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THE TAU PROTEINS IN NEURONAL GROWTH AND DEVELOPMENT

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TABLE OF CONTENTS

1. ABSTRACT

The neuronal microtubule-associated protein tau has been implicated as having a role in the outgrowth of neural processes and the development of neuronal polarity. *In vitro*, tau promotes microtubule assembly, stabilizes cellular microtubules, and affects their dynamic behavior. Antisense experiments using cultured neurons provided evidence for an essential role of tau in the development of axons. However, tau knockout mice turned out to be surprisingly healthy and developed neurons which were functionally and structurally almost normal. This raises the question of how essential tau is for neuronal development. In the first part of this paper, data on the function of tau as a microtubule assembly-promoting and stabilizing factor are reviewed. Then, studies investigating the role of tau in the development of neuronal polarity are discussed. In the last part, recent results which provide evidence for a role of tau not directly related to its activity on microtubule assembly are summarized.

2. INTRODUCTION

Neurons are one of the most extreme cell types in that they contain processes which can reach a meter or longer and encompass more than 99% of the cellular volume. This requires the presence of a sophisticated molecular machinery in order to establish and maintain such a morphology. From electron microscopic studies, it had been inferred that an extensive array of linear polymers confers the stability of the processes and is required for their formation. It became increasingly clear from the three cytoskeletal elements capable of forming such an array - actin filaments, intermediate filaments, and microtubules - that microtubules had a very important role due to their abundance in neurons, their characteristic morphological and biochemical organization during neuronal development, and their highly dynamic behavior (reviewed in 1).

The assembly of microtubules is regulated largely by microtubule-associated proteins (MAPs). The neuronal MAPs, tau and MAP2, have attracted particular interest due to their polar distribution in the axonal versus somatodendritic compartment and, mainly in the case of tau, for its potential role in some neurodegenerative disorders (for reviews, see ref. 2-5).

The focus of this review will be on the role of tau in neuronal development and process formation. First, studies on the molecular structure and functional organization of tau with respect to its activity to promote microtubule assembly and stability will be reviewed. Next, studies on the subcellular localization of tau *in situ* and in neuronal culture will be summarized. Then, apparently contradictory results on the role of tau obtained through gene-knockout and gene-transfer techniques will be discussed. In the final section of this review, increasing evidence for a role of tau beyond its function in promotion of microtubule assembly will be presented.

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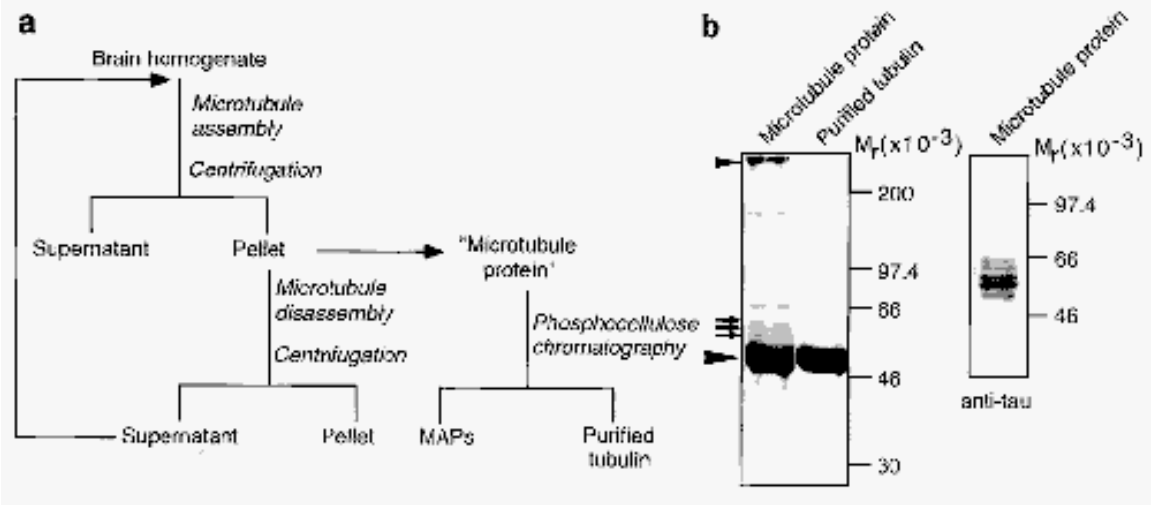

