
56 Phosphorylation of Protein tau and Rescue of Protein tau-induced Axonopathy by GSK-3 β in GSK-3 β \times htau40 Double Transgenic Mice

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INTRODUCTION

Biochemical and structural analysis of the phosphorylation sites of human protein tau of paired helical filaments (PHF) in brain of Alzheimer's disease (AD) patients revealed that many sites consist of serine or threonine residues followed by a proline residue. This observation focused attention on proline-dependent kinases (Hasegawa et al., 1992). In the brain of AD patients, NFT were demonstrated to be immunoreactive for glycogen synthase kinase-3 β (GSK-3 β), referring to a potential association with protein tau (Yamaguchi et al., 1996; Shiurba et al., 1996), and identifying GSK-3 β as one of the possible kinase candidates. In addition, active GSK-3 β is found to accumulate in pre-tangle and tangle-bearing neurons in AD (Pei et al., 1999). Phosphorylation by GSK-3 β of bovine (Ishiguro et al., 1992) and human protein tau (Mandelkow et al., 1992) in cell-free systems, resulted in phosphorylation patterns of protein tau that resembled those of the protein isolated from PHF from AD brain. Further evidence for GSK-3 β as a potential protein tau kinase has been obtained in transfected cells, in which co-transfection of GSK-3 β with protein tau increased its phosphorylation concomitant with loss of prominent bundles of microtubules (Wagner et al., 1996).

The involvement of GSK-3 β in the hyperphosphorylation of protein tau, both in cultured neurons and *in vivo* in brain, was indirectly supported by the finding that lithium, as an inhibitor of GSK-3 β , caused tau dephosphorylation at the sites recognized by antibodies Tau-1 and PHF-1, which are two of the

major epitopes typically associated with PHF in AD brain (Muñoz-Montaño et al., 1997; Hong et al., 1997).

Despite the wealth of data *in vitro*, convincing evidence demonstrating the phosphorylation of protein tau by GSK-3 β and the functional repercussions it causes *in vivo*, is lacking. Solid support for the hypothesis that GSK-3 β is an effective protein tau kinase *in vivo*, is presented here in single human GSK-3 β [S9A] transgenic mice and in double transgenic mice, additionally expressing human protein tau.

MATERIALS AND METHODS

GENERATION OF TRANSGENIC MICE

A constitutively active form of the human kinase, i.e. GSK-3 β [S9A], with serine at position 9 replaced by alanine, was used to design an expression cassette that was microinjected into 0.5 day-old FVB/N pre-nuclear mouse embryos. The GSK-3 β transgenic mice were crossed with mice that overexpressed the longest isoform of human protein tau (htau40) (Spittaels et al., 1999) to obtain double transgenic animals. In both constructs, the transgene cDNA was placed under the control of an adapted mouse thyl1 gene promoter to steer expression to central neurons. Using the same promoter to overexpress both cDNAs achieved the co-localization of both transgene products.

SENSORIMOTOR TESTS

Homozygous htau40 transgenic mice, denoted as htau40 HH, and double htau40 HH \times GSK-3 β transgenic mice, were subjected to three sensorimotor tests to assess muscle strength, endurance, coordination and equilibrium (Lamberty and Grower, 1991). Single and double transgenic mice, 2–4 months old, were used in the ‘forced swimming’ and ‘inverted wire-grid hanging’ test, as described previously (Spittaels et al., 1999). In the ‘uprighting reflex’ test, we scored the time that the mice needed to return to the upright position after being forced to lie on their backs.

TISSUE EXTRACTIONS AND WESTERN BLOTTING

To prevent phospho-epitopes from being destroyed by dephosphorylation, all tissues were stored at -70°C immediately after dissection, homogenized on ice and centrifuged at 4°C . The supernatant was rapidly stored at -70°C after snap-freezing in liquid nitrogen.

Brain hemispheres and spinal cords were homogenized as described (Spittaels et al., 1999). After centrifugation, portions of the supernatant

were denatured and reduced before separation on Tris-glycine buffered polyacrylamide gels (8% SDS-PAGE) and transferred to nitrocellulose filters (Spittaels et al., 1999). To eliminate reaction of the secondary antibody with mouse immunoglobulins in Western blotting, brain homogenates were treated with immobilized protein G at 4 °C for 2.5 h and centrifuged before use.

