Transgenic Mouse Models with Tau Pathology to Test Therapeutic Agents for Alzheimer's Disease

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Abstract: The deposit of two proteins in the brain characterizes Alzheimer's disease: deposits of β-amyloid protein to form senile plaques and tau protein in neurofibrillary tangles. This review discusses transgenic animals overexpressing normal or mutated tau protein as well as kinases involved in tau hyperphosphorylation. These animals hold a great potential as tools to test the effects of forthcoming therapeutical agents for Alzheimer's disease.

1. MICROTUBULE ASSOCIATED TAU PROTEIN AND TAUOPATHIES

Several neurodegenerative diseases which include Alzheimer's disease, Down's syndrome, progressive supranuclear palsy, amyotrophic lateral sclerosis / parkinsonism-dementia complex of Guam, Pick's disease, corticobasal degeneration, and frontotemporal dementias grouped as "frontotemporal dementia and parkinsonism linked to chromosome 17" (FTDP-17), present filaments composed of tau protein as a neuropathological characteristic. These diseases are collectively called tauopathies and support the hypothesis that tau plays a causative role in neurodegeneration.

Tau is a microtubule associated protein predominantly expressed in neural cells where it promotes microtubule assembly and stability. Tau is a cytosolic protein that can also be found associated to cell membrane [1] and is present mainly, but not exclusively, in axons. The binding of tau to microtubules, as well as to cell membranes, is regulated by phosphorylation [1,2]. Hypophosphorylated tau binds with high affinity to microtubules, whereas hyperphosphorylated tau, like that present in AD, shows a very low capacity to bind microtubules [3, 4].

The protein kinases known to use tau as a substrate are: protein kinase A [5], MARK kinase [5], casein kinase I [6], calcium/calmodulin-dependent kinase II [7], MAPK [8], JNK [9], stress-activated kinase p38/RK [10], Cdk5 [11] and GSK-3β [12]. Of these, GSK-3β (or tau kinase I) is the enzyme which modifies the largest number of sites [13] thus being the kinase which has received the most attention (see below). Tau does not seem to be an essential protein for mouse development since disruption, by homologous recombination, of the *tau* gene [14] results in no appreciable alterations when homozygous mice were compared with heterozygous and wild type littermates. The only morphological change observed was a reduction in the number and density of axons in parallel fibers from the cerebellum [14].

Tau is not a single protein. It is composed of a family of isoforms derived from alternative splicing of mRNA transcribed from a single gene located on chromosome 17 [15]. Six isoforms of tau are expressed in an adult brain . The various isoforms differ from each other in the microtubule-binding domain which consists of three or four repeats of 31 or 33 amino acids each, and also near the amino terminal end, by the absence or presence of one or two inserts. In addition, a high molecular weight tau has been described in the peripheral nervous system [16] containing an extra exon [17]. Two proline-rich regions whose phosphorylation affect the ability of tau to bind microtubules flank the microtubule-binding domain.

In Alzheimer's disease (AD), tau protein forms aggregates called paired helical filaments (PHFs). Inside the brain, tau filaments accumulate in dystrophic neurites as fine neuropil threads or as bundles of PHFs in neuronal bodies forming the neurofibrillary tangles (NFTs) which become extracellular ghost tangles after the death of the neuron [18, 19]. The severity of dementia has been correlated with accumulation of NFTs in different brain regions [20] while in the case of the other hallmark of AD, the senile plaques (extracellular aggregates formed by the amyloid peptide), such correlation has not been demonstrated [21].

A major biochemical modification of PHF-tau is abnormal hyperphosphorylation, mainly in the C-terminal end [22, 23]. As a consequence of this hyperphosphorylation, tau shows a loss of microtubule binding capacity [22] and accumulates in neuronal bodies. AD-tau undergoes posttranslational modifications such as ubiquitination [24], proteolysis [25], glycation [26, 27] and oxidation [28, 29]. The structure and morphology of PHFs show a twisted ribbon structure [30-32]. Another type of filaments (about 5%) can also be found in AD samples. These so-called "straight filaments" [33] are not twisted and do not show variations in width along their length.

