Glycogen Synthase Kinase-3β Phosphorylates Protein Tau and Rescues the Axonopathy in the Central Nervous System of Human Four-repeat Tau Transgenic Mice*

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Protein tau filaments in brain of patients suffering from Alzheimer’s disease, frontotemporal dementia, and other tauopathies consist of protein tau that is hyperphosphorylated. The responsible kinases operating in vivo in neurons still need to be identified. Here we demonstrate that glycogen synthase kinase-3β (GSK-3β) is an effective kinase for protein tau in cerebral neurons in vivo in adult GSK-3β and GSK-3β × human tau40 transgenic mice. Phosphorylated protein tau migrates slower during electrophoretic separation and is revealed by phosphorylation-dependent anti-tau antibodies in Western blot analysis. In addition, its capacity to bind to re-assembled paclitaxel (Taxol®)-stabilized microtubules is reduced, compared with protein tau isolated from mice not overexpressing GSK-3β. Co-expression of GSK-3β reduces the number of axonal dilations and alleviates the motoric impairment that was typical for single htau40 transgenic animals (Spittaels, K., Van den Haute, C., Van Dorpe, J., Bruyneels, K., Vandecasteele, K., Laenen, I., Geerts, H., Mercken, M., Sciot, R., Van Lommel, A., Loos, R., and Van Leuven, F. (1999) Am. J. Pathol. 155, 2153–2165). Although more hyperphosphorylated protein tau is available, neither an increase in insoluble protein tau aggregates nor the presence of paired helical filaments or tangles was observed. These findings could have therapeutic implications in the field of neurodegeneration, as discussed.

Protein tau represents a family of neuronal phosphoproteins, which were originally identified as proteins that co-purify with microtubules (MT)1 and that assemble tubulin dimers into microtubules (1). Six low molecular weight isoforms are known, which contain none, one, or two N-terminal inserts of unknown function and three or four C-terminal repeat domains that are essential for binding to microtubules (2–4). The binding to microtubules involves both the protein tau repeat domains and some of the flanking regions (5, 6).

Phosphorylation of serine and threonine residues within or flanking these MT-interacting regions reduces the interaction with microtubules considerably (7–11). Among other kinases, GSK-3β is identified as a potent proline-dependent protein tau kinase (12–15), also known as “tau protein kinase I” (16). GSK-3β phosphorylates protein tau in cell-free systems (17) and in transfected cells, and thereby reduces its ability to initiate microtubule nucleation (18–23). Detailed biochemical and structural analyses demonstrate that GSK-3β phosphorylates analogous epitopes on protein tau in vitro and in cell paradigms, as on protein tau embedded in paired helical filaments (PHF) of Alzheimer’s disease patients (AD) (12). PHF are composed of hyperphosphorylated protein tau (24–28), and antibodies directed to GSK-3β decorate PHF, indicating a direct association between GSK-3β and hyperphosphorylated protein tau (29, 30). Moreover, active GSK-3β accumulates in pre-tangle and tangle-bearing neurons in AD brain (31), and AD-related presenilin-1 clinical mutants are proposed to enhance the inter-reaction between protein tau and GSK-3β (32).

Unlike in Alzheimer’s disease, in frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), dementia segregates with mutations in the tau gene. Intronic mutations and missense mutations S305N and N279K induce a preponderance of the four-repeat over the three-repeat isoforms of protein tau (33–35), and most clinical missense mutations located in the microtubule binding domains reduce the association of protein tau with microtubules (36–39). No matter through which mechanism (phosphorylation or mutation) the MT-binding and MT-stabilizing function of protein tau is compromised, filamentous intraneuronal inclusions consist of hyperphosphorylated protein tau in both dementing illnesses,

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The abbreviations used are: MT, microtubule; GSK-3β, glycogen synthase kinase-3β; WT, wild type; AD, Alzheimer’s disease; PHF, paired helical filament; RAB, reassembly buffer; RIPA, radioimmuno-precipitation assay buffer; FA, formic acid; FTDP-17, frontotemporal dementia; and parkinsonism linked to chromosome 17; GFAP, glial fibrillary acidic protein; htau40-1, -2, and -5, transgenic mice of strain 1,2, and 5, respectively, overexpressing human tau; htau40-1HH, homozygous mouse of strain htau40-1; TPK-I, tau protein kinase I.
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Molecular Biochemicals, Mannheim, Germany) recognizes an epitope comprising non-phosphorylated Ser-199 and Ser-202 (50). Dephosphorylation of protein tau in brain homogenates by pretreatment with alkaline phosphatase was performed as described (42).

