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Axonal transport rates *in vivo* are unaltered in htau mice

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Abstract

Microtubule-based axonal transport is believed to become globally disrupted in Alzheimer's disease in part due to alterations of tau expression or phosphorylation. We previously showed that axonal transport rates along retinal ganglion axons are unaffected by deletion of normal mouse tau or by overexpression of wild-type human tau. Here, we report that htau mice expressing 3-fold higher levels of human tau in the absence of mouse tau also display normal fast and slow transport kinetics despite the presence of abnormally hyperphosphorylated tau in some neurons. In addition, markers of slow transport (neurofilament light subunit) and fast transport (snap25) exhibit normal distributions along optic axons of these mice. These studies demonstrate that human tau overexpression, even when associated with a limited degree of tau pathology, does not necessarily impair general axonal transport function *in vivo*. This investigation is contributed for the issue of Journal of Alzheimer's Disease dedicated to the memory of Inge Grunke-Iqbal and to the celebration of her contributions to Alzheimer's disease research.

Keywords

Tau; tauopathy; Alzheimer's disease; neurofilament; slow axonal transport; fast axonal transport

INTRODUCTION

Tau proteins bind to microtubules (MT) and stabilize MT in a polymerized state that is essential for MT-dependent axonal transport of membranous organelles and proteins – a process key for the elaboration and maintenance of axons [1, 2]. Overexpressing tau in fibroblasts induces axon-like processes containing polarized bundles of MTs [3], while knock-down of tau expression with antisense oligonucleotides decreases axonal outgrowth and retracts existing neurites [4].

The abnormal somatodendritic accumulation of tau in neurofibrillary tangles is one of the two hallmark pathologies in Alzheimer's disease (AD) and correlates strongly with the extent of neuronal cell loss [5] and the degree of cognitive decline [6]. Tau levels in brain homogenates from Alzheimer's disease may be elevated Khatoon, 1992 #7}, but this is still controversial [7]. Although there are no known mutations of tau in AD, tau mutations cause

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FTDP-17 and are associated with development of corticobasal degeneration, progressive supranuclear palsy and Pick's disease [1, 8]. These observations unequivocally establish a pathogenic role of tau in neurodegenerative diseases. Interference with MT-dependent axonal transport is one possible mechanism by which altered tau exerts neurotoxicity [9, 10]. High levels of htau40 overexpression in neuroblastoma and primary neurons have been reported to block the trafficking of membranous organelles and neurofilaments, suggesting that rates of fast and slow transport are impaired [11]. However, other studies have shown that monomeric tau at levels up to 20-fold higher than physiological ones do not affect in vitro axonal transport rates in squid axoplasm [12] and 2–4 fold overexpression of wild-type human tau in 8c mice did not affect *in vivo* axonal transport rates in optic pathway [13]. More recent reports suggested pathogenic form of tau filaments instead of monomeric tau inhibit axonal transport in squid axoplasm [14, 15].

Because there is no neuropathology in 8c mice [16] whereas human tau overexpression in the absence of endogenous tau in htau mice induces Alzheimer-like pathology, with hyperhposphorylated tau accumulating as aggregated paired helical filaments in the cell bodies and dendrites of neurons [17]. We investigated the competence of axonal transport *in vivo* in this tauopathy model. We find that pathologically high levels of tau alone are insufficient to impair general axonal transport function *in vivo.* These studies underscore the value of direct analyses of axonal transport *in vivo* to evaluate potential pathogenic influences on transport efficiency.

MATERIALS AND METHODS

Generation of transgenic animals

The htau mice were generated as described before [17], by crossing 8c line [16] with tau knockout mice [18].

Axonal transport studies

The retinal ganglion cells of adult htau and their age-matched wild type controls were radio-labeled by intravitreal injection of [^{35}S]-methionine as previously described [19]. Five hours or up to 21 days post-injection, optic pathways were dissected and processed as described [19]. These experimental protocols were approved by NYU/NKI IACUC Committees under the guidelines of the Institutional Animal Care and Use Committee of the United States.

Tissue preparation, SDS-PAGE, and immunoblot analysis

Retina or each optic pathway segment pooled from 3 mice was homogenized in 125 μl of cytoskeleton extraction buffer and processed as described previously [19]. Total protein extracts from nervous tissues were homogenized in buffer containing 1% SDS and processed as described before [20]. Antibodies used were polyclonal anti-tau antibody LK recognizing only mouse tau and polyclonal anti-tau antibody JM recognizing both human and mouse tau [21].

