

Coexpression of GSK-3 β Corrects Phenotypic Aberrations of Dorsal Root Ganglion Cells, Cultured from Adult Transgenic Mice Overexpressing Human Protein tau

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Coexpression of constitutively active GSK-3 β [S9A] rescued the axonal pathology induced by overexpression of human tau in transgenic mice (Spittaels *et al.*, (2000) *J. Biol. Chem.* 275, 41340–41349). We isolated dorsal root ganglion (DRG) neuronal cultures from adult tau4R- and tau4R \times GSK-3 β -transgenic mice to define the mechanisms at the cellular and subcellular level. DRG from tau4R-transgenics showed a reduced sprouting capacity while density and stability of microtubules in the axonal processes were significantly increased. Video-enhanced contrast microscopy demonstrated a dramatic inhibition of fast axonal transport. Coexpression of GSK-3 β increased tau phosphorylation and reversed the effects on microtubule stability and saltatory motion. In DRG from GSK-3 β single transgenics, increased tau phosphorylation was evident without any major effects on microtubule stability or axonal transport. These observations support the hypothesis that excess tau competed with motor-proteins for binding to microtubules and/or that a rigid microtubular system inhibits axonal transport. © 2002 Elsevier Science (USA)

Key Words: tau; GSK-3 β ; microtubules; dorsal root ganglion neuron; transgenics; axonal transport.

INTRODUCTION

Protein Tau is a microtubule associated protein with a primary neuronal localization. In humans, six isoforms originate from a single gene by alternative mRNA splicing and the isoforms differ by presence or absence of 1 or 2 N-terminal inserts and 3 or 4 C-terminal repeats. The binding of tau to microtubules is regulated by the C-terminal microtubule binding domains (Gustke *et al.*, 1994; Preuss *et al.*, 1997) and these interactions are implicated in a number of neurodegenerative diseases, i.e., Alzheimer's disease (AD) and other tauopathies (Vogelsberg *et al.*, 1999). The association of tau with microtubules and the stabilizing effect appears to be regulated through a complex interplay of phosphorylation-dephosphorylation processes (Trinczek *et al.*, 1995).

In AD brain, hyper-phosphorylated tau is present as paired helical filaments (PHF) that accumulate in neurofibrillary tangles (NFT), a diagnostic neuropathological hallmark of AD. Many kinases have been proposed as key players in this process, among which GSK-3 β is a prime candidate to regulate microtubular dynamics through phosphorylation of tau, both *in vitro*, in cellular systems and in transgenic mice (Billingsley and Kincaid, 1997; Leroy *et al.*, 2000; Spittaels *et al.*, 2001; and references therein).

Alterations in microtubule dynamics can have profound effects on normal physiological cell functions. In neuronal cells, one important function of microtubules is the maintenance of axonal stability and transport. In addition to its role in microtubule stabilization, tau can affect motorproteins, i.e., kinesin and dynein, in their binding to microtubules (Hagiwara *et*

al., 1994; Trinczek *et al.*, 1999). This was observed *in vivo*, by overexpression of the longest isoform of human tau, here denoted as tau4R, in mice induced axonal degeneration in the brain and spinal cord (Spittaels *et al.*, 2000). Coexpression of GSK-3 β alleviated the phenotypical defects caused by the increased amount of tau, which was paralleled by increased phosphorylation of tau on AD-specific epitopes (Spittaels *et al.*, 2000). Ongoing studies on these double transgenic mice, and especially ageing will allow further analysis and definition of the contribution to the tau-pathology as in AD.

In the current less time-consuming study, we optimized procedures for primary neuronal cultures derived from dorsal root ganglia from adult transgenic mice, i.e., from single tau and GSK-3 β mouse strains and from tau \times GSK-3 β double transgenic mice. We report observations at the single cell level, allowing detailed analysis of neuronal cell processes and their dynamics, and on the role of the cytoskeleton. The results demonstrate that GSK-3 β reduced the phenotypic changes caused by high levels of tau and rescued at the cellular level the cytoskeletal aberrations. The phenotypic rescue was paralleled by increased tau phosphorylation.

MATERIALS AND METHODS

Transgenic Mice

Generation of the transgenic mice overexpressing htau40 and htau40 \times GSK-3 β [S9A] has been described (Spittaels *et al.*, 1999, 2000). In all strains the engineered and modified mouse thy1 gene promoter was used, driving either the longest human tau isoform or GSK-3 β [S9A], to assure specific coexpression in neurons only (Spittaels *et al.*, 1999, 2000).

Primary Cultures of Dorsal Root Ganglion Neurons

Adult mouse DRG neurons were isolated essentially as described (Delree *et al.*, 1989). Transgenic mice of 6 months were euthanized and the spinal column isolated and sagittally transected. The spinal cord was discarded and the ganglia dissected using microforceps. After removal of connective tissue, the roots were cut close to the ganglia and enzymatically digested with collagenase (5 mg/ml, 45 min) followed by incubation with trypsin and collagenase (each 2.5 mg/ml, 30 min). The ganglia were then suspended in

DMEM /10% FCS, collected by centrifugation, and mechanically dissociated using glass pipettes with decreasing tip diameters. The resulting cell suspensions were plated on serum-coated petri dishes to allow the nonneuronal cells to adhere. After 90 min the remaining neurons in suspension were collected and plated onto poly-L-lysine-coated coverslips. After 24 h, cytosine arabinoside (10 μ M) was added to inhibit proliferation of nonneuronal cells.