The solubility of protein tau in brain and spinal cord from wild type, single, and double transgenic mice was determined by sequential extraction with high salt reassembly buffer (RAB), detergents containing radioimmunoprecipitation assay buffer (RIPA) and 70% formic acid (FA), as described (55). Four httau40-1 and httau40-1 × GSK-3β transgenic mice were used, quantitative Western blot analysis was carried out using Tau-5 antibody and the amount of protein tau in the different buffers was determined, with the total transgenic human protein tau taken as 100%.

GSK-3β Protein and Enzymatic Activity—The GSK-3β (389A) protein levels in brain and spinal cord extracts were estimated by Western blotting with the monoclonal antibody TPK-I/GSK-3β (Affinity, Nottingham, United Kingdom). The GSK-3β enzymatic activity was measured in brain homogenates after immunoprecipitation and fractionation by cation exchange fast protein liquid chromatography on a Mono S column (Amersham Pharmacia Biotech, Uppsala, Sweden) as described (56).

Quantification of Asonopathy in the Central Nervous System—Non-transgenic (n = 4), GSK-3β (389A) (n = 4), httau40-1 (n = 8), httau40-2 (n = 8), httau40-1 × GSK-3β (n = 4), and httau40-2 × GSK-3β (n = 4) mice were transcardially perfused with paraformaldehyde (4% in phosphate-buffered saline). Brain and spinal cord were immersion-fixed overnight, and vibratome sections (40 μm) were cut and processed (42). Axonal ditions were detected and quantified as described (42). The Krukal-Wallis test was applied to evaluate the differences.

Binding of Protein Tau to Isolated Microtubules—Polyaxial-depend-ent isolation and re-assembly of microtubules and isolation of microtu- bule-associated proteins from brain and spinal cord was performed as described (57). To increase the yield of microtubule preparations from mouse spinal cord, 3 mM CaCl2 was additionally added to the MT buffer to depolymerize cold-stable microtubules in situ (58).

Quantification of Axonopathy in the Central Nervous System—Two 50-μm-thick sections were cut and processed as described (42). To increase the yield of microtubule preparations from mouse spinal cord, 3 mM CaCl2 was additionally added to the MT buffer to depolymerize cold-stable microtubules in situ (58). Equal amounts of proteins were loaded on 8% SDS-polyacrylamide gels after removal of microtubeprototibulin. Densitometric quantitation of the different bands of protein tau was as described (42), and results were normalized for tubulin and Tau-5. The plots for tubulin were developed with an antibody specific for neuronal β-III anti-tubulin (Promega, Madison, WI).

The Wilcoxon signed-rank test was used to evaluate the differences.

Histochemistry and Immunohistochemistry—Mice were anesthetized and transcardially perfused with paraformaldehyde (4% in phosphate-buffered saline). Brain and spinal cord were immersion-fixed overnight and vibratome sections (40 μm) were cut and processed as described (42). The Krukal-Wallis test was applied to evaluate the differences.

Neuronal and Axonal (HT-7) and for GSK-3β (TPF-1) (Affinity), a polyclonal antisera against synaptophysin (Dako, Glostrup, Denmark), the monoclonal antibody SMI-32 for detection of neurofilament proteins (Innogenetics, Gent, Belgium), and an anti-antiserum to GFAP (Dako). Other procedures were performed as described:

standard hematoxylin/eosin staining for muscle and Bielschowsky's silver impregnation and thioflavine S staining for central nervous sys-

Ultrastuctural Analysis—For transmission electron microscopy, 4–5-μm-thick sections of 6-month-old httau40-1HH (n = 4) and wild-type (n = 4) mice were perfused with 4% glutaraldehyde in phosphate-buffered saline or 4% paraformaldehyde and 0.1% glutaraldehyde. Areas of necortex, hippocampus, and spinal cord were excised from 40-μm-thick vibratome sections, postfixed with OsO4, and embedded in epon.

For immunoelectron microscopy areas of cerebral neocortex, hip-

in addition to other tauopathies.

Despite the wealth of in vitro data, convincing evidence for any functional repercussion of the phosphorylation of protein tau by GSK-3β in vivo is lacking and the requirement of protein tau phosphorylation for PHF formation is still a matter of debate. We generated transgenic mice that overexpress a constitutively active form of the human kinase, i.e. GSK-3β(S9A) with serine at position 9 replaced by alanine to prevent its inactivation by phosphorylation (40, 41). The GSK-3β transgenic mice were crossed with transgenic mice that overexpress the longest isoform of human protein tau, i.e. containing 2 N-terminal inserts and 4 microtubule binding domains (htau40), extensively characterized previously (42). Both recombiant transgenic constructs were based on the adapted mouse thy1 gene promoter, warranting co-expression specifically in neurons of the central nervous system (42–45).