We used Tau-5 as a phosphorylation-independent monoclonal antibody against protein tau. Monoclonal antibodies directed against phosphorylated protein tau epitopes were AT-8, AT-180, PHF-1 and AD-2.

BINDING OF PROTEIN tau TO ISOLATED MICROTUBULES

(Spittaels et al., 2000)

Taxol-dependent isolation and re-assembly of microtubules and microtubule-associated proteins was performed essentially as described (Vallee, 1982). Briefly, mouse brain hemispheres were homogenized on ice in microtubule assembly buffer (MT-buffer) and the supernatant cleared by centrifugation. Taxol (20 M final) and GTP (1 mM final) were added to the supernatant. In some experiments, LiCl was added to inhibit GSK-3 β activity during tissue processing. The mixtures were incubated at 37 °C for 60 min and the microtubules collected by centrifugation through a cushion of 5% sucrose in MT-buffer containing 20 M taxol. The pellets containing the taxol-stabilized microtubules and associated MAPs were washed in MT-buffer with taxol, re-pelleted and dissolved in MT-buffer.

Equal amounts of proteins were loaded on 8% SDS PAGE gels after removal of mouse immunoglobulins. Densitometric quantification of Western blots of protein tau was performed as described (Spittaels et al., 1999) and the results were normalized for neuron-specific tubulin and Tau-5.

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

The mice were anaesthetized with nembutal and transcardially perfused with paraformaldehyde (4%, v/v). The brain and spinal cord were immersion-fixed overnight, cut sagittally into two hemispheres or transversally into four tissue blocks of 9 mm, respectively, dehydrated and embedded in paraffin wax.

ULTRASTRUCTURAL ANALYSIS

For transmission electron microscopy, see Spittaels et al. (1999).

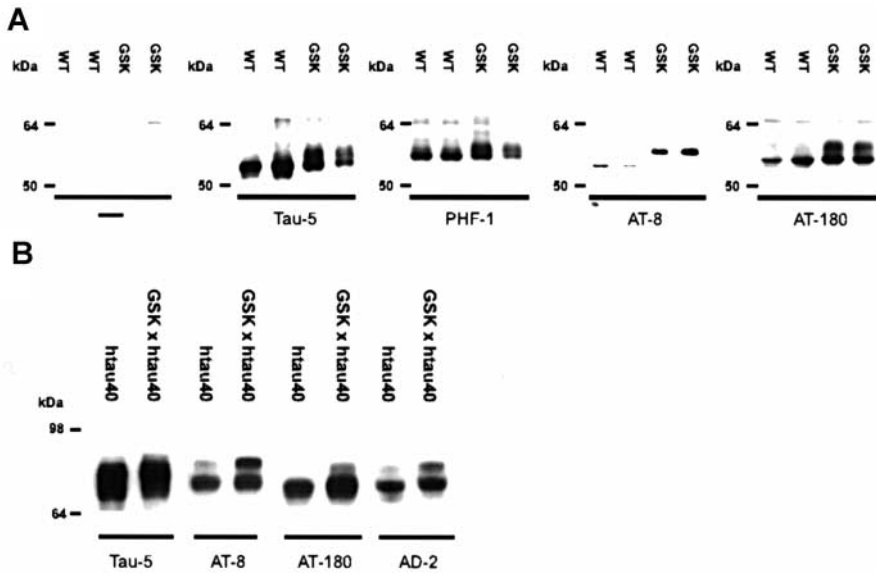


Figure 56.1. Protein tau is hyperphosphorylated in brain of GSK-3 β [S9A] transgenic mice. (A) and (B) Western blotting of brain extracts of single GSK-3 β [S9A] (A) and double GSK-3 β [S9A] \times htau40 (B) transgenic mice. In (A) each panel compares brain extracts from two wild-type (WT) and two GSK-3 β [S9A] transgenic (GSK) mice. Immunoblots demonstrate retardation in electrophoretic mobility (Tau-5 and PHF-1) and increased immunoreaction with phosphate-dependent antibodies (AT-8 and AT-180) of phosphorylated protein tau. Brain homogenates were purified from mouse IgG prior to electrophoresis, as demonstrated by incubation of the blot with only secondary antibody (-). (B) Brain extracts from htau40 transgenic mice and double htau40 \times GSK-3 β [S9A] transgenic littermates show increased phosphorylation of human protein tau in double transgenic animals when immunoblotted with the specified monoclonal antibodies