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Mutations in tau protein have not been described in AD. However, several tau mutations have been found in another type of tauopathy called FTDP-17 [34-37] . Several of these mutations map within the microtubule-binding repeats or close to them and, as a consequence, a partial loss of microtubule-binding function has been reported, providing a mechanism by which unbound tau might be more prone to accumulate and thus facilitate aggregation [38, 39]. Since missense mutations in tau have not been described in AD, a likely hypothesis is that aberrant phosphorylation can imitate the tau mutations found in FTDP-17 patients. Interestingly, FTDP-17 mutations cluster at the C-terminal end and aberrant phosphorylation is also located in this region.

In the majority of FTDP-17 patients, the polymers assembled from tau only contain the 4-repeat isoforms [40] and in several FTDP-17 families, the only tau mutations found have been those which affect the splicing of exon 10 to increase the ratio of 4-repeat with respect to 3-repeat isoforms [40]. Taken together, these observations indicate that the 4-repeat forms of tau may favor fibril formation compared to the 3-repeat forms. In this context it should be stressed that 4R/3R ratio appears to be subject to developmental regulation. Thus, in young animals only the 3R tau isoforms are expressed while in adult life all six isoforms (3R and 4R) can be found.

Several approaches have been undertaken in order to model tauopathies. Thus, animals transgenic for genomic human *tau*, as well as mice carrying cDNAs encoding either the largest or the smallest central nervous system isoform of human tau have been generated. In addition, transgenic mice carrying *tau* cDNA with missense mutations found in FTDP-17 patients have also been obtained. Finally, there has been an additional strategy for generating animal models of tauopathies to overexpress the kinases responsible for tau hyperphosphorylation.

Tau Transgenic Mice to Model Tauopathies

Mice that overexpress the genomic human *tau* containing coding sequences, intronic regions, and regulatory regions of the gene [41] present all six isoforms of human tau at the mRNA and protein level. Human tau is distributed in neurites and at synapses, but is absent from cell bodies; no neuropathology lesions were reported in mice up to 8 months of age.

Another approach has been to generate mice carrying cDNAs encoding either the largest or the smallest central nervous system isoform of human *tau*. Thus, several transgenic animals overexpressing the shortest isoform have been obtained but with different transgene promoters. Using the murine 3-hydroxy-methyl-glutaryl CoA reductase promoter [42] hyperphosphorylated somatodendritic transgenic tau was detected although NFTs were absent in these animals. The level of expression of the same tau isoform was increased by using the murine PrP [43]. Transgenic lines with high overexpression were not viable while lines with less than 10-fold overexpression present inclusions in cortical and brainstem neurons. These inclusions were most abundant in spinal cord neurons and correlate with axon degeneration, diminished microtubules, reduced axonal transport in ventral roots, spinal cord gliosis and motor weakness. NFT-like inclusions (detected by histochemical dyes such as Congo red and Thioflavin S) were detected in the same transgenic mice at 18 to 20 months of age [44]. In addition, filaments were isolated from detergent-insoluble tau fractions [44].

Transgenic animals with four repeat tau have also been generated. In favor of this approach is the finding that in several FTDP-17 families, the 4-repeat isoforms are overrepresented suggesting that they may favor fibril formation compared to the 3-repeat forms.