Here we demonstrate that human GSK-3β(S9A) hyperphosphorylates murine and human protein tau in the brain of single and double transgenic mice. The demonstrated phosphorylation of protein tau in the double transgenic mice reduces the binding of protein tau to isolated microtubules. Although more MT-unassociated protein tau is available, neither PHF nor neuro fibrillary tangles are formed and the amount of insoluble protein tau remains unaltered. Interestingly, protein tau hyperphosphorylation correlates with a strong reduction in the number of axonal dilations and with a nearly complete alleviation of the motoric problems observed in htau40 transgenic mice. A 2-fold increase in GSK-3β activity, relative to the endogenous enzymatic activity, thereby rescues nearly all neuropathological symptoms of the single htau40 transgenic mice (42). The conclusion that hyperphosphorylation of protein tau by GSK-3β reverses such a severe phenotype could have therapeutic implications in the field of neurodegeneration, as discussed.

MATERIALS AND METHODS

Generation of GSK-3β Transgenic Mice—cDNA coding for human GSK-3β (S9A) (Refs. 40 and 41; gift of J. Woodgett) was ligated in the adapted mouse thy1 gene (42, 43) and was microinjected into 0.5-day-old FVB/N nuclear mouse embryos. Transgenic founders were identified by Southern blotting and genotype of transgenic offspring, bred into the FVB/N genetic background, was performed on tail biopsy DNA by polymerase chain reaction. The htau40 transgenic mice have been described elsewhere (42). Three founder strains, i.e. htau40-1, htau40-2, and htau40-5, which transmitted the transgene in a stable Mendelian fashion, were used to generate double transgenic mice by cross-breeding with GSK-3β(S9A) animals.

Sensorimotor Tests—Single and double transgenic mice at the age of 2–4 months were subjected to three classical sensorimotor tests (46), i.e. the forced swimming test, the rod walking test, and the inverted wire-grid test, performed as described previously (42). In addition, we scored the time that the mice needed to return upward after being forced on their back. Four groups of mice were tested: 21 wild-type FVB mice (WT), 23 homozygous htau40-1 (1HH), 17 heterozygous GSK-3β(S9A) (GSK), and 7 htau40-1 × GSK-3β(S9A) double transgenic mice (1HH × GSK). The contingency x2 test was used to evaluate the difference.

Tissue Extractions and Western Blotting—To prevent dephosphorylation during post mortem delay (47, 48) all tissues were rapidly dissected and tissue homogenates were processed on ice and immediately stored at −70 °C after freezing in liquid nitrogen. Tissue extraction and Western blotting was performed as described previously (42). Reaction of the secondary antibody with mouse immunoglobulins in Western blotting was eliminated as described previously (42).

Phosphorylation-independent monoclonal antibodies against protein tau were HT-7 (Innogenetics, Gent, Belgium) and Tau-5 (PharMingen, San Diego, CA). Monoclonal antibodies directed against phosphorylated tau were HT-7 (Innogenetics, Gent, Belgium) and Tau-5 (PharMingen, San Diego, CA). Monoclonal antibodies directed against phosphorylated protein tau epitopes were AT-8, AT-180 (Innogenetics, Gent, Belgium), PHF-1 (P. Davies, New York), AD-2 (B. Pau, Lille, France), and 12E8 (P. Seubert, Elan Pharmaceuticals, San Francisco, CA). Their respective epitopes are: Ser(P)-199 and Ser(P)-202 (49, 50), Thr(P)-231 (51), Ser(P)-396 and/or Ser(P)-404 (PHF-1; see Ref. 52; AD-2, see Ref. 53), Ser(P)-262 and Ser(P)-356 (44). Monoclonal antibody Tau-1 (Roche
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**RESULTS**

*Generation of Single GSK-3β(S9A) and Double GSK-3β(S9A) × hTau40 Transgenic Mice—* We have generated transgenic mice that express a mutant form of human GSK-3β, denoted GSK-3β(S9A), containing alanine in position 9 instead of the wild-type serine, to prevent inactivation by phosphorylation (60). The cDNA was incorporated in a recombinant DNA construct based on the mouse *thy1* gene promoter (Fig. 1a), and transgenic mice were generated by micro-injection, in the FVB mouse strain (42–44).