Electron microscopy and Immunocytochemistry

Mice were anesthetized and retina and optic pathway were processed as described previously [20]. Paraffin-embedded retina was dissected and 6-μm-thick vibratome sections were processed for immunocytochemistry using antibody PHF1 (Ps 396/404) [20].

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RESULTS

Human tau is over-expressed in optic axons and hyperphosphorylated in retinas of htau mice

To determine if human tau is overexpressed in retinal ganglion cells in htau mice, we carried out SDS-PAGE analyses on optic axons and retina from htau and non-transgenic mice. Optic axons and retina have been shown to contain several tau isoforms in the 50–60 kDa (low molecular weight, LMW tau) and 90–95 kDa range (medium molecular weight, MMW tau) [21]. Tau isoforms were identified with polyclonal anti-tau antibodies LK and JM. As shown in Figure 1, htau mice expressed both MMW and LMW human tau, but did not express mouse tau. As expected, htau mice expressed about 3-fold more tau in the optic axons than did non-transgenic mice (Figure 1A, B and graph), consistent with previous findings in the brains [16] and retina ganglion cells / optic axons [13]. The steady-state levels of insoluble and soluble tau in htau retinas were about 2-fold higher than the corresponding levels in wild-type mice (n = 4, Figure 1C, D). The tau in the retina of htau mice also exhibited a partial shift in electrophoretic mobility (Figure 1C, D) consistent with hyperphosphorylation, indicating that the optic system displays aspects of tau pathology previously reported in the brains of the htau mice [17, 22]. Quantitative immunoblotting of retinal proteins with anti-NF-M and snap25 antibodies revealed no alteration in steady-state levels of these molecules (n = 4, Figure 1E, F), indicating no significant accumulation of neurofilament and synaptic vesicles, respectively, in the retinal ganglion cell bodies in retinas of htau mice. Immunocytochemistry using antibody PHF1, a marker for later stage tangles [23] that is specific for phosphorylation at serines 396 and 404 (Ps 396/404) detected higher levels of this tau phosphoepitope in many, but not all retinal ganglion perikarya in htau mouse (Figure 1H) and occasionally, retinal ganglion cell retinas (<1%) displayed exceptionally strong PHF1 labeling. The neuronal perikaryal filaments in this mouse model have been previously shown to contain epitopes (tau serine 202 and serines 396/404) of paired helical filaments [17, 22]. Although most retinal ganglion cells exhibited normal ultrastructure, a number of these neurons corresponding to that of strongly PHF1-positive cells exhibited masses of aggregated filaments (arrows) in the basal portion of the perikaryon, displacing other cytoplasmic organelles to the periphery (Figure 1J). These fibrous inclusions are presumably composed of tau filaments readily distinguishable from neurofilaments because of their larger diameters (15 nm vs 10 nm), lack of sidearms, uniform orientation and variable inter-filament spacing (Figure 1J inset, tau filament). Apart from these occasional fibrous inclusions, there was no evidence of organelle or protein accumulation.

Slow axonal transport of proteins in htau mice

To determine the effect of moderate tau over-expression and hyperphosphorylation on slow transport, we analyzed the rate and cargo composition of slow transport in optic axons from htau and non-transgenic controls after intravitreal injection of ^{35}S-methionine as previously described [20]. At 3, 7 and 14 days after injection, the optic pathways were cut into 1-mm segments, which were fractionated into cytoskeleton and soluble fractions with a Triton X-100–containing buffer. Fractionated proteins were separated on 5–15% SDS-polyacrylamide gels, transferred to nitrocellulose, and visualized by x-ray film and phospho-imaging. As shown in Figure 2, the transport rates of cytoskeletal proteins such as NF-M and tubulin at 3 days after injection were identical in htau mice and their controls (Figure 2E, F). The same result was also obtained at 7 (Figure 2G, H) and 14 days (Figure 2I, J). The calculated peak rates at 14 days were 0.14 mm/day for NF-M and 0.07 mm/day for tubulin in both htau and wild-type mice, consistent with previously published results [19]. The front rate of NFM transport in htau mice was slightly faster than that in wild –type mice (Figure 2E). To determine whether or not the distributions of cytoskeletal proteins at steady-

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state were altered in htau mice, we carried out quantitative immunoblotting of optic axons from htau and control mice using antibodies to NF-L, a protein component of slow axonal transport. The optic pathways were cut into 1-mm segments, separated into cytoskeleton and soluble fractions, and subject to 5–15% SDS-polyacrylamide gels, followed by immunoblot analysis using anti-NF-L antibody (Figure 2K). Quantitative analyses of these immunoblots revealed similar steady-state levels and distributions of NF-L in htau and wild-type mice (Figure 2L).