Tissue Extraction for Western Blotting

DRG were dissected without enzymatic treatment in cold RIPA buffer, containing protease and phosphatase inhibitors, with all incubations on ice. The tissue was mechanically homogenized, and proteins denatured and reduced (SDS 2%, 100°C, 5 min). Extracts were cleared by centrifugation and samples were separated by SDS-PAGE on 4–20% acrylamide gels. Proteins were transferred to nitrocellulose sheets and blocked by incubation in PBS/0.5% Tween/10% milk. Incubation with primary antibody diluted in PBS/0.05% Tween/5% milk was overnight at 4°C. The blots were washed three times in PBS/0.5% Tween, incubated with secondary antibodies labeled with horse radish peroxidase, and the resulting immune complexes were visualized by chemoluminescence (Amersham, UK).

Immunofluorescence

DRG cultures were fixed with 0.5% glutaraldehyde/0.5% Triton X-100 in PHEM buffer for 10 min (Schliwa and Van Blerkom, 1981) with additional permeabilization by incubation in 0.5% Triton-X100 in PHEM for 30 min. Remaining aldehydes were reduced (1 mg/ml NaBH₄ in PHEM, 10 min) and all subsequent incubations were in Tris-buffered saline (TBS) containing 0.1% BSA.

Primary antibodies used were anti-acetylated tubulin and anti-tubulin (Sigma, St. Louis, MO), anti-GSK-3 β (Becton Dickinson Benelux, Erembodegem, Belgium), AT8 and AT270 (Innogenetics, Gent, Belgium), and anti-tau (Dakopatts, Denmark). Secondary antibodies were FITC- or Cy-3-labeled. Images were obtained on a Zeiss LSM510 confocal microscope.

Image Analysis

Cells, labeled with the respective antibodies were imaged with a ZEISS Axiovert 35 microscope at excitation wavelengths 485 and 512 (Carl Zeiss Jena

GmbH, Jena, Germany). The images were analyzed by SCIL Image package (Van Balen *et al.*, 1994) with an algorithm for ridge detection (Steger, 1998; Geusebroek, 2000) applied to the images in the FITC channel. The binary mask of the neurites detected was applied to the Cy3 channel with interactive, blind correction for over-segmentation. For each neurite the average intensity, standard deviation and coefficient of variation was calculated while overall neurite length per neuron was determined interactively on phase-contrast microscopic images, analyzing at least 25 neurons for each genotype and for each condition.

Axonal Transport in DRG Neurons

For high resolution video microscopy experiments, the cells were plated on 24×32 -mm glass coverslips. After 3 days in culture a microchamber was constructed by inverting the coverslip on a microscope slide using Parafilm strips as spacers, sealed with Valap, i.e., equal amounts of vaseline, lanoline, and paraffin. The cells were transferred to the microscope stage (Zeiss Axioplan) and kept at 37°C in an air-curtain incubator. Observations were made in differential interference contrast (DIC) microscopy using a $100\times$ objective and 1.4 NA oil immersion condenser. Images captured by the Chalnicon video camera were processed in a C1966 video enhancement system (Hamamatsu) and recorded on U-matic video tape (Sony V05850P). Motion was analyzed interactively from the recorded videotapes. For the determination of the jump frequency a line was drawn crossing an axon and the number of vesicles passing this line was counted.

Statistical Analysis

Comparison between different groups was done using Mann-Whitney test.

RESULTS

Western Blot of Expressed Transgenes

The expression levels of the human proteins in the brain of transgenic mice were determined in previous studies (Spittaels *et al.*, 1999, 2000). The level of expression in freshly dissociated DRG neurons originating from mice aged 6 months was determined by Western blot of total cell extracts (Fig. 1). Immunolabeling with a polyclonal antibody against protein tau illustrated

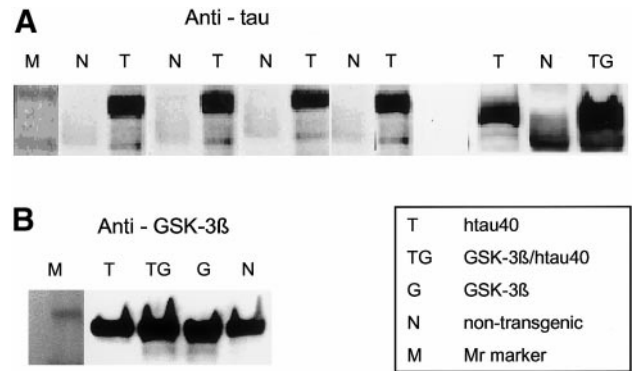


FIG. 1. (A) Western blot for protein tau in DRG cells derived from transgenic mice with the specified genotype. Cultures from four nontransgenic littermates and 4 httau40 transgenic mice illustrate the minimal variation between cultures (T = httau40, code TG = httau40 \times GSK-3 β [S9A], code G = GSK-3 β [S9A], code N = FVB wild-type). It also shows the tau expression levels in double transgenics and recombinant human tau as a reference. (B) Western blot from the different transgenic strains using anti-GSK-3 β antibody. The higher levels of protein are apparent in both the single GSK-3 β [S9A] and double httau40 \times GSK-3 β [S9A] expressing lines.

the very similar expression in DRG cells isolated from 4 distinct tau transgenic mice (Fig. 1A). In addition, it shows the expression of tau in a double Tau \times GSK-3 β transgenic mouse compared to wild type FVB and recombinant human tau samples.

Western blot analysis of neurons isolated from the different transgenic mice and labeled for GSK-3 β (Fig. 1B). In both the single and double, GSK-3 β overexpressing lines, there is a higher level of protein when compared to tau transgenic or to wild-type FVB mouse samples.

Morphology and Axonal Growth

Wild-type DRG neurons develop in culture a few axons that originate at the cell body and form, over time, a highly branched network (Fig. 2). In cells derived from tau40 transgenic mice, the process of neurite formation was dramatically inhibited and the complexity greatly reduced, with the reduced plasticity further illustrated by the lack of active growth-cones.

The visual inspection was confirmed by interactive measurements of the total length of the axonal processes for individual cells. The total neurite length per cell was significantly reduced from 1319 ± 199 pixels to 728 ± 124 pixels, ($P < 0.05$ over $n = 25$), respectively, for DRG isolated from wild-type and tau transgenic mice.