RESULTS

GSK-3 β PHOSPHORYLATES MURINE AND HUMAN PROTEIN tau *IN VIVO*

Extensive analysis of mouse brain extracts was performed by Western blotting with a battery of well-characterized antibodies, to reveal the typical slow-migrating isoforms of protein tau. Antibodies known to recognize epitopes on protein tau that are phosphorylated by GSK-3 β *in vitro* were evidently included (Sperber et al., 1995). Extracts of brain from GSK-3 β transgenic mice (6–7 months old) reacted with both phosphorylation-dependent antibodies AT-8 and AT-180, and revealed the presence of protein tau

isoforms with slower electrophoretical mobility. These isoforms were also detected with antibodies Tau-5 and PHF1 (Figure 56.1A).

Further studies were mainly devoted to double transgenic mice. These mice were heterozygous for both transgenes and expressed both human protein htau40 and GSK-3 β [S9A] in the CNS, in the same neurons. Western blotting demonstrated hyperphosphorylation of human protein tau in brain extracts of double transgenic mice of 5 weeks. Antibodies AT-8, AT-180 and AD-2 reacted with slowly migrating human protein tau isoforms, and these were virtually absent in the brain extracts of the single transgenic littermates (Figure 1B).

GSK-3 β REDUCED BINDING OF PROTEIN tau TO RE-ASSEMBLED TAXOL-STABILIZED MICROTUBULI.

We examined whether GSK-3 β affected the binding of protein tau to microtubules in brain and spinal cord extracts. In the presence of taxol, extracts of tubulin from mouse brain and spinal cord can still assemble into microtubular structures, despite unfavorable *in vitro* conditions. These re-assembled microtubular preparations are suitable for estimating the association of different MAPs (Vallee, 1982).

The binding of protein tau to reassembled microtubules was significantly reduced in preparations derived from brain and spinal cord of htau40 \times GSK-3 β double transgenic mice, compared to their htau40 littermates. The addition of lithium ions in all buffers during the isolation and reassembly process did not affect the reduced binding of protein tau to the microtubules, except that reduced binding was due to phosphorylation of protein tau by GSK-3 β *in vitro*. We conclude, therefore, that phosphorylation of protein tau had occurred *in vivo* and that these *in vitro* findings reflected the *in vivo* conditions of the brain of double transgenic mice (Figure 56.2). Reduced binding to microtubules was related to phosphorylation of protein tau, which was further supported by the hyperphosphorylation (especially at the AD-2 epitope) of unbound protein tau that remained in the supernatant after microtubule assembly and isolation (Spittaels et al., 2000).

Although more MT-unassociated hyperphosphorylated protein tau is available, neither an increase in insoluble protein tau aggregates nor the presence of paired helical filaments or tangles was observed in the CNS of any of our single and double transgenic mice up to the age of 14 months (Spittaels et al., 2000).

GSK-3 β RESCUED THE AXONOPATHY AND MOTORIC IMPAIRMENT OF htau40 TRANSGENIC MICE

The pathological hallmark of the htau40 transgenic mice, i.e. the dilated axonal segments in brain and spinal cord (Spittaels et al., 1999), were