The first transgenic mice published carrying cDNAs encoding the largest human brain tau isoform showed low levels (10%) of overexpression [45]. Transgenic human tau protein was present in nerve cell bodies, axons and dendrites. Tau was phosphorylated at sites that are hyperphosphorylated in paired helical filaments although filaments were not detected. The murine Thy 1.2 promoter has been used by two groups to express the same cDNA encoding the longest human brain tau isoform, although with different genetic background. In the first case [46] the mice developed axonal degeneration in brain and spinal cord. Axonal dilations with accumulation of neurofilaments, mitochondria, and vesicles were documented. The axonopathy and the accompanying dysfunctional sensorimotor capacities were transgene-dosage related. Mice generated by the second group [47] contained numerous abnormal, tau-immunoreactive nerve cell bodies and dendrites. In addition, large numbers of pathologically enlarged axons containing neurofilament- and tau-immunoreactive spheroids were also present, especially in the spinal cord.

The third approach to model tauopathies in animal model has come from the discovery that *tau* is mutated in FTDP-17 [34-37]. Recently two groups have reported the generation of transgenic mice expressing mutant human tau containing the P301L mutation [48, 49] that is located in the tubulin-binding domain and reduces the affinity of tau for microtubules. Mice generated by the first group develop spinal cord pathology and motor dysfunction .These animals developed NFTs mainly in spinal cord and like the previous transgenic models these mice developed some neuropathological symptoms encouragingly reminiscent of the human disease. The second transgenic model published presented short filaments of tau, filaments that could be isolated from the brains of the transgenic mice [49].

In summary, much of the previous data demonstrates that a large excess of normal or mutated human Tau , a situation which does not occur in AD, can provoke some of the cellular changes observed in tauopathies but is insufficient for the formation of the mature neurofibrillary aggregates observed in the human diseases, although some transgenic lines develop NFTs as well as short PHF-like filaments. What these approaches seem to indicate is, firstly, that tau is linked to neurodegeneration, and secondly, that neurons with long axons, such as those present in spinal cord, seem to be especially susceptible. In the near future, an additional

approach to reproduce a more pronounced tauopathic phenotype will consist in generating transgenic mice of tau containing several FTDP-17 mutations simultaneously, aiming for a cumulative effect stronger than that using only a single mutation, similar to the strategy previously used to generate transgenic mice expressing mutant APP [50].

Tau Transgenic Mice to Study the Formation of PHFs.

We do not yet understand how tau can polymerize to form PHFs and SFs in different tauopathies. After the discovery that tau protein was the main component of PHFs, several attempts were made to obtain PHFs *in vitro*. Tau protein purified from brain extracts is able to aggregate *in vitro* although only at high concentrations [51-54]. This observation suggested that other molecules might contribute to tau assembly. Sulfo-glycosaminoglycans (sGAG) have received particular attention mainly because they are present in NFTs [55] and tau polymerization is facilitated in the presence of sGAG or other anionic compounds [56-59]. PHFs contain some unidentified sGAG as evidenced by the fact that heparinase and condroitinase treatments of PHFs result in untwisting of these filaments [60]. How the interaction between tau and sGAG can take place in neurons is a matter of controversy [61]. Transgenic animals will aid investigation of whether these negatively charged polysaccharides or another anions are involved in tau polymerization. These models should also help clarify if fatty acids are involved in tau aggregation [62-65]. A mechanism for increased aggregation induced by fatty acids in oxidative stress is that fatty acids can be oxidised to yield toxic products as 4-hydroxynonenal [66] which in turn stimulate tau assembly [67]. In addition, transgenic animal models will also aid to clarify the contribution of proteolysis or other tau modifications as glycation and oxidation in tau polymerization.

From transgenic tau models a pretangle state has been obtained although it is difficult to obtain real PHFs. Thus, it seems that additional biological triggers play a major role in neurodegeneration. Neurofibrillary tangles are composed of highly phosphorylated forms of the microtubule-associated protein tau. Phosphorylated tau proteins accumulate early in neurons, even before formation of neurofibrillary tangles, suggesting that an imbalance between the activities of protein kinases and phosphatases is an initial phenomenon in AD. Among the protein kinases involved in tau aggregation, cdk5 and GSK-3β have received special attention mainly because both copurified with microtubules [68, 69]. Transgenic mice that overexpress p25, an activator of cdk5 under the control of the neuron-specific enolase promoter [70] develop hyperphosphorylated tau as well as cytoskeletal alterations localized in the amygdala, thalamus/hypothalamus and cortex. However, one of the best candidate enzymes for generating the hyperphosphorylated tau that is characteristic of PHFs is GSK-3β [12]. Three transgenic mice that overexpress GSK-3β in the nervous system have been generated. These are discussed in the following section.