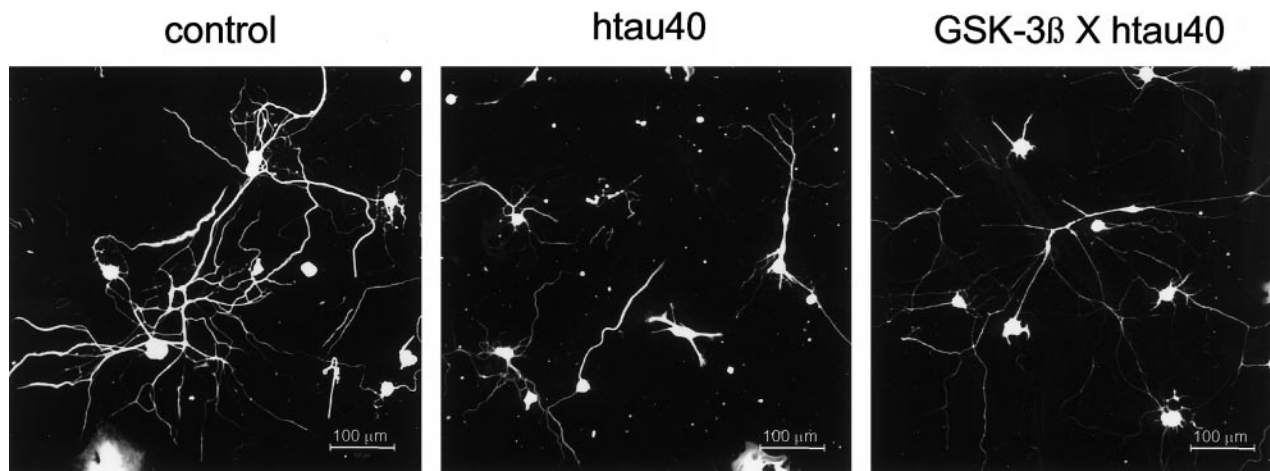


FIG. 2. Morphology of individual cultured DRG neurons derived from different transgenic mice after 7 days *in vitro*. Low magnification images of tubulin labeled cells representative for the different cultures. Control cultures showed a nice neuritic extension, whereas cultures derived from single htau40 transgene mice have a much reduced neuritic arborisation. GSK-3 β [S9A] single expressing cells are not different when compared to the nontransgenic cultures. Coexpression of htau40 \times GSK-3 β [S9A] substantially reduced the morphological alterations seen in the dorsal root ganglion neurons derived from htau single transgenic mice.

On the other hand, DRG cells derived from Tau \times GSK-3 β double transgenic mice developed neurites normally with the length completely normalized, i.e., averaging 1367 ± 189 pixels. In addition, the overall growth and complexity of their neuritic tree was highly comparable with those of nontransgenic DRG cultures.

These data demonstrate that overexpression of the microtubule-associated protein tau compromised the plasticity of DRG neurons and furthermore that phosphorylation by GSK-3 β restored the normal situation. This *in vitro* paradigm thereby faithfully reflected the *in vivo* situation (Spittaels *et al.*, 1999, 2000).

Phosphorylation of Protein Tau

Analysis of single cells by immunofluorescence and confocal microscopy was used to study tau phosphorylation in DRG from mice with the different genotypes. After 7 days in culture DRG neurons were prominently labeled with an antibody for all forms of protein tau, while the phosphorylation dependent AT270 signal was strongly dependent on the genotype (Fig. 3). DRG from nontransgenic mice were hardly labeled with phospho-tau specific antibodies. In DRG from GSK-3 β and from Tau single transgenic mice the signal was weak and primarily confined to the distal parts of the axons close to the growth-cones. In DRG from Tau \times GSK-3 β double transgenic mice, the robust staining with AT270 demonstrated extensive phosphorylation of protein tau of this epitope that

includes threonine 181 (Goedert *et al.*, 1994). In most instances the entire cell stained positively for phosphorylated tau with AT270 (Fig. 3). These findings were confirmed and extended quantitatively using antibody AT8, which is specific for an epitope on tau, including the phosphorylated Serine residues 202–205 (Mercken *et al.*, 1992). In DRG cultures from nontransgenic and from tau transgenic mice, about 8% of the cells reacted with AT8. In DRG cultures from GSK-3 β transgenic mice about 15% of the cells were stained with AT8, whereas this increased to 27% of positive DRG cells when derived from double Tau \times GSK-3 β transgenic mice.

Analysis of Microtubular Distribution

Since the binding of protein tau to microtubules is supposed to be its most important physiological function, we compared the cytoskeletal arrangement in cultured DRG derived from single and double transgenic and from nontransgenic mice. By double label immunofluorescence the differential expression of human tau was analyzed using an antibody against tau that is phosphorylation-independent, and anti-tubulin (Fig. 4). In DRG cultures from nontransgenic mice, protein tau is primarily located in the axonal shaft and mostly associated with microtubules, while neurons from tau transgenic mice contained substantial higher levels of tau, residing both in the cell body and in the axons (Fig. 4A). In cells from tau transgenic mice, the