The human GSK-3β protein was demonstrated by Western blotting in brain and spinal cord (Fig. 1, b and c). The transgene was enzymatically active on a synthetic peptide substrate, resulting in a doubling of the total GSK-3β kinase activity in brain homogenates of GSK-3β transgenic mice toward the GS-1 peptide (p < 0.01). Data are mean with S.D. of four independent determinations.

Transgenic mice that express a mutant form of human GSK-3β and human protein tau (Fig. 1, e and i). Both transgenes were thus expressed in the same cells of brain and spinal cord.

GSK-3β Phosphorylates Murine and Human Protein Tau in Vivo—A panel of antibodies known to recognize epitopes on protein tau that are phosphorylated by GSK-3β (61) were used for Western blot analysis of brain homogenates of transgenic mice. Murine protein tau was not more phosphorylated in young GSK-3β(S9A) transgenic mice (4–8 weeks) compared with age-matched wild type mice (data not shown). In brain of older GSK-3β transgenic mice (6–7 months), however, slower migrating protein tau isoforms reacted with phosphorylation-dependent antibodies AT-8 and AT-180, which were absent in brain of wild type mice. In addition, protein tau isoforms with slower electrophoretical mobility were also demonstrated with antibodies Tau-5 and PHF-1, only in brain of GSK-3β transgenic animals (Fig. 2a). On the other hand, human protein tau was already hyperphosphorylated in brain extracts of young (5 weeks) double htau40-1 and htau40-5 × GSK-3β transgenic mice. Antibodies AT-8, AT-180, and AD-2 reacted with slow migrating human protein tau isoforms, and these were virtually absent in brain extracts of single transgenic littermates (Fig. 2b). Dephosphorylation of protein tau by pretreatment with alkaline phosphatase of brain extracts of single and double transgenic mice prior to electrophoresis, yielded identical
protein tau patterns on Western blotting with antibody Tau-1, and reduced AT-8 immunoreactivity of murine and human protein tau (Fig. 2c). In addition, dephosphorylation of protein tau resulted in more distinct bands (Fig. 2c, Tau-1 panel).

To determine if hyperphosphorylation by GSK-3β affected protein tau binding to microtubules, we examined its binding to paclitaxel-mediated reassembled MT. The amount of protein tau bound to MT was significantly reduced in preparations derived from brain and spinal cord of htau40-1 and htau40-5 transgenic mice and the respective double htau40 × GSK-3β(S9A) transgenic littermates (Fig. 3). Addition of lithium in all buffers during the isolation and assembling procedure was necessary to reduce the binding of protein tau to the microtubules. Thus, the reduced binding was not due to phosphorylation of protein tau by GSK-3β in vivo during the isolation and assembling procedure (62), but occurred in vitro (Fig. 3, a–f). The soluble protein tau that remained in the supernatant after microtubule assembly and isolation was also hyperphosphorylated, as demonstrated by reaction with antibodies Tau-1, AT-180, and AD-2. Semiquantitative estimations identified the epitope recognized by AD-2 and PHF-1 to be most abundant (Fig. 4c). Quantification by densitometric scanning and normalization to reaction with antibody Tau-5 demonstrated a 4-fold increase of phosphorylation at the epitope defined by AD-2 (Fig. 4b). Antibodies AT-180 and Tau-1 revealed a more moderate increase in the phosphorylation of their respective epitopes in protein tau in the double transgenic mice, relative to the single htau40 transgenic mice.

As phosphorylation at the AD-2 epitope is significantly increased in brain of GSK-3β transgenic mice, its effect on the MT-binding capacity of protein tau was assessed. The amount of MT-associated human protein tau that was phosphorylated at the AD-2 epitope was only about half of the amount in the MT-unbound fraction, relative to the total amount of human protein tau in each fraction separately, detected by the Tau-5 antibody. Even more prominent was the at least 4-fold reduction in the amount of murine protein tau phosphorylated at the AD-2 epitope that bound to microtubules (Fig. 4c).

These data demonstrate that both murine and human protein tau are hyperphosphorylated in brain of GSK-3β transgenic mice and that their ability to associate with microtubules is thereby reduced.