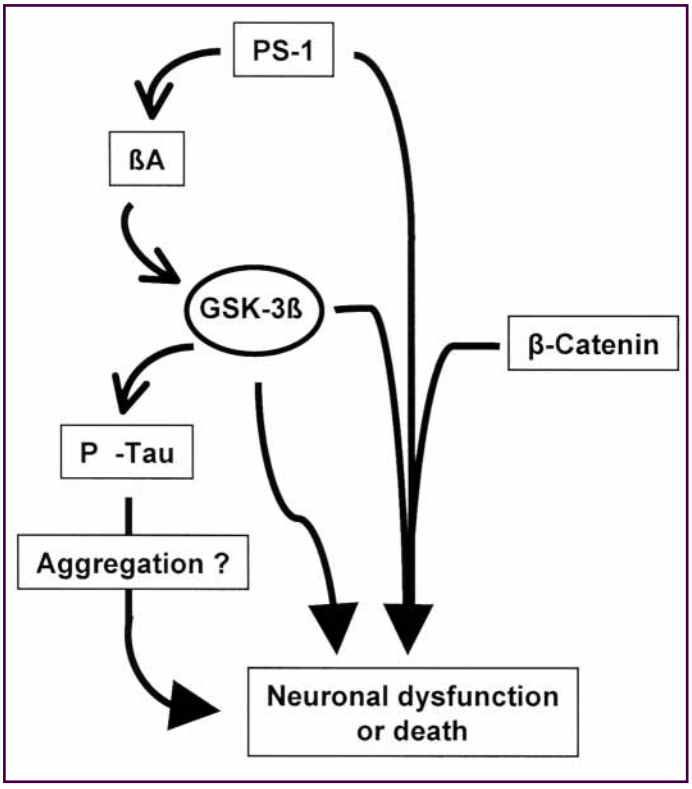

Fig. (1). GSK-3β as an enzyme found at the convergence of the pathways involved in AD-like tau hyperphosphorylation, β-amyloid induced toxicity and PS-1 mutations. For discussion of the figure see the text.

Transgenic Animal Models of GSK3β. Target to Pharmacological Intervention

GSK-3 is a proline directed kinase originally identified due to its role in glycogen metabolism regulation and this kinase is most abundant in the CNS [71]. GSK-3β has been shown to phosphorylate tau in most sites, hyperphosphorylated in PHFs both in transfected cells [72] and *in vivo* [73, 74]. Furthermore, GSK-3β accumulates in the cytoplasm of pretangle neurons and its distribution in brains staged at AD neurofibrillary changes is coincident with the sequence of development of these changes [75, 76]. GSK-3β is implicated in insulin and IGF-1 mediated signal transduction as well as in the wnt/wingless signaling pathway as the key enzyme regulating β-catenin stability and, as a consequence, its translocation to the nucleus and its transcriptional activity [77, 78].

Exposure of cortical and hippocampal primary neuronal cultures to Aβ induces activation of GSK-3β [79], tau hyperphosphorylation [80, 81], and cell death [80, 82]. Blockade of GSK-3β expression by antisense oligonucleotides [82] or activity by lithium [83], induces neurodegeneration of cortical and hippocampal primary cultures.