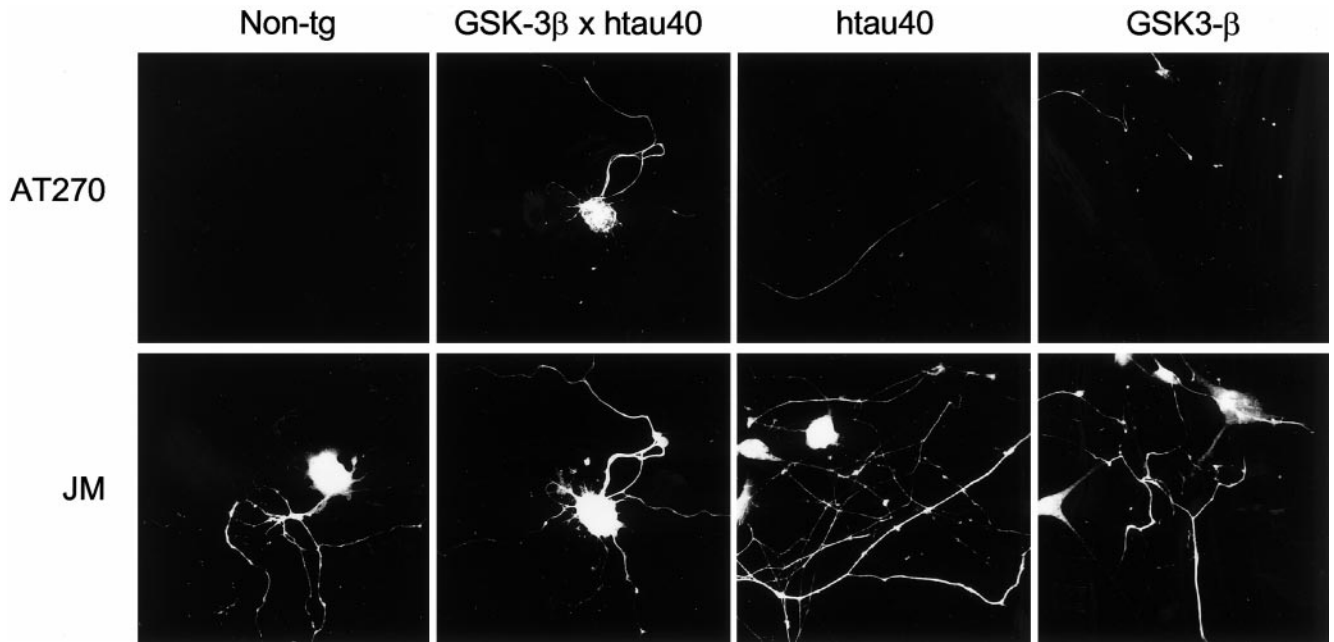


FIG. 3. Double label immunocytochemistry for phosphorylated tau with AT270 antibody (1) and total tau (2). Expression of the httau40 and GSK-3 β [S9A] slightly increases the degree of tau phosphorylation, but the most prominent degree of tau hyperphosphorylation occurs in the case of double httau40 \times GSK-3 β [S9A] derived DRG cultures.

microtubular system was much more pronounced and appeared very dense and compact, making it difficult to discern individual microtubules as opposed to those in nontransgenic neurons (Fig. 4B).

Analysis of Acetylated Microtubules

Posttranslational modifications of microtubules are indications for the stability of the cytoskeleton. Therefore, we evaluated the degree of acetylation of microtubules in the cell-bodies and in the axons by double immunofluorescence for total and for acetylated tubulin. Acetylated tubulin was primarily localized in the axonal shaft, consistent with its role in axonal rigidity, while in general much less acetylated microtubules were observed in the cell-bodies (Fig. 5). Overexpression of human tau severely increased levels of acetylated tubulin, which was most pronounced in the cell-bodies, resulting in a dense array of acetylated microtubules (Fig. 5A, middle panel). In axons, the high tau levels also increased the density of acetylated microtubules even into the very distal, outer parts of the growth-cones, as opposed to nontransgenic axons in which acetylated microtubules do not extend into the growth-cone (Fig. 5B). In contrast, in cultures from tau \times GSK-3 β double transgenic mice, the distribution

of acetylated microtubules was very similar to non-transgenic cultures, both in the cell body and the axons (Fig. 5).

Image analysis of double immunofluorescence yielded a measure of the ratio of acetylated over total tubulin in the axons of DRG neurons originating from the different transgenic mouse strains. Relative to non-transgenic DRG cultures, the ratio was 115% in tau transgenic mice as opposed to 90% in cultures from GSK-3 β [S9A] transgenic mice. In DRG from tau \times GSK-3 β double transgenic mice, the ratio was 99%, thus GSK-3 β normalized also the level of microtubular acetylation.

The effect of GSK-3 β on cytoskeletal alterations in DRG cultures from double transgenic mice was illustrated by the effect of Li⁺-ions, known to inhibit GSK-3 β . DRG neurons from tau \times GSK-3 β double transgenic mice treated with LiCl (10 mM) for 2 days, reverted their microtubular organization toward that of tau transgenic mice, including the reappearance of stable acetylated microtubules in the cell body (Fig. 6).

Axonal Transport

Fast axonal transport is dependent on a functional microtubule cytoskeleton, therefore we evaluated fast