Figure 1: Isolation of neuronal MAPs. a. Schematic representation of the procedure of "cycled assembly". A bovine or porcine whole brain homogenate is prepared and microtubule assembly is promoted by addition of GTP, glycerol, and incubation at 37°C. Microtubules are collected by centrifugation, disassembled in the cold, and cleaned from particulate material by a second centrifugation step. The whole procedure is repeated a second time, yielding a "microtubule protein" fraction which, in addition to tubulin, contains several associated proteins including the MAPs. The MAPs can be chromatographically separated from tubulin by exploiting their positive charge at neutral pH. Tau can then be gel chromatographically separated from the high-molecular weight MAPs. b. Gel electrophoretic separation of microtubule protein and purified tubulin. Electrophoretic separation was done in a 10% polyacrylamide gel in the presence of SDS. 10µg of microtubule protein or purified tubulin was loaded and stained by Coomassie (left). Immunodetection employed Tau-1 antibody (42) and an alkaline-phosphatase coupled secondary antibody. Note the presence of several proteins other than tubulin (large arrowhead) in the "microtubule protein fraction". Under the employed conditions, the high molecular weight MAPs (MAP1A, MAP1B, MAP2) are retained at the top of the gel (small arrowhead). The tau isoforms range in size from 50-70 kDa (arrows) and can be identified by immunodetection using an anti-tau antibody (right).

3. DISCUSSION

3.1. Tau, a protein factor required for microtubule assembly.

The classical technique for the isolation of neuronal MAPs is the procedure of cycled assembly (Figure 1). With this procedure, a "microtubule protein" fraction enriched in tubulin and microtubule-associated proteins is prepared by several reversible assembly/disassembly cycles starting from bovine or porcine brain homogenates. The procedure of cycled assembly yields as major MAPs, several high-molecular weight proteins (MAP1A, MAP1B(MAP5), and MAP2) with molecular weights >200 kDa, and the tau proteins, a family of closely related low molecular weight phosphoproteins with a mass between 50 and 70 kDa (6, 7).

Purified tau protein promotes microtubule nucleation and elongation from purified tubulin which would not assemble in the absence of MAPs (8, 9). Tau modulates the dynamic instability of tubulin assembly by affecting the rates of polymerization, transition into catastrophic depolymerization, and depolymerization (10, 11). It should be noted that tau (and MAP2) can also be classified as F-actin-binding proteins since they interact with actin filaments *in vitro* (12, 13). However, until now no evidence exists that tau interacts with actin filaments also in cells.

Based on the *in vitro* activities of tau, it has been suggested that tau's major role is to regulate neuronal microtubule assembly and stability. Such a role is supported by the time course of tau expression during process formation where neurite extension is paralleled by an increase in the amount of expressed tau (14-16). In addition, gene transfer experiments have shown that the level of expressed tau influences microtubule assembly, neurite outgrowth, and neuritic stability in cultured neural cells (17).

Molecular cloning of tau from different species has revealed that the tau proteins are produced from a single gene by alternative splicing and posttranslational modification (Figure 2). Expression of the tau isoforms is developmentally regulated and differs in the central and peripheral nervous system. In rat brain, tau is first expressed as early as on embryonic day 13. At this time, only the shortest isoform (fetal tau) is expressed. During postnatal development, a transition to the expression

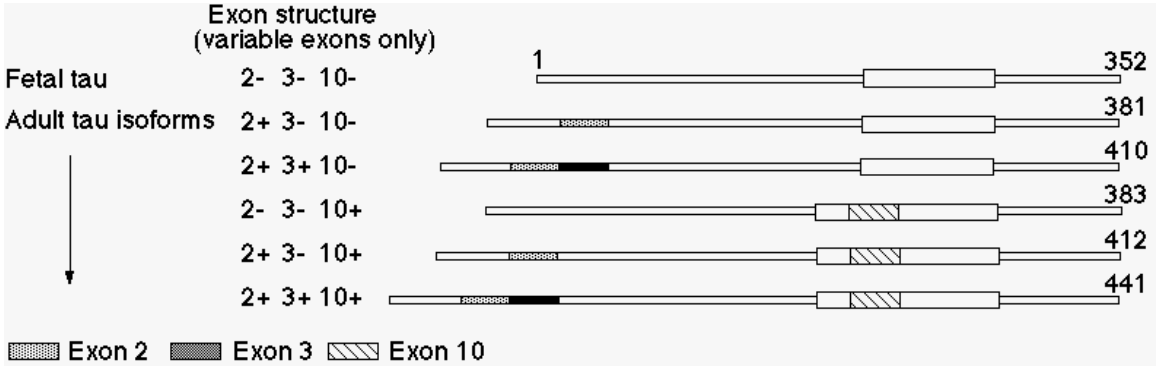

Figure 2: Schematic representation of isoforms of human tau. Fetal and adult isoforms of tau and adult-specific exons are shown. The microtubule-binding domain at the carboxy-terminal half of the proteins is indicated by the open box. Note that one of the exons (exon 10) codes for an additional repeat. The high molecular weight species of tau in peripheral nerves contain two additional exons (4A and 6; (110)).

of all low-molecular weight tau isoforms occurs (18) with an abrupt decline in the expression of fetal tau beginning on postnatal day 8 (19). In the adult peripheral nervous system, a high molecular weight tau isoform (110 kDa) which contains two additional exons is expressed (20, 21).