Fast axonal transport of proteins in htau mice

To further determine whether or not the rate of fast transport is altered by moderate tau overexpression and hyperphosphorylation, pulse-radiolabeling analyses were carried out in htau mice. Fast transport was measured in optic axons of hatu and non-transgenic controls as described above for slow transport, except that mice in fast transport studies were analyzed at 5 hours after isotope injection. As shown in Figure 3, the transport rates of either cytoskeletal and soluble proteins in htau mice were not significantly different from those in wild-type controls. Densitometry of autoradiographs revealed that the rates of three different fast transport cargoes (represented by protein p135, p85 and snap25, n=3, mean ± SD) were not altered in htau mice (Figure 3E–J). The calculated peak and front rates for p135 were 9.6 and > 38.4 mm/day, respectively (Figure 3E, F) while the calculated peak and front rates for p85 were 9.6 and 28.8 mm/day, respectively (Figure 3G, H). The transport wave peak of snap25, the most heavily labeled fast transport protein marker [24] (Figure 3A–D) moved out of the 8mm window at 5hr, indicating a peak rate of more than 38.4 mm/day (Figure 3I). We carried out additional quantitative immunoblotting of snap25 to determine the steady-state distribution of a representative fast transported protein in htau and wild-type mice. Optic pathways cut into 1-mm segments were fractionated into cytoskeleton and soluble fractions, which were separated on 5–15% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained with anti-snap25 (Figure 3K). Quantitative analyses of these immunoblots revealed similar steady-state levels and distributions of snap25 in htau and non-transgenic controls (Figure 3L, mean ± SD, n = 4).

DISCUSSION

We have examined fast and slow axonal transport *in vivo* in mice after replacing mouse tau with human tau expressed at levels moderately higher than endogenous levels and demonstrate that transport rates in CNS axons of mice are not altered by substantial modulations of tau expression. The findings are notable given the further observation that both MMW and LMW tau isoforms [21] were abnormally phosphorylated, as evidenced by slower mobility on SDS gels and increased PHF1 immunoreactivity. Some retinal ganglion cell neurons also showed perikaryal redistribution and aggregation of tau as previously reported in the brain [17, 22].

The results of present studies are consistent with our previous report that axonal transport rates *in vivo* are unaffected by deletion of endogenous tau or moderate overexpression of human tau in mice [13]. These results are also consistent with a recent report that axonal transport rates are not affected by 3–5 fold tau overexpression and hyperphosphorylation in mice measured with Manganese Enhanced MRI [25, 26]. These findings contrast with earlier proposals that tau over-expression impedes axonal transport, which were based on *in vitro* studies of neuronal cells showing massive perikaryal accumulation of organelles after high tau over-expression [11, 27]. Our results are consistent with report that monomeric tau at levels even 20-fold higher than physiological ones did not affect axonal transport rate in squid axoplasm [12]. At levels 50-fold or higher, all tau proteins non-specifically inhibit axonal transport, independent of their microtubule-binding activity [12]. Our findings are also compatible with other reports that neurofilament transport rate is not disturbed in

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transgenic mice overexpressing 8–10 fold R406W mutant human tau over endogenous mouse tau [28]. In one study of 12-month-old T44 tau transgenic mice that over-express the shortest human tau isoform, fast transport rates were slowed in the L5 ventral roots of the spinal cord [29]. However, in a later study by this group, the rate of fast transport in 13-month T44 mice exceeded that previously seen in 12-month-old wild-type mice [30]. Therefore, no conclusive evidence exists for changes in axonal transport rates after tau overexpression *in vivo.*

There are conflicting reports on tau levels in AD brain [7, 31] and soluble tau levels may actually be reduced 38–65% in affected brain regions such as frontal and temporal lobes [7]. More recent studies show that total and phosphorylated tau are increased while non-phosphorylated tau are decreased in AD brain [32]. Our finding that the presence of hyperphosphorylated tau in retinal ganglion cell bodies did not influence rates of either fast or slow transport in optic axons, is consistent with other data showing that cytoskeletal protein hyperphosphorylation in neuronal perikarya is common in stressed cells and is not necessarily a consequence of general transport defects [33].