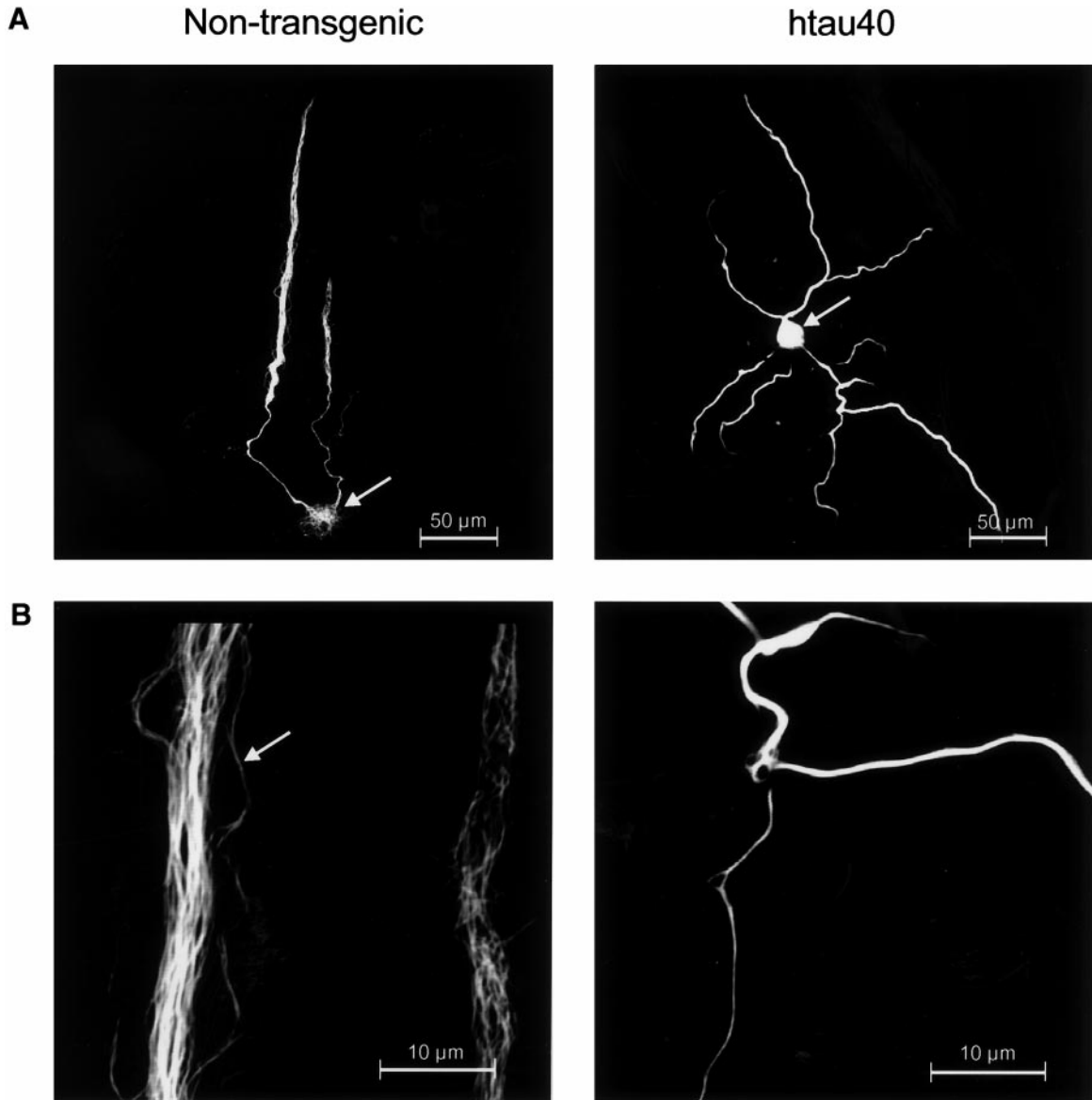


FIG. 4. Immunofluorescence for total, phosphorylation-independent, tau (A) and tubulin(B) in dorsal root ganglion neurons from non-transgenic mouse and htau40 overexpressing mouse. Notice the different levels of tau, both in the cell body (arrows in A) and the axons. In DRG cultures, derived from control mice individual microtubules are resolved (B). Tau overexpression induces alterations in the microtubule organization and the increased microtubule density in the processes makes it impossible to discern individual microtubules.

axonal transport in primary dorsal root ganglion neurons from transgenic mice with the different genotypes. Video enhanced contrast microscopy with Nomarski differential interference contrast allowed us to follow and quantify fast axonal transport of sub-micron vesicles in living neurons. Video-sequences of movement in individual neurites were recorded over periods of 5 min and the number of vesicles passing a virtual boundary (“jumps”) were counted in 9 sepa-

rate axons in DRG cultures derived from mice for each genotype. In DRG derived from the tau-transgenic mice the number of passes was reduced to 18% relative to those in nontransgenic DRG cultures, while coexpression of GSK-3 β largely restored the decrease in jumps induced by tau (86%) (Table 1). In cells from GSK-3 β transgenic mice, this index of axonal transport was considerably higher (156%) than in cells from nontransgenic mice (Table 1).