Absence of Paired Helical Filament Formation in GSK-3β × htau40 Transgenic Mice—Bielschowsky’s silver impregnation and thioflavine S staining (data not shown) did not reveal the presence of tangles or protein tau filaments in central nervous system of double transgenic mice (Figs. 5 and 6). Ultrastructurally, polyclonal antibodies to protein tau strongly labeled the paired helical filaments in the hippocampus of the Alzheimer’s patient. In the transgenic htau40-1 and htau40-5 × GSK-3β mice, however, these antibodies reacted with protein tau in cell bodies, dendrites, axons, and dilated axons, but reactivity was much weaker than with paired helical filaments.
in AD brain. Paired helical or straight filaments similar to those in Alzheimer’s patients or other diseases with protein tau pathology were not present (Fig. 5, a–d). In addition, monoclonal antibody AT8 reacted with filamentous protein tau in the Alzheimer’s disease patient, but no AT8 reactive filaments were present in brain tissue from transgenic mice (data not shown).

The solubility of protein tau in brain and spinal cord of htau40-1 and htau40-1 × GSK-3β transgenic 7-month-old mice was compared by differential extraction (see “Materials and Methods”). Although the majority of protein tau is soluble in the high salt RAB, a substantial amount of RAB-insoluble transgenic protein tau was present in the RIPA-soluble fraction, and even in the FA-soluble fraction. The amount of RIPA-soluble (htau40-1 = 23 ± 1.9%; htau40-1 × GSK-3β = 19 ± 1.7%) and FA-soluble (htau40-1 = 1.25 ± 0.14%; htau40-1 × GSK-3β = 1.12 ± 0.20%) protein tau was similar (p > 0.05) in brain of double compared with single htau40 transgenic animals. Similarly, in spinal cord, the amount of human protein tau dissolved in the RIPA (htau40-1 = 17 ± 0.9%; htau40-1 × GSK-3β = 16 ± 0.9%) and FA buffer (htau40-1 = 2.5 ± 0.20%; htau40-1 × GSK-3β = 2.3 ± 0.15%) was not elevated in htau40-1 × GSK-3β transgenic mice (Fig. 5e).

GSK-3β Rescues the Axonopathy and Motoric Impairment of hTau40 Transgenic Mice—Transgenic mice that overexpress human protein tau at moderate levels have no severe phenotype (63, 64), whereas considerable overexpression of protein tau results in transgenic mice that develop axonopathy and motoric problems (42, 55, 65).

In brain and spinal cord of single htau40 transgenic mice, Bielschowsky’s silver impregnation, anti-neurofilament, and anti-synaptophysin immunohistochemical stainings revealed dilated axons, the number of which was reduced when transgenic GSK-3β was coexpressed (Figs. 6 and 7, Table I). This was observed for two different parental mouse strains (htau40-1 and htau40-2), transgenic for human protein tau at different expression levels (Table I) (42). Moreover, the disorganisation of the microtubule cytoskeleton was reversed in the central nervous system of double transgenic mice compared with single htau40 transgenic animals. In a number of dilated axons of htau40 single transgenic mice, the cytoskeleton was disrupted and numerous microtubules, randomly oriented, engirdled accumulations of pleomorphic vesicles, dense-cored ves-

**Fig. 3.** Determination of protein tau bound to paclitaxel-stabilized microtubules. *a*, Western blots of tubulin and microtubule-associated human and murine protein tau in paclitaxel-stabilized microtubule pellets from brain of single and double transgenic mice. Tubulin and protein tau was determined by immunoblotting with neuron-specific β-III anti-N-tubulin and Tau-5 antibody, respectively. *b*, quantification by densitometric scanning of the amount of associated protein tau/tubulin for each mouse individually, normalized to htau40-1 transgenic mice (*n* = 5). *c*, Western blotting as in *a* with LiCl (10 mM) added during the *in vitro* assembly of microtubules. *d*, quantification of the LiCl-treated samples of panel *c* as in *b* (*n* = 3). *e*, Western blotting as in *a* of spinal cord of single and double transgenic mice. Even though the yield of re-assembled microtubules and associated protein tau derived from spinal cord was improved (see “Materials and Methods”), the number of protein tau/tubulin was similar (*Materials and Methods*), murine protein tau remained undetectable. *f*, quantification of Western blots displayed in *e* (*n* = 3). The reduction in amount of protein tau associated with microtubules in the central nervous system of double compared with single transgenic mice is significant, both in LiCl-treated (*p* < 0.001) and untreated (*p* < 0.05) conditions. Panels *a*, *c*, and *e* are representative experiments. Black and gray boxes represent human protein tau/tubulin and murine protein tau/tubulin, respectively. Error bars represent S.E. *IH*, heterozygous htau40-1 mouse; *n*, number of htau40-htau40 × GSK-3β couples used. All mice were older than 4 months.