The primary structure of the tau isoforms and its sequence in different species has been extensively reviewed in the past (3, 22, 23). A striking feature of tau's sequence is the presence of imperfectly repeated short stretches of 31 or 32 residues which constitute the microtubule-binding domain (24-26). Regions close to this domain affect tau's interaction with microtubules and its nucleation activity (27, 28). Tau has a relative weak affinity for microtubules (about 10^{-7} M). A series of weak interactions (25) makes it possible for tau to "migrate" on the surface of microtubules. Interestingly, one of the alternatively spliced exons (exon 10; see Figure 2) constitutes an additional repeat. This may lead to a stronger binding of adult-specific tau isoforms to microtubules, which in turn results in the formation of more stable and less dynamic microtubules in the axons of adult neurons.

In addition to isoform variation, phosphorylation of tau may be an important factor in regulating tau's interaction with microtubules. Tau isolated from brain is phosphorylated at multiple sites and many kinases are capable of phosphorylating tau *in vitro* (Figure 3). Some phosphorylation events change tau's conformation (29), lead to a decreased microtubule binding (30), lower its activity to promote microtubule assembly (31), and increase the dynamic instability of microtubules (10, 11). Since phosphorylation can differentially affect tau's activity to promote microtubule growth and nucleation (32), it may have a role in fine tuning tau's activities on the assembly of microtubules. As expected from the *in vitro* data, tau binds to cellular microtubules, promotes their assembly, and stabilizes them against drug-induced disassembly when microinjected or transfected into cultured cells (33-35). Interestingly, in insect cells (*Spodoptera frugiperda*), the expression of tau expression induces formation of long and thin cellular processes which morphologically reflect neurites (36). Process formation by the expression of tau can also be induced in other cell types. However, most of these cells require a prior treatment with the actin filament disrupting drug cytochalasin (37). Most likely, cytochalasin acts by weakening the cortical actin network which normally prevents process extension (38). It should be noted that the specificity of these effects for tau is unclear since MAP2 displays similar effects on cellular microtubule assembly. Even the microtubule stabilizing drug taxol promotes process formation in cytochalasin treated cells (37, 38). It is possible that MAPs generally act by stiffening the microtubules which allows them to induce processes (39). When discussing a specific role for tau, it should also be kept in mind, that tau is not only present in neurons but is also clearly present in oligodendroglia and astrocytes (40, 41). Therefore a unique role for tau in neuronal development remains to be shown.

3.2. An axon-specific function for tau?

Speculations about a compartment-specific role of the neuronal MAPs were primed by the finding that tau is localized to the axonal compartment *in situ* (42-45) whereas MAP2 has a somatodendritic localization (46, 47). The axonal localization of tau is a conserved feature across species since it is also present in quail and *Xenopus* (48). In the rat central nervous system all axons stain for tau as judged by immunohistochemistry. However, a gradient of staining intensities is present within the axons. Small axons (*i.e.* mossy fibers in the