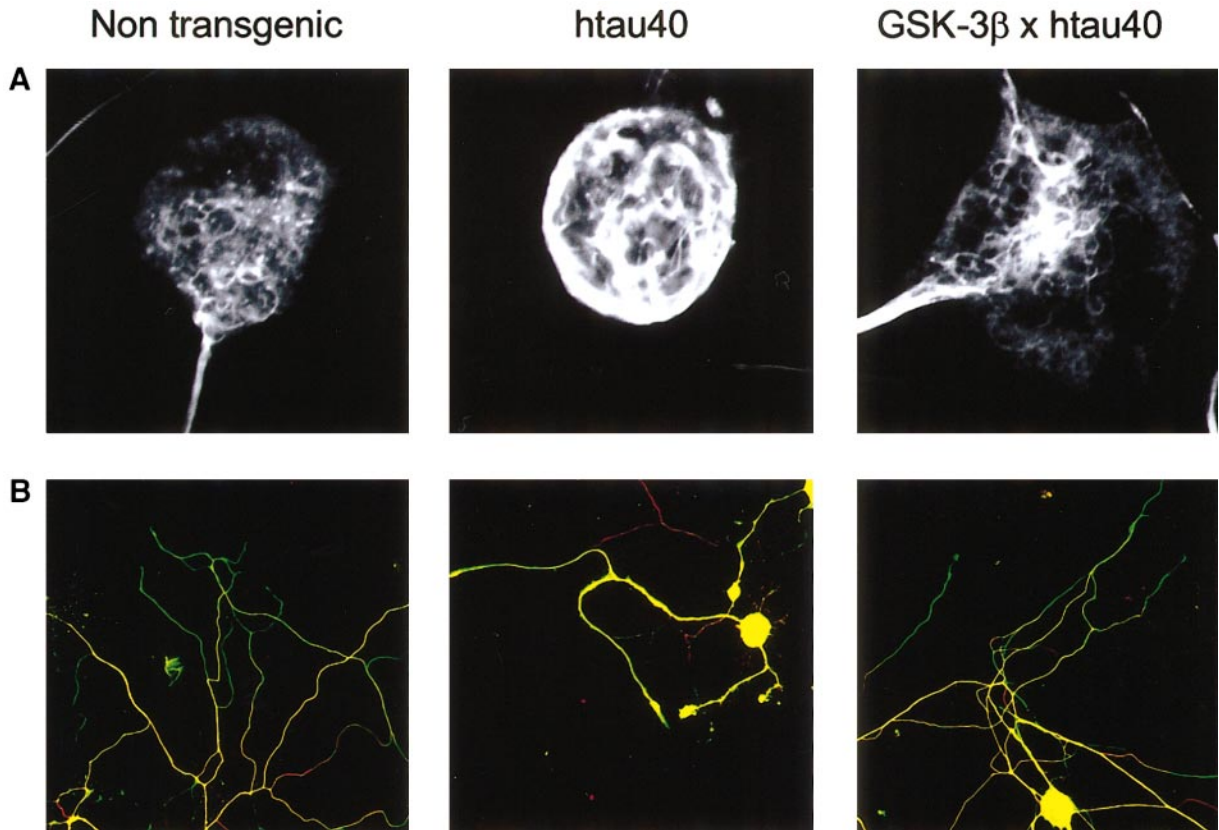


FIG. 5. Double label immunofluorescence for acetylated tubulin (red) and total tubulin (green) in dorsal root ganglion neurons from non-transgenic mouse, htau40 overexpressing and htau40 \times GSK-3 β [S9A] transgenic mice, cell bodies (A) and axons (B) are shown. Overexpression of tau induced a substantial increase of acetylated tubulin in the cell body (A) when compared to control cultures. Similar increases in this ratio are observed in the axonal extensions (B). Composite images show a shift from the green signal (total tubulin) toward the red (acetylated tubulin) with increased tau levels. Coexpression of GSK-3 β [S9A] significantly reduces the abnormal amount of stable microtubules, both in the cell body and in the neuronal processes.

Fast axonal transport proceeds by an average of about 1 to 2 $\mu\text{m/s}$ (Coy and Howard, 1994). In DRG from nontransgenic mice, the measured average speed of 1.8 $\mu\text{m/s}$ was within this range, while in cultures derived from tau transgenic mice the speed was reduced to about 0.5 $\mu\text{m/s}$. Coexpression of GSK-3 β with tau again restored, although not completely, the fast axonal transport (1.1 $\mu\text{m/s}$). Despite the higher number of jumps in DRG derived from single GSK-3 β transgene mice, the jump velocity (0.9 $\mu\text{m/s}$) was less than in control cultures (Table 1).

A final parameter that was measured and shown to be affected by the different transgenes was the distance travelled during individual saltatory jumps. In DRG cultures from tau transgenic mice, the average length for each jump was decreased to 35% relative to those in nontransgenic mice. Again coexpression of GSK-3 β [S9A] with tau restored the decrease to only

about 50% of nontransgenic values, while GSK-3 β by itself also reduced the jump length to about 80% of that in wild-type mice (Table 1).

DISCUSSION

To our knowledge, this is the first study to use primary DRG cultures derived from adult transgenic mice that overexpress one or more human proteins. The aim was to investigate the interaction of protein tau and of its kinase GSK-3 β at the cellular and sub-cellular level as a novel paradigm to corroborate biochemical and histological observations made *in vivo* in the parent transgenic mice (Spittaels *et al.*, 1999, 2000). Tau transgenic mice suffer axonopathy and motor problems, evidently due to impairment of axonal transport as indicated by the occurrence of the large