**Fig. 4.** Determination of protein tau phosphorylation when unassociated with paclitaxel-stabilized microtubules, and distribution of protein tau isoforms phosphorylated at the AD-2 epitope between MT-bound and unbound fraction. *a*, Western blots of unbound protein tau with antibodies indicated. *b*, quantification of unbound protein tau detected by the respective antibodies, normalized to the appropriate reaction with Tau-5 for each mouse individually, and relative to htau40-1 mice (*n* = 4). Differences are significant for AD-2 (*p* < 0.001), AT-180 (*p* < 0.05), and Tau-1 (*p* < 0.05). Black and gray boxes represent human and murine protein tau, respectively. *c*, distribution of the phospho-epitope defined by antibody AD-2 on protein tau bound to (MT) or un-associated with (supernatant) microtubules, derived from htau40-1 × GSK-3β transgenic mice (*n* = 4). Three (1–3; 13, 6.5, 3.25 μg, respectively) dilutions of the corresponding supernatant were applied. Panels *a* and *c* are representative experiments. Error bars represent S.E. *IH*, heterozygous htau40-1 mouse; -, blots solely incubated with secondary antibody; *n*, number of htau40-htau40 × GSK-3β couples used. All mice were older than 4 months.
transgenic human protein tau in brain and spinal cord of wild type mice and htau40-1 littermates, respectively.

Insoluble protein tau represented in the RIPA and FA fractions did not co-precipitated with RAB buffer, RIPA buffer, and FA. The amount of RAB-precipitated protein tau was significantly higher over the paired helical filaments.

The axonopathy and motoric problems in htau40 mice were closely related (42). The effect of co-expression of GSK-3\(\beta\) on the motoric phenotype was evaluated by four different tests on htau40-1 HH \times GSK-3\(\beta\) double transgenic mice, relative to single transgenic animals expressing either protein tau (htau40-1 HH) or GSK-3\(\beta\)(S9A), and relative to wild-type mice (Fig. 8).

Overall, the double transgenic mice behaved in all tests significantly better than their single parental strains, with the exception of the rod-walking test. In the “uprighting reflex,” the impairment of the single htau40 transgenic mice was nearly completely corrected in the double transgenic mice by co-expression of GSK-3\(\beta\) (Fig. 8a). In the forced swimming test and inverted grid-hang test, the double transgenic mice performed equally well as wild-type mice and significantly better than single htau40 or GSK-3\(\beta\) transgenic mice (Fig. 8, b and c). The number of mice that were unable to walk on the rod, however, did not differ between single htau40 and double transgenic mice, demonstrating that all htau40 transgenic mice were unable to remain on the rod, a characteristic not affected by GSK-3\(\beta\) co-expression (data not shown). Interestingly, increased GSK-3\(\beta\) activity itself negatively affected motoric capacities in the swimming and grid-hanging test when compared with wild type mice, but co-expression with human protein tau rescued to a large extend or even completely, the phenotype of GSK-3\(\beta\) transgenic mice.

**DISCUSSION**

The kinases that phosphorylate protein tau *in vivo* have not been identified yet. The hypothesis that GSK-3\(\beta\) is such a kinase was tested here experimentally by generating transgenic mice that overexpress in the central nervous system a constitutively active kinase, *i.e.* GSK-3\(\beta\)(S9A). Obvious overexpression was obtained, resulting in a 2-fold increase of GSK-3\(\beta\) kinase activity. In depth characterization of the GSK-3\(\beta\) transgenic mice is still ongoing, but no overt pathology has been observed.

Experimental *in vivo* evidence is limited to a recent report describing somewhat augmented phosphorylation of mouse protein tau in brain of 18-month-old GSK-3\(\beta\) transgenic mice (66). Indirectly, phosphorylation of protein tau at the Tau-1/AT-8 site was suggested to be dynamically regulated by GSK-3\(\beta\) (67). There is no evidence that GSK-3\(\beta\) activity is increased in AD brains, but GSK-3\(\beta\) activity is elevated in other neurodegenerative conditions, such as Huntington’s disease (68). The activation of GSK-3\(\beta\) in AD brain is likely to be mediated by another GSK-3\(\beta\)-activating factor (69). In our study, the ability of GSK-3\(\beta\) to phosphorylate protein tau was evident in GSK-3\(\beta\) transgenic mice, and even more so in double transgenic mice, generated by cross-breeding with transgenic mice that overexpress in the central nervous system a constitutively active kinase, *i.e.* GSK-3\(\beta\)(S9A). Obvious overexpression was obtained, resulting in a 2-fold increase of GSK-3\(\beta\) kinase activity. In depth characterization of the GSK-3\(\beta\) transgenic mice is still ongoing, but no overt pathology has been observed.