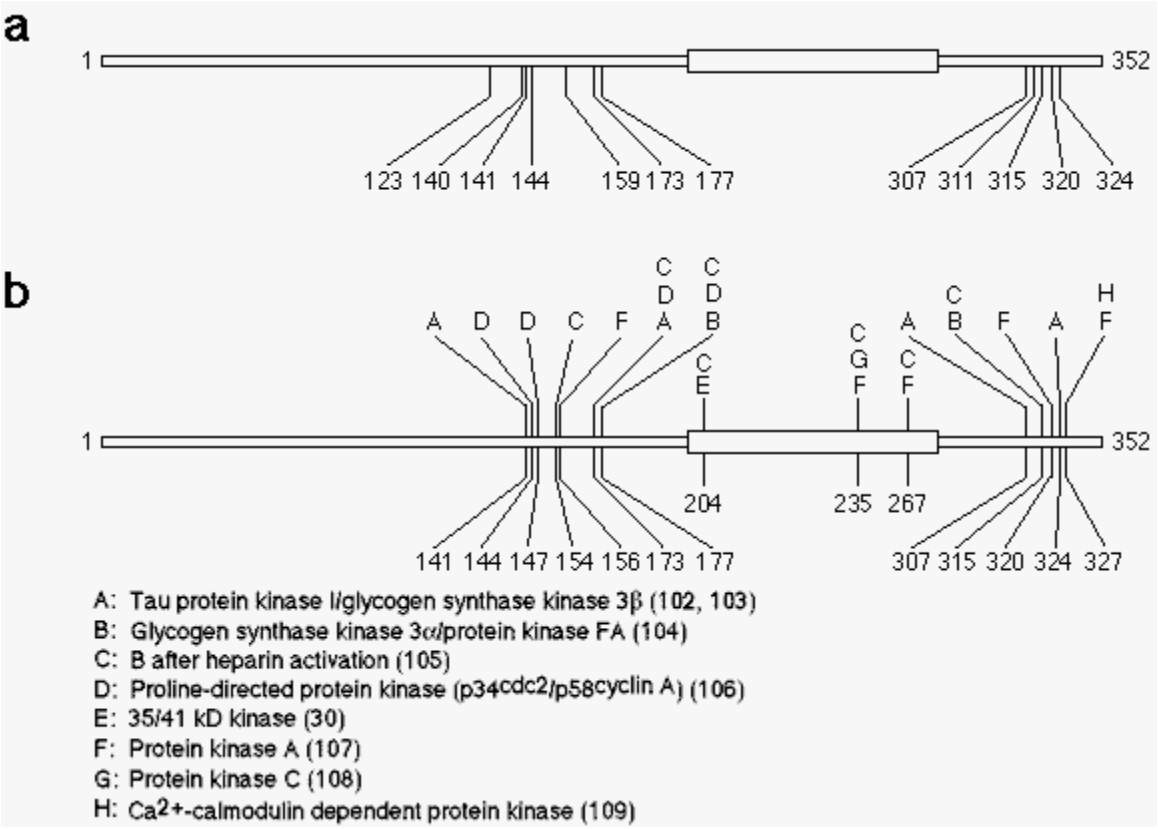

Figure 3: Phosphorylation of tau. a. Phosphorylation sites in fetal rat tau (111). b. Phosphorylation of tau as determined from *in vitro* reactions using purified kinases. Note that most phosphorylation sites are clustered in domains which flank the microtubule binding domain. References are shown in parentheses.

hippocampus) are most intensely stained and large axons (*i.e*. fasciculi gracilis and cuneatus in posterior columns) are lightly stained (40). This suggests that the concentration of tau is inversely proportional to the cross-sectional area of the axon. It should be noted that tau's localization *in situ* may not be exclusively axonal since the originally used antibody turned out to be phosphorylation sensitive and some somatodendritic tau has also been found when other tau antibodies were used (40).

Axonal microtubules have unique features which distinguish them from their somatodendritic counterparts. They are organized into a uniformly oriented array of discontinuous microtubules with their fast-growing (plus) ends pointing toward the axonal tip. Dendritic microtubules have a mixed orientation (49-51). Typically, axonal microtubules exhibit a much smaller mean intermicrotubule distance (25-40 nm) than the intermicrotubule distance in dendrites (60-70 nm) (52, 53). From its axon-specific distribution *in situ*, tau has been implicated as having a role in the unique organization of axonal microtubules but direct evidence is still lacking. When tau is expressed in insect cells, microtubules are uniformly oriented like they are in the axon (54), but the same uniform orientation of microtubules results when MAP2 is expressed in this system (53). This may indicate that the uniform orientation of microtubules is the default state during process formation. This is also consistent with the sequence of microtubule orientation in developing neurons, which is uniform in the initial processes and later becomes mixed when the dendrites develop (55).

Unfortunately, tau's axon specific localization could never be fully reproduced in cell culture. In cells maintained *in vitro*, a ubiquitous or a cell-body immunostaining in conjunction with axonal staining was observed (16, 56-59) (Figure 4). For this reason, experiments aimed to study the function of tau's axonal localization and the mechanisms responsible for its axonal segregation are difficult to design. As far as tau's axon specific localization is concerned, it would have to be established post-translationally, since ribosomes are excluded from most of the axon (60). The axonal transport rate of tau in rat retinal ganglion cells is significantly slower than that for tubulin (61). This indicates that tau is