PS-1 has been shown to directly bind GSK-3β and tau in coimmunoprecipitation experiments from human brain samples [84]. Thus, the ability of PS-1 to bring GSK-3β and tau into close proximity suggests that PS-1 may regulate phosphorylation of tau by GSK-3β. Mutant forms of PS-1 in transfection experiments result in increased PS-1/GSK-3β association and increased phosphorylation of tau [84]. Furthermore, PS-1 has also been shown to form a complex with the GSK-3β substrate β-catenin in transfected cells [85, 86] and *in vivo* [85, 87] and this interaction increases β-catenin stability [87]. Pathogenic PS-1 mutations reduce the ability of PS-1 to stabilize β-catenin, which in turn results in decreased β-catenin levels in AD patients with PS-1 mutations [87]. All these data point towards GSK-3β as an enzyme found at the convergence of the pathways involved in AD-like tau hyperphosphorylation, β-amyloid induced toxicity and PS-1 mutations (see Fig. (**1**)).

In order to establish the *in vivo* functions of GSK-3, molecular genetic, approaches have been undertaken. Up to the present three transgenic mice that overexpress GSK-3β in the nervous system have been reported. The first lines of GSK-3β transgenic animals described [88] used either ubiquitous (murine sarcoma virus) or CNS-specific promoters (murine neurofilament light gene). Although the authors were able to find a slight increase in tau phosphorylation at the AT8 epitope, overexpression could not be detected using transgenes encoding either wild type GSK-3β or a mutant GSK-3β(S9A) which is not inhibited by phosphorylation. The authors speculate that the low levels of expression of the kinase were obtained probably because high levels of GSK-3 are lethal. Conversely, GSK-3β knockout mice die during embryonic life [89].

The second transgenic animal established also expressed a constitutively active form of the kinase, i.e. GSK-3β(S9A) but using the *thy1* gene promoter [90]. An increase in phospho-tau was demonstrated by Western blot analysis but only in older transgenic mice (6-7 months). These GSK-3β transgenic mice performed normally in the Morris water maze test [91]. Interestingly, when GSK-3β(S9A) transgenic mice were cross-bred with transgenic mice that overexpress the longest human protein tau isoform [46], the number of axonal dilations present and the motor impairment typical of these tau transgenic animals were reduced. In the double transgenic animal an increase in human tau phosphorylation was observed although neither an increase in insoluble tau aggregates nor the presence of paired helical filaments was observed.

Taking into account the lethality of GSK-3β observed in the first transgenic animals [88] as well as the known role of GSK-3β in development, the third GSK-3β transgenic mouse described [92] used a conditional transgenesis approach to achieve substantial overexpression of the kinase. Transgene switching is achieved using the tetracyclin-regulated system. By using these transgenic animals (tet/GSK-3β), it was found that GSK-3β overexpression was restricted to some neurons in the cortex and neurons of the dentate gyrus and CA2 regions in the hippocampus. *In vivo* overexpression of GSK-3β results in increased phosphorylation of tau in tet/GSK-3β animals, as detected with antibodies raised against tau present in Alzheimer's disease. Hyperphosphorylated tau was found in the somatodendritic compartment, similar to the localization of tau previously observed in tau transgenic mice. However, the change in subcellular localization of tau that was observed was due exclusively to the hyperphosphorylation of tau by GSK-3β as there is no change in the total level of tau. Somatodendritic hyperphoshorylated tau in the conditional tet/GSK-3β transgenic mice resulted in an increase in tau levels in this neuronal compartment since immunoreactivity of 7.51, an antibody that recognizes the tubulin-binding domain of tau, was also increased. These data suggest that an increased phosphorylation of tau results in decreased affinity of tau for microtubules reproducing two of the characteristics of AD tau: hyperphosphorylation and decreased interaction with microtubules. However, the aberrant tau aggregation found in AD was not observed in these GSK-3β transgenic mice.