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Associated with microtubules was reduced by 50% in preparations from brain and spinal cord of double transgenic mice compared with single htau40 transgenic animals. Moreover, unbound protein tau was hyperphosphorylated. Especially at the AD-2 epitope, free protein tau from brain of double transgenic mice was even more phosphorylated, exceeding the phosphorylation level of unbound protein tau of single htau40 transgenic mice about 4 times. In addition, the amount of protein tau isoforms phosphorylated at the AD-2 epitope was enriched in the unbound protein tau fraction at the expense of protein tau bound to the microtubules. Hence, phosphorylation at the AD-2 epitope is directly or indirectly, i.e. in concert with other phosphorylated residues recognized by antibodies AT-8 and AT-180, related to the reduced binding of protein tau to microtubules. However, since AD-2 immunoreactive isoforms are still detectable when bound to microtubules, the phosphorylation of this epitope does not eliminate the binding of protein tau to microtubules, but contributes negatively to this binding. The phosphorylation of the AD-2 epitope involves serine 396 and 404 in protein tau. These are thereby identified as essential determinants governing protein tau-microtubule interaction in vivo, corroborating studies in transfected cells (9). Also in extracts of brain biopsies from AD patients, protein tau phosphorylated at serines 396 and 404 was significantly hampered in binding to microtubules (54). Our findings are the in vivo correlate of the reduced binding of protein tau to microtubules in NT2N cells, transfected with GSK-3β (68), and corroborate the capacity of GSK-3β to hyperphosphorylate protein tau in neurons in the central nervous system of mice.

As hyperphosphorylation of protein tau might be an upstream phenomenon of PHF formation or even a prerequisite for filament formation, we scrutinized the central nervous system of transgenic mice for tangle-like structures. Neither Bielschowsky’s silver impregnation, nor thioflavine S staining, nor ultrastructural analysis, nor immunogold labeling revealed signs of neurofibrillary tangles or protein tau filamentous aggregates in brain and spinal cord of single or double transgenic mice. Moreover, although more unbound protein tau is available, the amount of insoluble protein tau was not increased in brain and spinal cord of double transgenic mice compared with

![Fig. 6. Comparison of central nervous system and quadriceps skeletal muscle from homozygous htau40-1 (1HH) and htau40-1 (1HH) × GSK-3β double transgenic mice. Panels a, c, and e are from htau40 transgenic mice; panels b, d, and f from htau40 × GSK-3β double transgenic mice. a and b, reduced number of argyrophilic dilated axons in the gray matter of the ventral horn and surrounding white matter (arrowheads) in double compared with single htau40 transgenic mice. c and d, low power view of the neocortex stained with monoclonal antibody SMI-32, showing dilated axons (arrowheads) only in the cortex of single htau40 transgenic mice. Inset in c displays a higher magnification of a dilated axonal segment. e and f, hematoxylin/eosin staining of quadriceps muscle. Atrophic fibers are absent in quadriceps of double transgenic mice. Bars, 100 μm. Mice are 2–4 months old.](image)

| Mouse | Spinal cord | Cerebral cortex |
|-------|-------------|----------------|
|       | n | No. dilated axons (± S.E.) | n | No. dilated axons (± S.E.) |
| WT | 4 | 0 | 4 | 0 |
| GSK | 4 | 0 | 4 | 0 |
| hTau40–1 | 8 | 9.9 ± 0.49 | 8 | 10.7 ± 1.15 |
| hTau40–1 × GSK | 4 | 2.2 ± 0.34 | 4 | 0.5 ± 0.08 |
| hTau40–2 | 8 | 3.6 ± 0.84 | 8 | 2.6 ± 0.21 |
| hTau40–2 × GSK | 4 | 0.3 ± 0.07 | 4 | 0.3 ± 0.05 |

TABLE I
Quantification of dilated axons in brain and spinal cord of heterozygous transgenic mice
Numbers of dilated axons in the spinal cord and cerebral neocortex are reduced by about an order of magnitude in mice coexpressing GSK-3β. All differences between single and double transgenic mice are significant (p < 0.05). Mice used were 3 months of age. n, number of mice analyzed.
single htau40 littermates. In this respect, our current and previous data (42) and those of others (65) are identical in that both studies fail to demonstrate protein tau filaments in transgenic mice overexpressing four repeat human protein tau. However, lampreys (69, 70) overexpressing three repeat human protein tau did form straight protein tau filaments. All data combined support the hypothesis that extra phosphorylation of protein tau is not directly responsible for the pathological aggregation into PHF, confirming recent in vitro data (71).