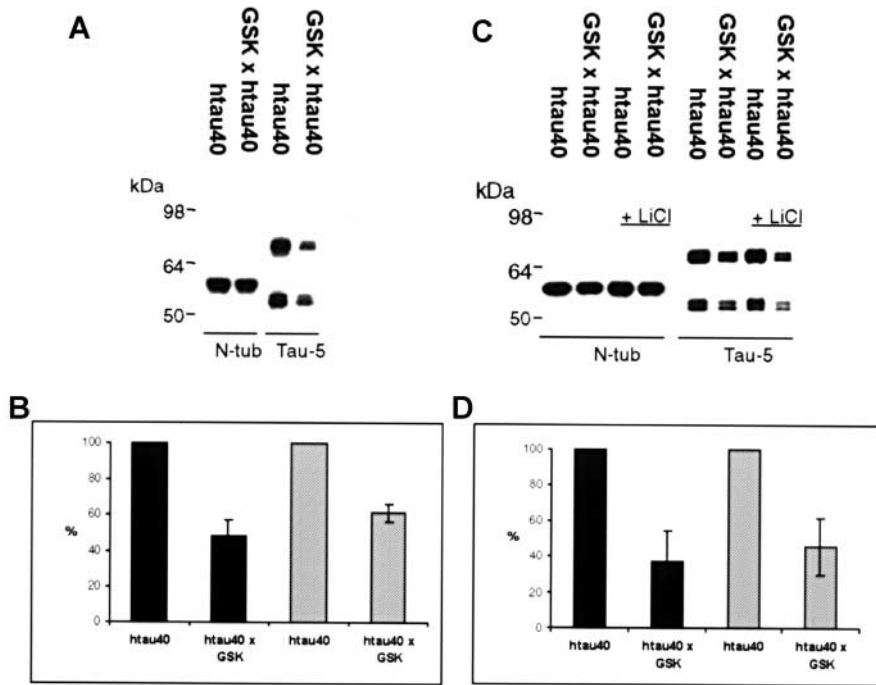


Figure 56.2. Determination of protein tau bound to taxol-stabilized microtubules. (A) Western blots of tubulin and microtubule-associated human and murine protein tau in taxol-stabilized microtubule pellets from brain of single and double transgenic mice. Tubulin and protein tau was determined by immunoblotting 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 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) ($n = 3$). The reduction in the amount of protein tau associated with microtubules in brain of double compared to single transgenic mice is significant, both in LiCl treated ($p < 0.001$) and untreated ($p < 0.05$) conditions. Panels (A) and (C) are representative experiments. Black and grey boxes represent human