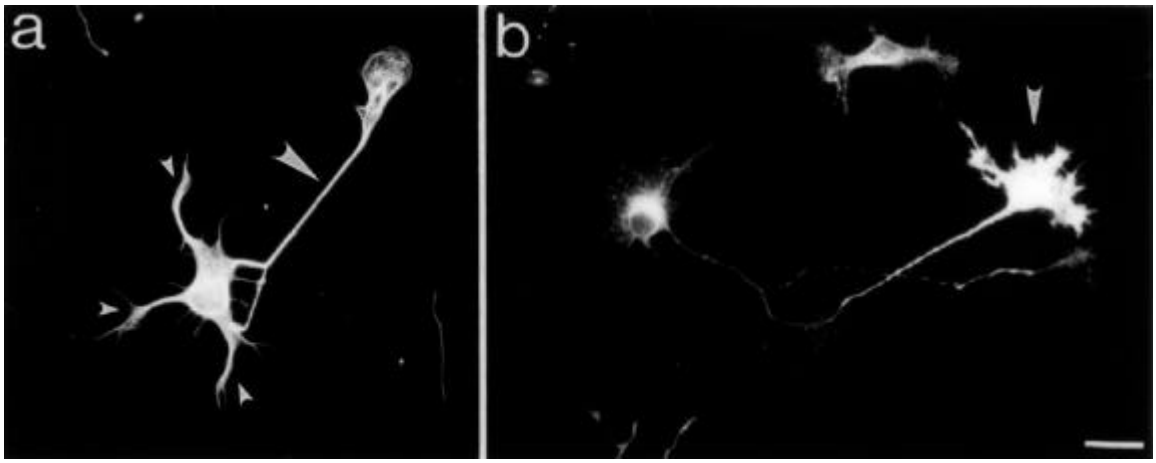

Figure 4: Distribution of microtubules and tau in cultured hippocampal neurons. The hippocampal neurons were immunostained as described earlier (71) employing monoclonal anti-tubulin (DM1A) and anti-tau (Tau-1) antibody. Scale bar, 20μm. a. Distribution of microtubules in hippocampal neurons which have been kept for 3 days in culture. Note the presence of a single axon (large arrowhead) and several minor neurites (small arrowheads) which will later develop into dendrites. Microtubules are abundant in the neuron and extend far into the axonal growth cone. b. Distribution of tau in 3 day culture of hippocampal neurons. Note the presence of tau in the cell body, the axon, and the giant growth cone (arrowhead).

not simply co-transported with microtubules into the axon.

Several mechanisms may participate in the localization of tau in axons (Figure 5). mRNA of tau is localized to the proximal axon in cultured neurons (59). This suggests that a high local concentration of tau builds up at the axonal hillock which may drive the assembly of axonal microtubules or cause the diffusion of tau into the axon. Additionally or alternatively, locally differing turnover of tau may cause its differential distribution (62, 63). Since some isoforms of tau differ in their stability against proteolytic digestion (64), this may result in an enrichment of certain isoforms in the axon. Phosphorylated tau is more stable against proteolytic digestion (65, 66) suggesting that locally differing turnover of tau is mediated by its phosphorylation state. Axonal localization of tau may also be caused by locally regulated microtubule binding (67, 68). This could again be mediated by the phosphorylation state of tau because some phosphorylation events reduce the affinity of tau for microtubules (69). This would be consistent with the intracellular distribution of tau where axonal tau is less phosphorylated than somatodendritic tau (40). Finally, binding of tau to an as yet unidentified axon-specific factor may contribute to its axonal segregation. Evidence for such a mechanism has been recently provided by the finding that tau interacts with neural plasma membrane components (70, 71).

3.3. Gene knockout contra antisense studies: How essential is tau?

Evidence for a specific functional role of tau in axonal development came from experiments in which developing neuronal cultures had been treated with antisense oligonucleotides to tau mRNA. Normally, after initial formation of exploratory neurites, one of these become an axon whereas others develop into dendrites (72). Cells with suppressed synthesis of tau fail to develop axon-like processes suggesting a role for tau in the development of neuronal polarity (73, 74). In contrast, treatment with MAP2 antisense inhibits the development of exploratory neurites (75). These results indicate distinct roles for tau and MAP2 in the development of neurons with MAP2 being required for initial process formation and tau being essential for the development of polarity.