In contrast, concomitantly with hyperphosphorylation of protein tau, co-expression of GSK-3β had a major effect on the pathology of the single htau40 transgenic mice, detailed previously (42). Indeed, practically all the pathological defects documented in the htau40 transgenic mice were nearly completely rescued by the mild overexpression of GSK-3β. This “restoration” comprised (i) the reduction by about an order of magnitude of the number of axonal dilations in brain and spinal cord, (ii) the reduction in axonal degeneration and muscular atrophy, and (iii) the alleviation of practically all the motoric problems. Moreover, the normalized motoric performances of GSK-3β transgenic mice when overexpressing htau40 underscore the subtle balance between microtubule-associated and free protein tau that is required to ensure proper nerve functionality. In addition, the remarkable abundance of microtubules in a number of dilations suggest that excess protein tau binds, stabilizes existing, and/or assembles new microtubules, mimicking responses of dorsal root ganglion neurons when incubated with the microtubule-polymerizing drug paclitaxel (72). The fact that mild overexpression of GSK-3β prevented formation of these axonal accumulations in brain and spinal cord of the double transgenic mice corroborated our observation that microtubules isolated from brain of double transgenic mice did bind less protein tau.

The mechanism of this rescue still needs to be unraveled, but...
model, could have therapeutic implications in the field of neurodegeneration. The success of treating manic depressive illness with lithium salts has invited propositions for its use and of drugs with similar effects to therapeutic interventions in Alzheimer’s disease (79) and other tauopathies. Although lithium ions protect neurons against excitotoxicity (80), it also attenuated protein tau phosphorylation (81), most likely by inhibiting GSK-3β (81, 82). Based on our current data, drugs that inhibit GSK-3β could even lead to more axon damage. On the other hand, the fact that GSK-3β is now proven to act as a protein tau kinase in vivo in neurons still qualifies it as a target for drug discovery.

In conclusion, we have generated and characterized double transgenic mice that overexpress constitutively active human GSK-3β and human protein tau. Extra phosphorylation of protein tau reduces its binding capacity to microtubules, but fails to cause protein tau filament formation. The alleviation of the psychomotoric defects and the axonopathy supports in vivo the necessity of a balanced phosphorylation of protein tau in the functionality of central nervous system neurons. Ongoing cross-breeding of the GSK-3β and htau40 transgenic mice with other transgenic mice that harbor other transgenes related to AD, i.e. amyloid precursor protein, presenilin, and apolipoproteinE4 (44, 45), will eventually provide additional information and will be suitable models for the many questions that remain open in the pathogenesis of AD.

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FIG. 8. Performance of single and double transgenic and wild type mice in four sensorimotor tasks. a, time (milliseconds, logarithmic scale) mice needed to return on four legs after being forced to lie on the back. 1HH mice were significantly slower than WT, GSK, and 1HH × GSK littermates (p < 0.001). b, swimming speed defined as distance traveled in 2 min. GSK and 1HH mice covered shorter distances than WT mice (p < 0.001). However, 1HH × GSK double transgenic and WT mice traversed the same distance (p > 0.05). Moreover, 1HH × GSK mice swam faster than 1HH and GSK animals (p < 0.05). c, inverted wire grid hanging, expressed as number of mice that did not remain suspended for the entire 1-min test period, relative to the number of mice tested in each group. Significantly more GSK (p < 0.05) and 1HH (p < 0.001) mice lost hold than WT and 1HH × GSK mice. The 1HH × GSK double transgenic and WT mice performed equally (p > 0.05) well. WT, wild-type mice; 1HH, single homozygous htau40-1 transgenic mice; 1HH × GSK, double homozygous htau40-1 heterozygous GSK-3β transgenic mice. All mice were 2–4 months old.

the amelioration of the axonal transport, inhibited by excess MT-associated protein tau in the single htau40 transgenic mice, is the most plausible explanation. We demonstrated that the axonal dilations accumulate besides mitochondria and vesicles (42), also the synaptic marker synaptophysin, reminiscent of synaptotagmin accumulations in motor neurons of protein tau overexpressing Drosophila (73). This further supports the hypothesis that excess protein tau inhibited kinesin-mediated anterograde transport (74, 75) by binding to axonal microtubules (42, 55, 76–78). That hyperphosphorylation of protein tau by GSK-3β is effectively preventing this pathological outcome is herewith demonstrated in vivo.

The importance of a subtle balanced phosphorylation of protein tau, as supported in vivo in our double transgenic mouse...