protein tau/tubulin and murine protein tau/tubulin, respectively. Error bars represent SEM. n, Number of htau40–htau40 \times GSK-3 β couples used

demonstrated to also contain synaptophysin. Synaptophysin is normally transported to the synapses by fast axonal transport mediated by kinesin. Moreover, in a number of dilated axons, the cytoskeleton was disrupted and numerous microtubules, randomly orientated, engirdled accumulations of pleomorphic vesicles, dense-cored vesicles and smooth endoplasmic reticulum (Spittaels et al., 2000). Surprisingly, in the brain and spinal cord of the double transgenic mice, the number of dilated axons was dramatically

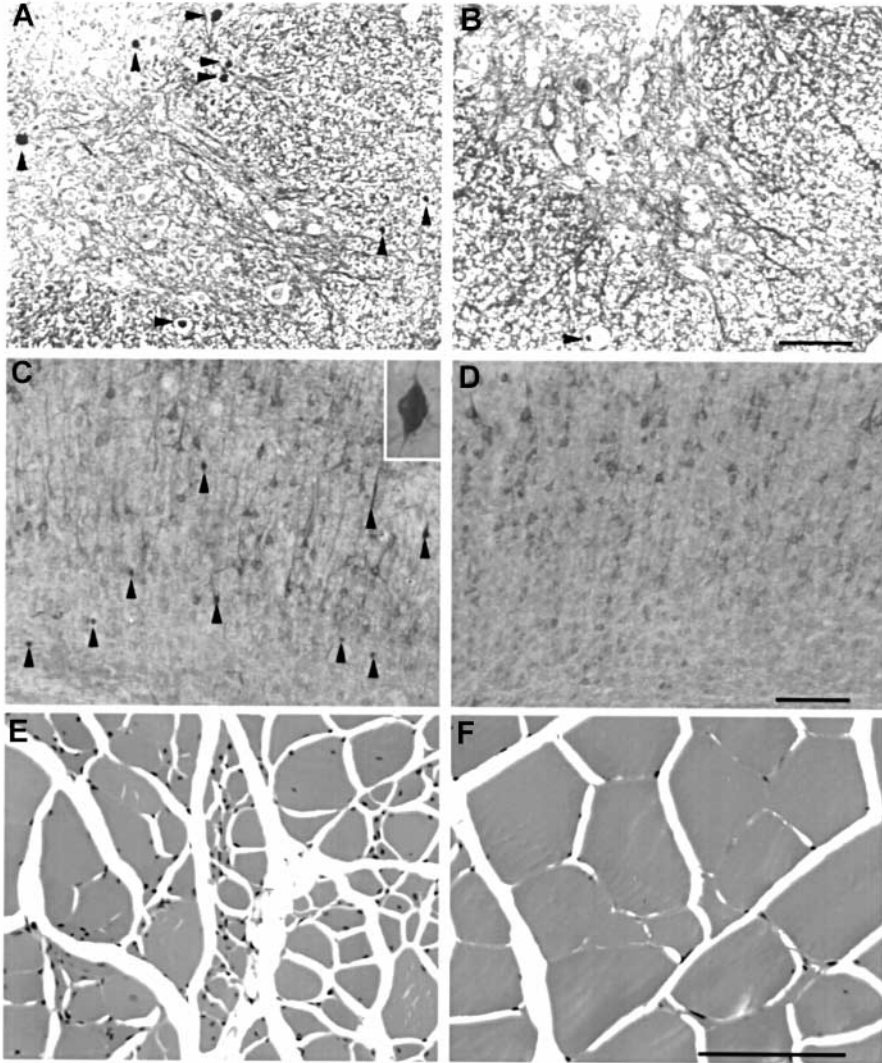
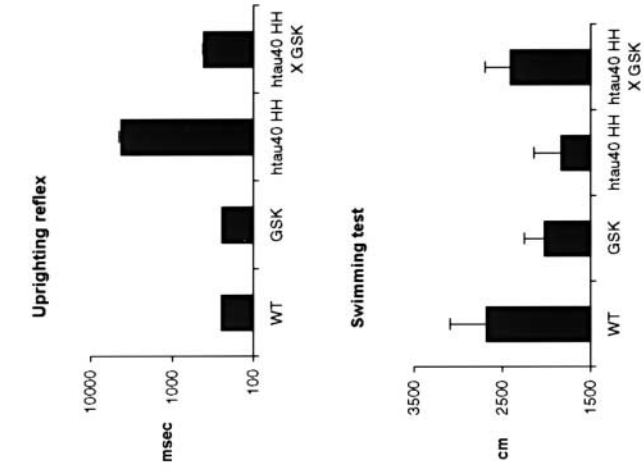
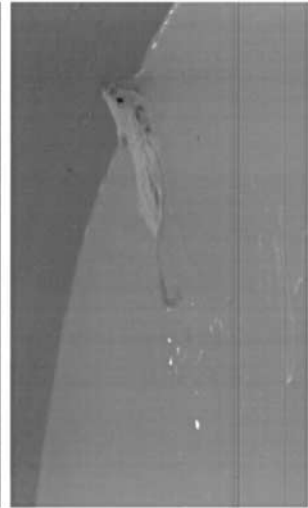


