processes, and vesicles are strongly reduced. Because diffusion is restricted, axonal compartments are more vulnerable than neuronal cell bodies or the non-neuronal cells studied previously. The inhibition of organelles, vesicles and neurofilaments is consistent with the view that these components are transported by a kinesin-dependent transport [13,22]. The inhibition does not apply to microtubules or their associated proteins (e.g. tau itself), presumably because their transport is achieved by a different type of mechanism, for example by dynein-dependent transport along actin filaments [3].

The inhibition of transport would be expected to have serious consequences for the growth and survival of cell processes. We have tested this for the growth of neurites and their vulnerability to oxidative stress. Tau-transfected cells have shorter neurites and become sensitive to oxidative stress (H_2O_2), consistent with the lack of peroxisomes (note that the alternative pathway of detoxification by glutathione peroxidase plays little role in neurons [9]). By the same argument, the exclusion of mitochondria from the cell processes implies a local depletion of ATP which would be equivalent to a loss of mitochondrial function in the neurites (but not in the cell body), leading to their gradual decay.

What do these results mean for the understanding of neurodegenerative disorders such as Alzheimer's disease and other tauopathies which are characterized by elevated and aggregated tau protein? AD is characterized by two types of protein deposits in the brain, the extracellular amyloid plaques, consisting largely of the peptide Aβ, a derivative of the membrane protein APP, and the intracellular neurofibrillary tangles, consisting mostly of tau protein. The progression of the disease correlates with the spreading of the neurofibrillary tangles, whereas the amyloid plaques have a more generalized distribution [1,5]. One of the earliest detectable signs is the loss of synapses and retrograde degeneration ("dying back") of neurons which appears to be accompanied by a decay of intracellular transport [32]. This may be linked to an elevation of non-fibrillar forms of Aβ [27]. APP is thought to have neurotrophic functions and is carried by kinesin-driven vesicles, and it appears that the cytosolic C-terminal tail of APP interacts directly with a kinesin light chain [13,17]. As shown here, once tau becomes elevated the APP-vesicles are mostly removed from the axon, and APP is found concentrated in the cell body. This is a locus for the generation of the Aβ peptides; in particular, both Aβ40 and the more toxic variant Aβ42 are generated in the trans-Golgi network [37]. If the residence time of APP increased by a tau-dependent slowing of traffic one would expect an increase in the production of Aβ, causing pathological aggregation and toxicity. This would be analogous to the increased accumulation of Aβ by other treatments which inhibit vesicle budding or transport [15]. Although trafficking of APP-vesicles is reduced by tau, the transport infrastructure is much more resistant than traffic itself: microtubule tracks survive for many hours after mitochondria, peroxisomes, neurofilaments, APP-vesicles and others have deserted the neurite and accumulated in the cell body, but eventually microtubules also disappear when the cell processes degenerate. One could imagine two potential causes for neuronal damage due to transport inhibition of APP. One is that APP becomes depleted at the synapse where it could no longer serve its neurotrophic function, the second is that APP is retained in the cell body where increased levels of Aβ would be produced (gain of toxic function).

How are our results linked to models of tau pathology in AD and other "tauopathies"? The most visible change for the pathologist is the aggregation of tau into neurofibrillary tangles which is accompanied by the accumulation of tau, hyperphosphorylation, loss of microtubule binding and microtubule decay in the affected neurons [18]. A current hypothesis holds that the physiological function of tau (stabilization of microtubules) is disrupted due to excess phosphorylation of tau, the unbound tau then aggregates and obstructs the cell interior, and the microtubules disassemble so that axonal transport is disrupted (Fig. 6a–c). This hypothesis is based on the view that tau's role is to promote neurite outgrowth by stabilizing microtubule bundles [10]. Thus, microtubule-bound tau would be beneficial for the neuron. However, other observations do not fit this scheme: Tau-deficient transgenic mice show no major phenotype since tau can be substituted by other cofactors (MAP1b) [31], mice overexpressing tau show transport defects even though microtubules are intact and tau aggregates are absent [14,16,19], and flies overexpressing tau also show defects in neuronal traffic without evidence of tau aggregation [36].

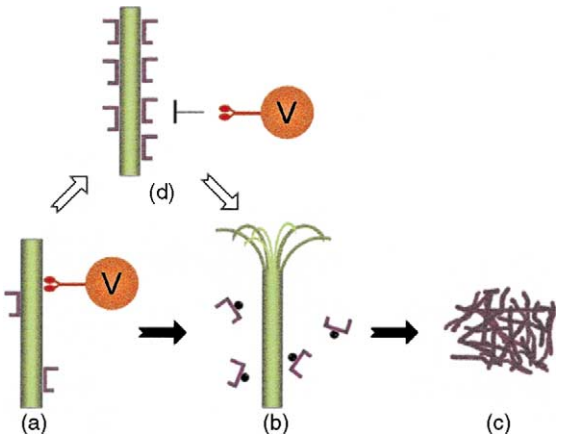

Fig. 6. Model of tau functions in neurodegeneration. (a) In a healthy axon, microtubules (green) are stabilized by tau (purple) and serve as tracks for axonal transport of vesicles and organelles (orange). (b) The common "tau hypothesis of AD" assumes that tau becomes "hyperphosphorylated" at an early stage of neuronal degeneration, detaches from microtubules, microtubules break down and thus no longer support axonal transport. (c) The detached and hyperphosphorylated tau is thought to aggregate into "paired helical filaments" (PHF) which coalesce into neurofibrillary tangles (NFT) and present further obstacles to intracellular transport. In this model, microtubule-bound tau is considered beneficial because it enables microtubule-dependent traffic. (d) An alternative model postulates that tau bound to microtubules is elevated early during neurodegeneration. Even though microtubules are stable, the excess tau blocks traffic into the axon because it interferes with motor proteins, long before microtubules break down or neurofibrillary tangles form. This makes axons vulnerable and causes the decay of synapses. Phosphorylation of tau is seen as the cell's attempt to clear the inhibitory tau off the microtubule tracks. In this view, excess microtubule-bound tau is considered detrimental to the cell.

These observations argue that even "normal" tau may be detrimental when it becomes elevated. A hint to explain this discrepancy comes from the fact that the tau/tubulin ratio is usually low, so that a few tau molecules may suffice to initiate a growing neurite. Thus, in a physiological environment tau stabilizes while its effect on transport is negligible, but it can become noticeable if tau becomes elevated in degenerating neurons. Once traffic jams are initiated, they are exacerbated by the disappearance of organelles. This could be the situation mimicked by the cell models described here. As a corollary, the "hyperphosphorylation" of tau seen in AD may be the cell's attempt to keep the transport tracks open, rather than initiating the neurofibrillary pathology. In conclusion, tau may be "good" for the neuron at physiologically low concentrations, but becomes "bad" at elevated concentration. The first victim of the subtle inhibition of axonal flow is likely to be the most vulnerable structure—the synapse.

4. Materials and methods

The methods of cell transfection and observation, quantification of axonal transport and other experimental procedures have been described elsewhere (for details see [11,30,34]).

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