In tet/GSK-3β mice, β-catenin, another GSK-3 substrate relevant for AD [87], was also analyzed. GSK-3 is a key enzyme involved in β-catenin stabilization and subsequent nuclear translocation [78]. Tet/GSK-3β mice demonstrate that GSK-3β is an effective kinase for β-catenin in brain neurons *in vivo* since nuclear β-catenin in hippocampal granular cells of tet/GSK-3β mice was found to be reduced about 75% [92]. Considering that the genes transactivated by β-catenin are poorly characterised, tet/GSK-3β mice may serve as a good tool to identify such genes.

Tet/GSK-3β mice also demonstrate neuronal stress and neuronal death as revealed by reactive glia, TUNEL and cleaved caspase-3 staining. These data are in good agreement with the known role of GSK-3β in the survival pathway as well as the neuroprotective effect of lithium, a relatively specific GSK-3β inhibitor [93].

In summary, from *in vivo* genetic approaches it can be assumed that GSK-3β is implicated in tau phosphorylation

in the nervous system, suggesting that GSK-3 deregulation can be relevant to the pathogenesis of Alzheimer's disease. In addition, implication of GSK-3 in neurodegeneration has also been firmly established from these studies. The possibility of crossing tet/GSK-3β with tau transgenic mice will improve chances of obtaining PHFs. Tet/GSK-3β mice are thus a good tool to test the neuroprotective effect of forthcoming GSK-3β specific inhibitors. Furthermore, their efficacy can be compared with the effect of silencing transgene expression by administering tetracycline analogs.

GSK-3, from a kinetic point of view, is an enzyme with at least two substrates: the protein to be phosphorylated and ATP, the donor of phosphate. Lithium, the inhibitor most often used, is a competitive inhibitor of GSK-3 with respect to magnesium, but not with respect to the protein substrate or ATP [94]. These data suggest that GSK-3 has a magnesium binding site additional to Mg-ATP and that lithium is a competitive inhibitor of that site. It is interesting that for another enzyme inhibited by lithium, inositol monophosphatase, a similar magnesium-binding site has been proposed [95]. Thus, several pharmacological interventions can be considered. Firstly, ATP analogs selective for GSK3 can be sought. An example seems to be indirubins which bind to the ATP binding pocket present in GSK-3 [96]. Secondly, analogs of lithium should be more selective than those of ATP. Rational design of lithium analogs will be possible when 3-dimensional macromolecular structure data of the enzyme are available. Currently two groups have deposited crystal structure coordinates in the PDB database (PDB ID are 1I09 and 1H8F) and the data will be released at the beginning of 2002. Yet another report has recently been partially published [97] suggesting a mechanism for autoinhibition in which the phosphorylated N-terminal end binds as a competitive pseudosubstrate. Finding analogs of this region may also be an interesting strategy to find effective inhibitors of the kinase.

CONCLUSIONS

Transgenic animals with high levels of amyloid deposition have been generated and as we have indicated, several lines of transgenic mice with hyperphosphorylated somatodendritic tau have already been obtained. In fact some of these mice present NFTs as well as short PHF-like filaments. In the near future, crossing of transgenic tau animals with amyloid transgenics as well as with tet/GSK-3β mice to obtain triple transgenic animals, will aid in the understanding of the contribution of NFT and senile plaques to AD pathology as well as of which pathways are altered. When the altered pathways are clarified, inhibitors of selected enzymes (i.e. GSK-3β) can be generated to interfere with and to delay the progress of neurodegeneration.

NOTE ADDED IN PROOF

We have recently established a transgenic mice of tau containing three FTDP-17 missense mutations: G272V, P301L and R406W. Ultrastructural analysis of mutant Tau-positive neurons revealed a pre-tangle appearance with filaments of Tau and increased numbers of lysosomes displaying aberrant morphology similar to those found in AD (FTDP-17 mutations in *tau* transgenic mice provoke lysosomal abnormatlities and Tau filaments in forbrain (2001) F. Lim, F. Hemández, J.J. Lucas, P. Gómes-Ramos, M.A. Morán and J. Ávila, Mol. Cell. Neurosci. 18, 702-714).

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