From these data, one would expect that suppression of tau during brain development would have severe effects and probably be lethal. Surprisingly, mice lacking tau protein developed normally, survived well, and did not develop major phenotypic changes (76). The nervous system of the tau-deficient animals was immunohistologically normal and axonal elongation was unaffected in cultured hippocampal neurons prepared from these mice. Only in some small-caliber axons (*i.e*. parallel fiber axons from the cerebellar molecular layer), microtubule density and stability were decreased. Interestingly, the highest staining intensity for tau was previously found in neurons with small caliber axons (40). This suggests a role for tau in the

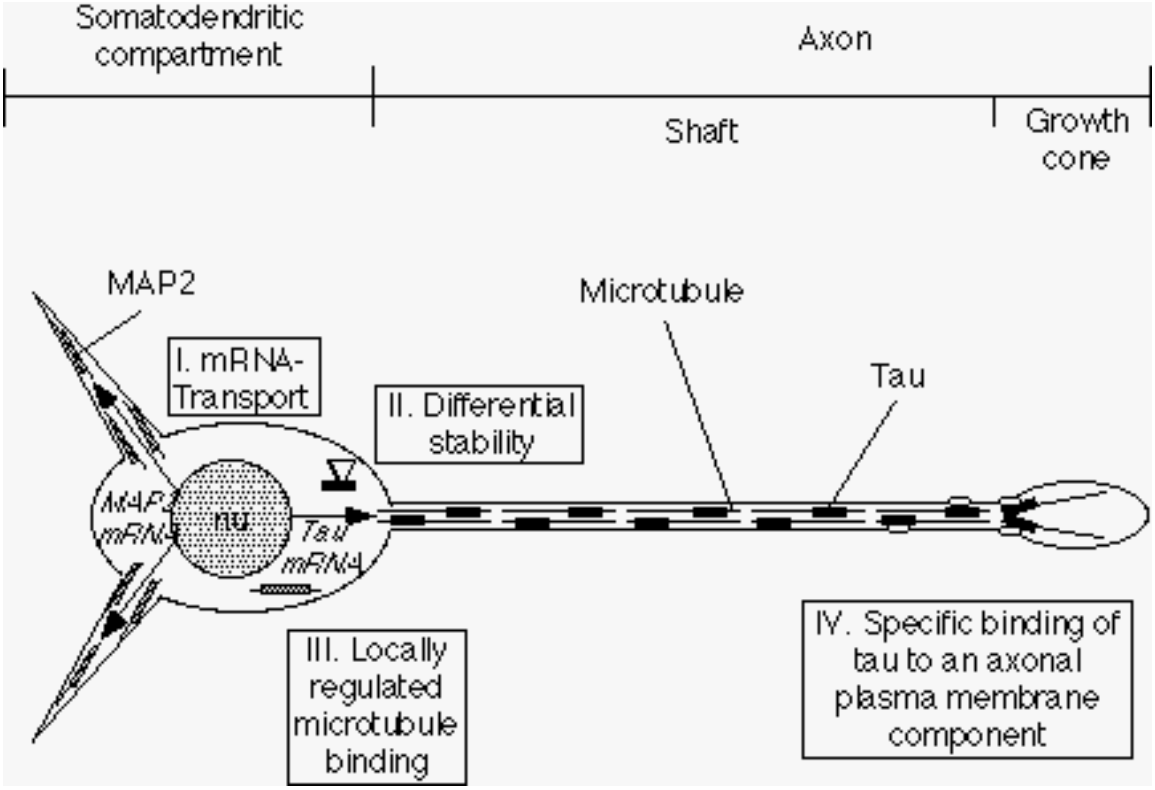

Figure 5: Schematic representation of mechanisms for axonal localization of tau. Mechanisms thought to be involved in the segregation of tau and MAP2 are: (I) a selective mRNA transport of mRNA of tau to the proximal axon and mRNA of MAP2 into the dendrites, (II) a differential stability of tau and MAP2 in the axon and somatodendritic compartment, respectively, against proteolytic degradation, (III) a locally regulated microtubule binding in the appropriate compartment, and (IV) a specific binding of tau to an axonal plasma membrane component. ∇; proteolytic degradation, nu: nucleus.

stabilization and organization of microtubules in this type of neurons. The overexpression of (human) tau in mice resulted, in addition to its axonal localization, in a somatodendritic distribution of tau without development of major phenotypic changes (77).

How can the apparent discrepancy of the antisense studies and the gene knockout animals be explained? In the past few years, the knockout of several proteins, which were previously thought to have important roles in the nervous system, resulted in much milder phenotypic effects than expected. Examples include mice deficient in amyloid precursor protein (78), mice with neurofilament-deficient axons (79), and mice deficient in certain extracellular matrix proteins (80). This may point to a higher functional redundancy or plasticity of the developing nervous system than previously anticipated. In fact, an increase in another MAP, MAP1A, has been found in tau-minus mice. This may compensate for the loss of tau during neuronal development (76). Functional compensation may not be possible in the antisense experiments due to the lack of time to adapt. In any case, the fact that other proteins are able to compensate for the function of tau argues against a unique role for this protein during axonal development.