The accumulation of tau in retinal ganglion cell perykarya in the htau mice suggests that at least a sub-population of tau is impaired in its export after synthesis. Tau itself is transported at a unique slow rate of transport, which on average is somewhat faster than tubulin and slower than other MAPs [21]. The rates of wild-type and mutant tau (various missense mutations in FTDP-17 including R406W) axonal transport in cultured cortical neurons are similar [34] whereas the axonal transport of R406W mutant tau has been shown to be retarded in mice over-expressing the longest human tau isoform with the R406W mutation [28]. Although the transport of mutant or otherwise modified forms of tau may be specifically affected in pathological situations, such alterations of a sub-population of tau do not necessarily alter general axonal transport function. Our data indicate that pretangle accumulation of hyperphosphorylated tau in some neurons is not indicative of a transport rate defect, but do not exclude defective export of a small proportion of tau from some neuronal perikarya. Also, our transport analyses, which are based on the transport behavior in the entire optic axon population, cannot exclude a possibility that transport in a small subpopulation of most affected (>angle bearing) neurons, may be impaired. The absence of organelle accumulations, in any RGCs, however, argues against this possibility. Abnormal intraneuronal accumulation of α-synuclein in Lewy bodies and Lewy neurites caused by familial Parkinson's disease (FPD)-linked mutations has suggested a defect in axonal transport of α-synuclein. In transgenic mice over-expressing FPD-linked mutant human α-synuclein, Lewy body inclusions develop in some spinal motor neurons but the transport rates of slowly transported proteins including α-synuclein, neurofilaments and tubulin are not obviously impaired [35]. Defective axonal transport has been implicated in spinal and bulbar muscular atrophy (SBMA) caused by an abnormal expansion of the CAG repeat in the androgen receptor gene on the X-chromosome, encoding a polyglutamine sequence in the protein product [36]. In a mouse model of SBMA recapitulating many aspects of the human disease, no overt axonal transport deficits were detected [36].

In conclusion, our studies directly demonstrate *in vivo* that axonal transport can proceed unimpeded despite high levels of human tau and the increased phosphorylation of tau species. In contrast to reports of perikaryal accumulations of transport cargoes in neuronal cell cultures with high tau over-expression, we find that pathologically high levels of tau alone in htau mice are insufficient to impair general axonal transport function *in vivo*. Our data do not preclude the possibility that tau aggregation or dysfunction induces neurodegenerative changes in Alzheimer's diseases that secondarily cause axonal transport dysfunction, which is a common consequence of neurodegeneration arising in many neuropathological states [37]. These results underscore the need to perform axonal transport

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in vivo to interpret the influences of local accumulations of protein and/or organelles on axonal transport.