Figure 56.3. Comparison of central nervous system and quadriceps skeletal muscle from homozygous htau40 HH and htau40 HH \times GSK-3 β double transgenic mice. (A), (C) and (E) are from htau40 transgenic mice, (B), (D) and (F) from htau40 \times GSK-3 β double transgenic mice. (A, B) Reduced number of argyrophilic dilated axons in the gray matter of the ventral horn and surrounding white matter (arrowheads) in double compared to single htau40 transgenic mice. (C, 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, F) Hematoxylin/eosin staining of quadriceps muscle. Atrophic fibres are absent in quadriceps of double transgenic mice. Bars, 100 m. Mice are 2–4 months old



A



B

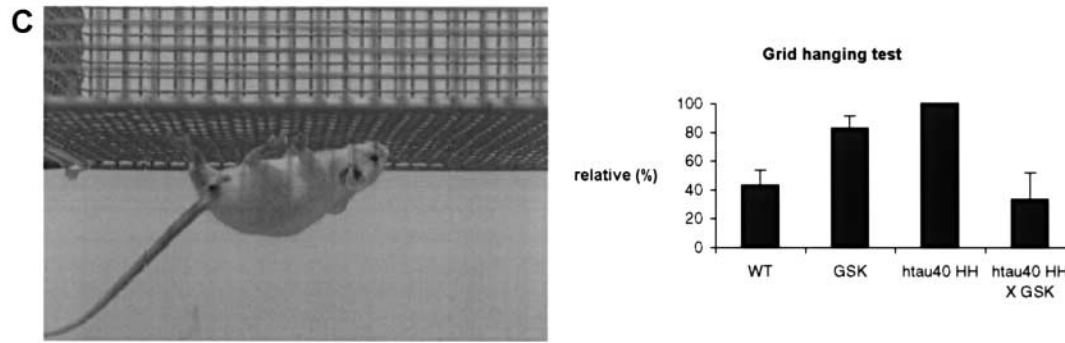


Figure 56.4 Performance of single and double transgenic and wild-type mice in four sensorimotor tasks. (A) Time (ms, logarithmic scale) mice needed to return to four legs after being forced to lie on their backs. httau40 HH mice were significantly slower than WT, GSK and httau40 HH \times GSK littermates ($p < 0.001$). (B) Swimming speed, defined as distance travelled in 2 min. Httau40 HH mice covered shorter distances than WT mice ($p < 0.001$). However, httau40 HH \times GSK double transgenic and WT mice traversed the same distance ($p > 0.05$). Moreover, httau40 HH \times GSK mice swam faster than httau40 HH 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 httau40 HH ($p < 0.001$) mice lost hold than WT and httau40 HH \times GSK mice. The httau40 HH \times GSK double transgenic and WT mice performed equally ($p > 0.05$) well. WT, wild-type mice; httau40 HH, single homozygous httau40 transgenic mice; httau40 HH \times GSK, double homozygous httau40 heterozygous GSK-3 β transgenic mice

reduced. Concomitantly, the quadriceps muscle of double transgenic mice was completely normal and devoid of any muscle wasting, which is a pathological hallmark of the ht40 single transgenic animals (Figure 56.3).

We previously demonstrated in three distinct ht40 transgenic founder strains that the severity of the axonopathy closely correlated with the motoric problem (Spittaels et al., 1999). The effect of co-expression of GSK-3 β on the motoric phenotype was evaluated by three different tests. Overall, the double transgenic mice behaved in all tests significantly better than the single ht40 parental strain. In the 'uprighting reflex' test, the evident impairment of the single ht40 transgenic mice was nearly completely corrected in the double transgenic mice by co-expression of GSK-3 β . In the forced swimming and inverted grid-hanging tests, the double transgenic mice performed equally as well as wild-type mice and significantly better than single ht40 transgenic mice (Figure 56.4).

DISCUSSION

The kinases that phosphorylate protein tau *in vivo* have not been identified. It remains imperative and important, both fundamentally and for the sake of patients suffering from protein tau-mediated dementia, to identify these neuronal kinases that phosphorylate protein tau and thereby control its function(s). The hypothesis that GSK-3 β is such a kinase was tested here experimentally by generating transgenic mice that overexpress a constitutively active kinase, i.e. GSK-3 β [S9A], in their CNS. Mild overexpression was obtained, resulting in a two-fold increase of GSK-3 β kinase activity in brain.

In this study, the ability of GSK-3 β to phosphorylate protein tau was revealed in the GSK-3 β transgenic mice, and even more so in double transgenic mice, generated by cross-breeding with transgenic mice that overexpress the longest isoform of human protein tau, characterized previously (Spittaels et al, 1999). Evidence for hyperphosphorylation was the appearance of slower migrating isoforms of protein tau, which reacted with specified monoclonal antibodies in Western blotting, i.e. AT-8, AT-180 and AD-2. These are known from *in vitro* studies to define phosphorylated epitopes on protein tau that are generated by GSK-3 β . Moreover, the binding of protein tau to MT was reduced by 50% when protein tau was extra-phosphorylated in brain of double transgenic mice. This is the *in vivo* correlate of the reduced binding of protein tau to microtubules in NT2N cells, transfected with GSK-3 β (Hong and Lee, 1997).

In addition, the co-expression of GSK-3 β had a major effect on the pathology of the single ht40 transgenic mice. Indeed, an important finding of the work presented here is the nearly complete rescue, by the mild overexpression of GSK-3 β , of nearly all the pathological defects documented in the ht40 transgenic mice. This 'restoration' comprised: (1) reduction by

an order of magnitude of the number of axonal dilations in brain and spinal cord; (2) reduction in axonal and muscular degeneration; and (3) alleviation of practically all the motoric problems.

The formation of dilated axons in single htau40 transgenic mice supports the hypothesis that excess protein tau inhibits kinesin-mediated anterograde transport (Feiguin et al., 1994) by binding to axonal microtubules (Ebnet et al., 1998; Trinczek et al., 1999). That mild overexpression of GSK-3 β prevented the formation of axonal dilations in the CNS of double transgenic mice suggested that the axonal transport was restored, since hyperphosphorylated protein tau binds less efficiently to MT. Nevertheless, the mechanism of this rescue needs further study. Our current data suggest that drugs that inhibit GSK-3 β —and subsequently increase the binding capacity of protein tau to microtubules—could even lead to more axon damage. On the other hand, the fact that GSK-3 β is a protein tau kinase *in vivo* in neurons still qualifies it as a target for drug discovery in Alzheimer's disease.

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