3.4. A role for tau beyond promoting microtubule assembly.

Evidence for a role of tau, beyond its activity in promotion of microtubule assembly, comes from experiments in which the association of tau with microtubules was analyzed in cultured neurons. Despite its ubiquitous presence in cultured hippocampal neurons, as judged by extraction protocols generally used to analyze cytoskeletal associations, tau is selectively bound to axonal microtubules (71). Interestingly, the binding of tau to microtubules is not uniform throughout the axon but shows a striking increase towards the distal axon both in hippocampal (Figure 6) (81) as well as sympathetic neurons (82). This increase is not paralleled by microtubule distribution which shows a slight decrease towards the distal axon. If the role of tau was to stabilize microtubules, an increase in

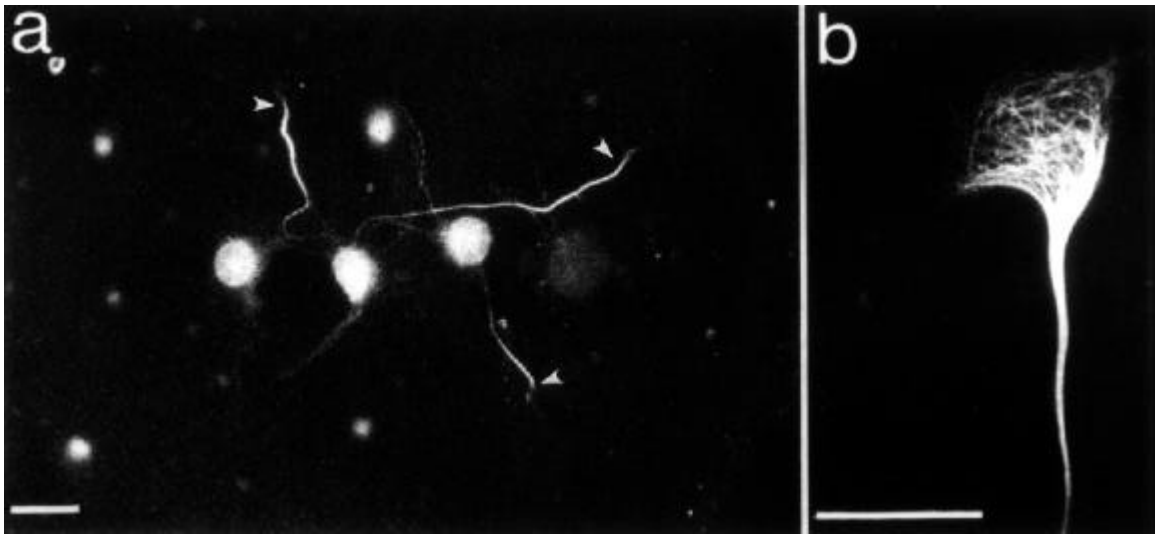

Figure 6: Localization of tau in extracted cultured hippocampal neurons. The culture and immunostaining of hippocampal neurons was performed as described earlier (71). The neurons were maintained in culture for 3 days. Immunostaining employed monoclonal anti-tau (Tau-1) antibody. Scale bar, 20 μm. a. Intracellular tau in cultured hippocampal neurons as revealed by an extraction protocol for analysis of cytoskeletal associations. Note that tau is enriched in the distal portion of the axons (arrowheads). b. Tau in the axonal growth cone of a hippocampal neuron. Note the filamentous staining indicative of association of tau with growth cone microtubules.

microtubule stability would be expected towards the distal axon as a result of increased binding of tau. However, the opposite is the case. Microtubules at the distal axon are most dynamic (83) and are most sensitive to drug-induced disassembly (81, 82). The main protein domain of tau responsible for its activity in axons is likely to reside in its amino terminal portion. This domain is unique for tau, contains two adult-specific insertions (see Figure 2), and its presence does not influence the activity of tau on microtubules *in vitro* (27, 28, 84). Since this domain projects from the microtubule surface about 20 nm when tau is associated with microtubules (85), it may independently interact with other neuronal components and specifically influence the axonal organization. Interestingly, expression of different MAPs in insect cells induces distinct patterns of process outgrowth. Whereas MAP2 induces the formation of processes with a proximo-distal taper resembling dendrites, tau induces processes with a smaller but uniform caliber which more closely resemble axons (86).