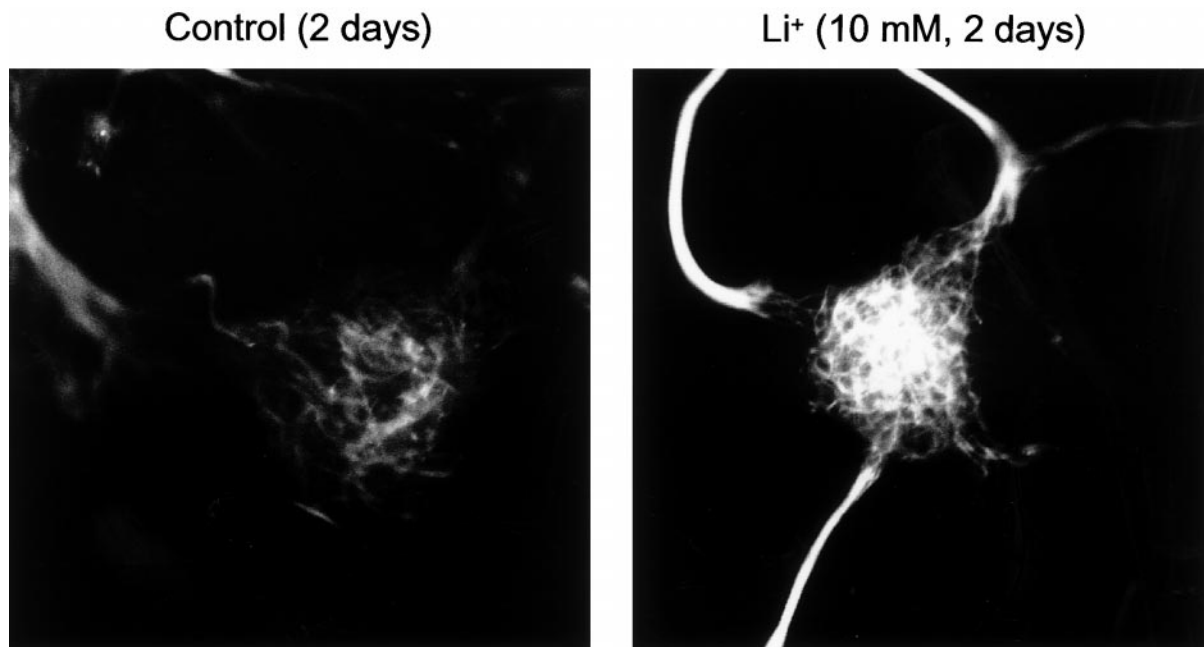


FIG. 6. Immunofluorescence of acetylated tubulin in dorsal root ganglion cells derived from double htau40 \times GSK-3 β [S9A] transgene mice, with and without LiCl treatment. Pharmacological inhibition of GSK-3 β [S9A] by lithium 10 mM for 2 days, clearly shifts the distribution of acetylated tubulin toward the phenotype observed in tau single transgenic cultures.

number of axonal dilatations in brain and spinal cord due to accumulation of transported proteins and vesicles (Spittaels *et al.*, 1999). This entire pathology was nearly completely alleviated by coexpression of GSK-3 β , i.e., in tau \times GSK-3 β double transgenic mice (Spittaels *et al.*, 2000). The parallel in restoration of the normal phenotype with the disappearance of axonal dilatations, was a surprising finding but made a very strong case for the hypothesis that tau affected the axonal transport directly. In addition it was the first strong argument that GSK-3 β was an efficient tau-kinase in vivo in brain. Finally, it demonstrated the essential role of GSK-3 β in controlling binding of protein tau to the microtubules and thereby of their integrity and stability (Spittaels *et al.*, 2000).

A cellular system was devised to allow us to study the functional details of the mechanisms that underlie the phenotype as revealed in vivo in the different transgenic mice. DRG neuronal cultures were anticipated to be interesting because these cells can be readily isolated from adult mice and cultured, in contrast to hippocampal or cortical neurons which need to be derived from embryonic tissue. DRG were particularly interesting for our transgenic mice given their motoric defects, but also in general when expression of the transgene is late in onset, as is the case for the

mouse thy1-gene promoter that we have used. Neuronal expression of authentic endogenous thy-1 is known to be maximal once neurons are established in their final positions, which is for mouse brain predominantly during the second and third weeks after birth (Caroni, 1997; own results not shown). By using DRG neurons derived from adult animals the natural selection of cells was omitted as these cells could be used immediately after isolation compared to a 2-week culture period of embryonic central nervous system necessary for the transgene to be expressed. Western blot analysis of transgene expression also illustrated that the interanimal variation was minimal.

The results demonstrate that the primary DRG neuron cultures were indeed effective and informative as cellular model-systems. Not only became the phenotypic effects caused by human protein tau and of GSK-3 β apparent, but most exciting also the rescue effect of GSK-3 β on the tau pathology was evident and subjected to further analysis, as discussed below. First, that the higher than normal levels of protein tau severely reduced the complexity of the axonal processes, was rather unexpected since published findings in non-neuronal cells indicate that these are forced to form processes after transfection with tau (Knops *et al.*, 1991). Since protein tau is not known to affect the

TABLE 1
Analysis of Fast Axonal Transport in DRG Cultures
from Tau Transgenic Mice

Mouse genotype	No. jumps (%)	Jump distance (μm) (%)	Jump velocity ($\mu\text{m/s}$) (%)
Nontransgenic	17.6 \pm 3.5 (100)	8.5 \pm 0.8 (100)	1.8 \pm 0.2 (100)
htau40	3.2 \pm 1.7* (18)	3.0 \pm 0.4* (35)	0.5 \pm 0.1* (28)
htau40 \times GSK-3 β	15.1 \pm 3.5† (86)	6.4 \pm 0.4† (75)	0.9 \pm 0.1† (50)
GSK-3 β	27.4 \pm 6.1 (156)	5.4 \pm 0.4 (64)	1.1 \pm 0.1 (61)

Note. *Statistically different from control. † Statistically different from htau40. Axonal transport was analyzed quantitatively in video-enhanced microscopic recordings in cultures after 3 days *in vitro*. The number of vesicles moving in a saltatory fashion, the distance of each jump and the speed was affected by the particular transgene. Overexpression of htau40 significantly reduced all parameters ($P < 0.001$ vs control in number of jumps, $P < 0.0002$ vs control in distance and $P < 0.0002$ vs control in jump speed) while coexpression of both htau40 and GSK-3 β [S9A] reverted both phenotypes to the control situation ($P < 0.005$ in htau40 vs GSK-3 β [S9A] \times htau40 in number of jumps, $P < 0.001$ in htau40 vs htau40 \times GSK-3 β [S9A] in distance of individual jumps and $P < 0.001$ in htau40 vs htau40 \times GSK-3 β [S9A] in jump speed). Expression of GSK-3 β [S9A], on the other hand, had a mild stimulating effect on the number of moving vesicles only (statistically insignificant), whereas the distance and speed of individual jumps were decreased slightly. Shown are mean with SEM of 10 individual movements in 9 axons for each genotype.

intrinsic capacity of neurons to form processes, we hypothesize that the stability of microtubules as affected by excess tau was responsible, and that neuronal cells are more sensitive to this effect than nonneuronal cells. Increased rigidity and loss of flexibility of the microtubules is thereby likely to compromise axonal outgrowth. It is known that stable, acetylated microtubules are primarily located in the axonal shaft of neuronal cells (Brown *et al.*, 1993). We demonstrate here that overexpression of tau dramatically increased this acetylated, stable microtubular population, with some reorganization toward the cell-body. Spatial reorganization was also observed in DRG from GSK-3 β transgenic mice although the density of acetylated microtubules decreased compared to that in nontransgenic DRG cells.