Microtubules may provide a scaffold with MAPs serving as anchors for other factors. In fact, the regulatory subunit of the cAMP-dependent protein kinase II (PKA) binds to the amino terminus of the MAP2 isoforms (87, 88). This may serve as a mechanism to localize PKA specifically to the somatodendritic compartment. Since tau binds to F-actin *in vitro* (12, 13), actin filaments are a candidate as an interaction partner for tau. But a role for tau as a linker between the microtubule and actin filament system seems unlikely since tau's actin-binding domain is identical to its microtubule-interaction unit and consequently is also shared by MAP2 (89). Until now, no specific interactions of the amino terminus of tau with cytosolic proteins have been reported. Recent molecular genetic approaches such as the yeast two-hybrid system may provide a sensitive tool to identify such factors.

Frequent cross-bridges between adjacent microtubules in quick-freeze, deep-etch electron micrographs from neuronal processes suggest a role for the neuronal MAPs in crosslinking microtubules and thereby organizing them into bundles (90). However, an active bundling activity is controversially discussed in the case of MAP2 (91). So far, other than the microtubule binding domain, no region of the tau protein has been identified as being required for the organization of cellular microtubules into bundles (34, 92). This indicates that tau may not bundle microtubules by actively bridging them together but that bundling *in vivo* is a consequence of stabilization of microtubules by tau. Tau's projection domain may therefore act as a spacer for axonal microtubules. *In vitro* studies have also suggested that the presence of tau's projection domain prevents lateral association of microtubules ("tight bundles"). This occurs when, in presence of peptides encoding single repeats or truncated MAP constructs, microtubules are assembled (93-95).

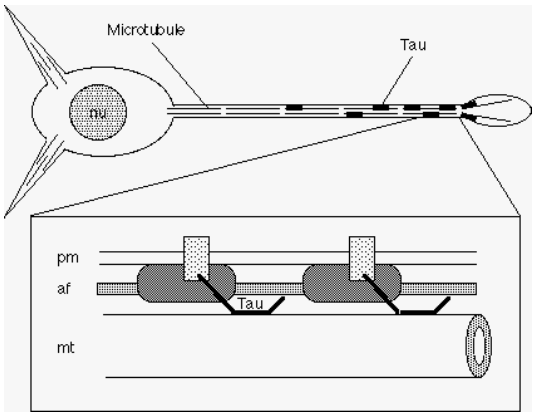

Figure 7: A model for a microtubule-tau-plasma membrane complex in the distal axon. Schematic representation of the binding of tau in the distal axon. Microtubule-associated protein tau interacts either directly or indirectly with a plasma membrane component through its amino terminal projection domain (longer arm which protrudes from the microtubule surface). Also actin filaments appear to participate in the formation of the complex since the F-actin depolymerizing drug cytochalasin abolishes the binding of tau in the distal axon (81). It should be noted that, to this point, none of the components with which tau interacts have been identified and that the features of binding partners of tau (*i.e.* integral or peripheral membrane proteins) are purely speculative.

Recent experiments point to another possible role for tau. When expressed in neural cells, tau and carboxy terminally deleted tau fragments lacking its microtubule-binding domain, interact with plasma membrane components (71). Thus, during neuronal development, tau may serve as a linker for connecting the microtubule system to the axonal plasma membrane. DiTella *et al.* (70) have shown that a microfilament-associated growth cone component relies on tau for its intracellular localization. It is possible that this component is part of such a microtubule-tau-plasma membrane complex. *In vitro*, tau interacts with spectrin (96), which may provide another link to the membrane skeleton. One may speculate that tau has a role in maintaining the axon-specific plasma membrane composition by anchoring the components of plasma membranes to the microtubule system (Figure 7). A similar mechanism has recently been suggested in postsynaptic membranes. Here, a protein named gephyrin has been implicated as having a role in anchoring the inhibitory glycine receptor to microtubules (97, 98). Since the addition of axonal plasma membrane components occurs preferentially by addition at the axonal tip (99, 100) tau may participate in preventing the intermixing of axonal and dendritic membrane components, which would occur with freely diffusible membrane proteins within a few hours (99, 100).

4. CONCLUSIONS AND PROSPECTS

More than twenty years after the isolation and identification of tau as a microtubule-associated protein, its function during neuronal development is still not fully understood. It is increasingly clear that the role of tau in neurons is more complex than initially thought. Recent data on the subcellular distribution of tau and the function of its amino terminal projection domain suggest that tau's prime role in neurons may not be to promote microtubule assembly or to stabilize axonal microtubules. Recent data raise the possibility that tau may be an integral part of the molecular machinery which underlies the development and maintenance of the polarity of neurons and may regulate the translation of extracellular cues into the structural changes during axonal outgrowth and pathfinding. For a complete understanding of the role of tau in neurons, it will be critical to identify the component(s) with which tau interacts at the distal axon and to analyze their interaction during neuronal development.

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