Expression of GSK-3 β [S9A] by itself had no apparent adverse effects on the neuritic outgrowth but in cultures from double transgenic mice, GSK-3 β reduced the tau associated phenotypic changes in the

neurons. This normalization in cultures derived from tau \times GSK-3 β double transgenic mice was clearly demonstrated, both in the amount of acetylated tubulin and in its cellular distribution. Moreover the compensating effect of GSK-3 β was further evident in all assays and test-systems as presented in the results section, thereby providing ample experimental evidence for the working-hypothesis and corroborating completely the observations made *in vivo* (Spittaels *et al.*, 1999, 2000).

Previous studies in nonneuronal cells (Wang *et al.*, 1998; Lovestone *et al.*, 1996) demonstrated a contribution of GSK-3 β to the phosphorylation of protein tau. Here we provide additional convincing evidence that GSK-3 β increased tau phosphorylation, and generated epitopes recognized or defined by the monoclonal antibodies AT270 (Thr 181) and AT8 (Ser202–205). This was also evident in DRG derived from GSK-3 β single transgene mice, thereby documenting the increased phosphorylation of endogenous mouse tau, very similar to phosphorylation of human tau in cultures from double transgene mice. In neurons derived from tau single transgenic mice, no AT8 nor AT270 reactivity was observed relative to nontransgenic littermates. These cell-biological findings thereby corroborate the findings in cerebral neurons *in vivo* (Spittaels *et al.*, 1999, 2000).

The specificity of GSK-3 β mediated phosphorylation of tau was further underlined by the inhibition with lithium ions, known to be a noncompetitive inhibitor of GSK-3 β (Kleine and Melton, 1996; Stambolic *et al.*, 1997; Munoz-Montano *et al.*, 1997; Hong *et al.*, 1997). Lithium ions might, however, also act via other kinases and phosphatases, as it is known to inhibit polyphosphate-1 phosphatase, inositol monophosphatase, casein kinase II, MAP kinase activated protein kinase-2, p38-regulated/activated kinase, and activation of PI3-kinase/PKB and c-jun N-terminal kinase (JNK) in cellular assays (Berridge *et al.*, 1989; Davies *et al.*, 2000; Manji *et al.*, 1999; Chalecka-Franaszek, 1999; Yuan, 1999). In DRG neurons derived from double transgenic mice, lithium ions annihilated the GSK-3 β induced rescue of microtubular organization, and reverted the cellular phenotype to that of tau transgenic cells. That is circumstantial evidence for a direct effect via GSK-3 β , rather than indirect via any or more of the other enzymes listed above. These findings thereby establish the DRG cultures as an important experimental neuronal paradigm, accessible and useful to develop and study specific inhibitors of GSK-3 β on the cytoskeletal phenotype *in vitro*, in dif-

ferent conditions and from different types of transgenic mice.

A unique feature of neuronal cells is the ability to transport many different types of proteins, vesicles and organelles over long distances. The fast axonal transport is driven by motor proteins and essentially require a functional cytoskeleton (for review see Goldstein and Yang, 2000).

By a combination of microscopic techniques, we studied the effect of the transgenic proteins on the axonal transport of membrane bound vesicles in DRG cultures. The data are in complete agreement with published *in vivo* studies but provide us with a more refined view on the intracellular dynamics as this approach allows the monitoring of individual vesicles.

Human protein tau impacted negatively on all parameters quantified, i.e., jump frequency, jump length, and jump speed. This corroborates studies in non-neuronal cell types (Ebner *et al.*, 1998; Trinczek *et al.*, 1999) and is consistent with data obtained in transgenic mice that overexpress the shortest human tau isoform (Ishihara *et al.*, 1999). The competition between tau and motor proteins for binding to microtubules (Hagiwara *et al.*, 1994) besides competition or interaction of endogenous mouse tau and overexpressed human tau, are the likely causes for the reduced microtubule density observed here and in transgenic models *in vivo* (Ishihara *et al.*, 1999; Spittaels *et al.*, 1999, 2000).

Intriguingly, overexpression of GSK-3 β itself positively effected the parameter of jump-frequency, while decreasing both jump-distance and jump-speed (Table 1). This is reminiscent of the positive effect of cAMP, via a Protein kinase A mediated activation of axonal transport (Nuydens *et al.*, 1993) involving enhanced phosphorylation of protein tau (Sato-Harada *et al.*, 1996).

We demonstrate that co-expression of GSK-3 β with protein tau, besides normalizing the pathological phenotype *in vivo* and *in vitro*, largely normalized the axonal transport parameters. If increased availability of binding sites for the motor proteins is involved directly, it must be due to the dissociation of tau from microtubules through enhanced phosphorylation of tau by GSK-3 β , as proposed from the *in vivo* observations (Spittaels *et al.*, 2000). Coexpression of GSK-3 β might in addition alter the density and/or the length of the microtubule arrays so that the vesicles might travel over longer distances. Whereas the combined findings demonstrate that regulation of axonal transport depends on competition between tau and motor-proteins, they additionally imply that the density of

the microtubular arrays might be an important factor. In the tau overexpressing neurons, very dense microtubule bundles were observed that could create mechanical barriers, hampering vesicles to travel. In line with this conclusion are the observations that a rigid microtubular network induced by taxol also inhibited transport in dorsal root ganglion neurons (Theiss and Meller, 2000).

We conclude that overexpression of human protein tau had a strong inhibitory effect on the cellular dynamics of adult primary DRG neurons. These effects were probably caused by the competition between tau and motor proteins for microtubule binding, resulting in inhibition of fast axonal transport. Phosphorylation of protein tau by introducing and neuronal coexpression of GSK-3 β , largely neutralized the tau-induced pathological changes, both *in vivo* and in the cells. The effects on fast axonal transport were closely correlated with reorganization of the microtubular system and with the cellular phenotype, and completely in line with the reported data and observations in transgenic mice.

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