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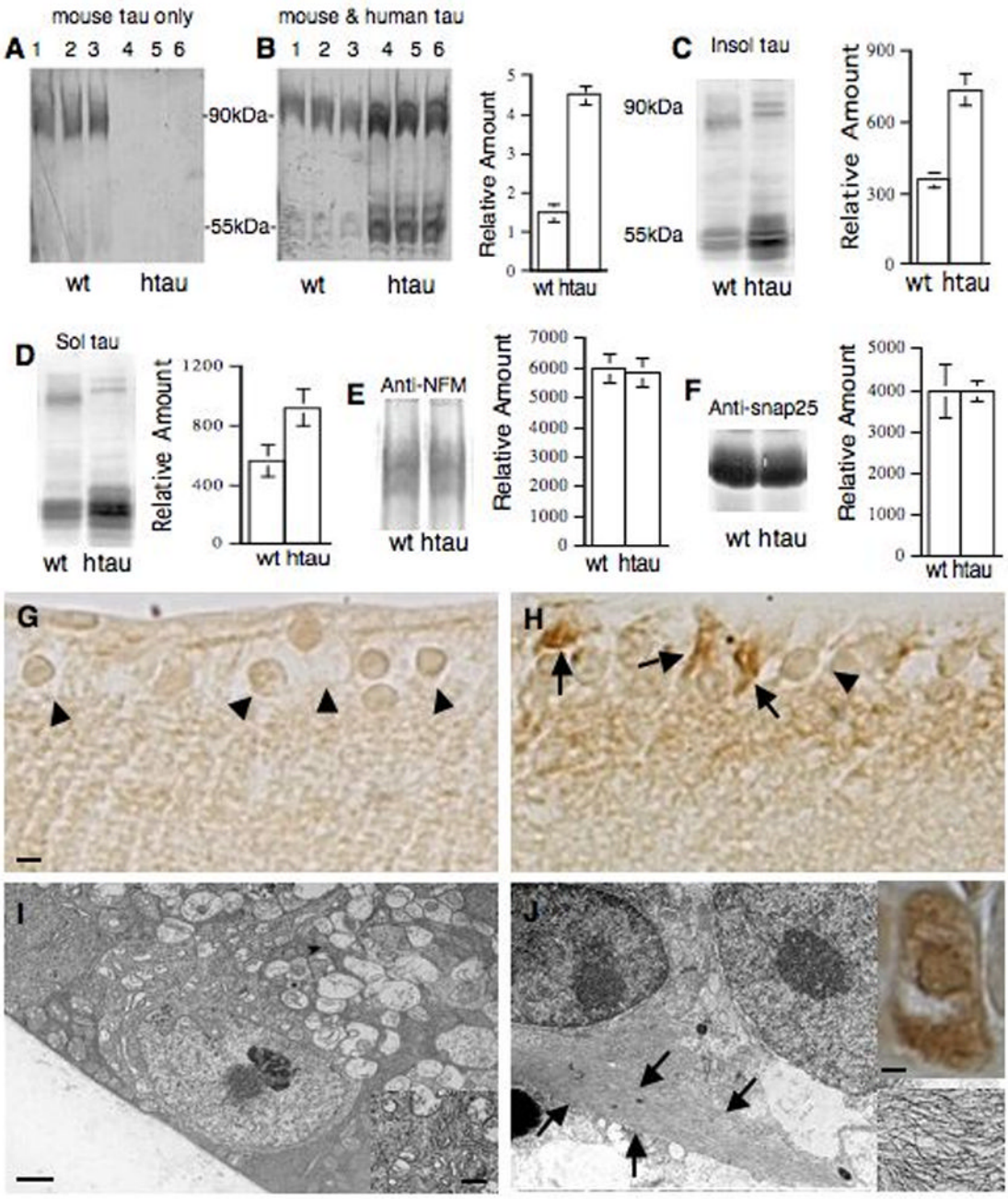

Figure 1.

Human tau over-expression and hyperphosphorylation in htau mice. A, B, Total optic nerve protein (20 μg) from mice after separation on 10% SDS gels were subject to immunoblot analysis with polyclonal anti-tau antibody LK recognizing only mouse tau (A) and polyclonal anti-tau antibody JM recognizing both human and mouse tau (B, C, D). Relative levels of tau isoforms on immunoblots determined by densitometry using NIH imaging were 3-fold higher in htau mice than in nontransgenic mice (B), consistent with elevations in brains from 8c mice. C, D, immunoblots of 100–200 μg of Triton-soluble and Triton-insoluble fractions from mouse retinas showing a partial shift in electrophoretic mobility of tau in htau mice, consistent with hyperphosphorylation. Levels of neurofilament protein (E)

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and snap25 (F) are unaltered. Immunocytochemistry with antibody PHF1 (Ps 396/404) detects accumulated hyperphosphorylated tau (arrows) in some retinal ganglion neurons of htau mice (H), but not in age-matched wild-type mice which exhibit no cytoplasmic staining (arrowheads) and pale non-specific nuclear staining (G). A fibrous inclusion (arrows) containing 15 nm straight filaments is seen in small numbers of retinal ganglion cells of htau mice (J and inset) but not in age-matched wild-type mice (I and inset). These lesions correspond in frequency to the incidence of very strong PHF1-positve neurons (J inset). Scale bars 7μm in G and H, 1μm in I and J, 3μm in J inset (top) and 200 nm in I and J insets (bottom).

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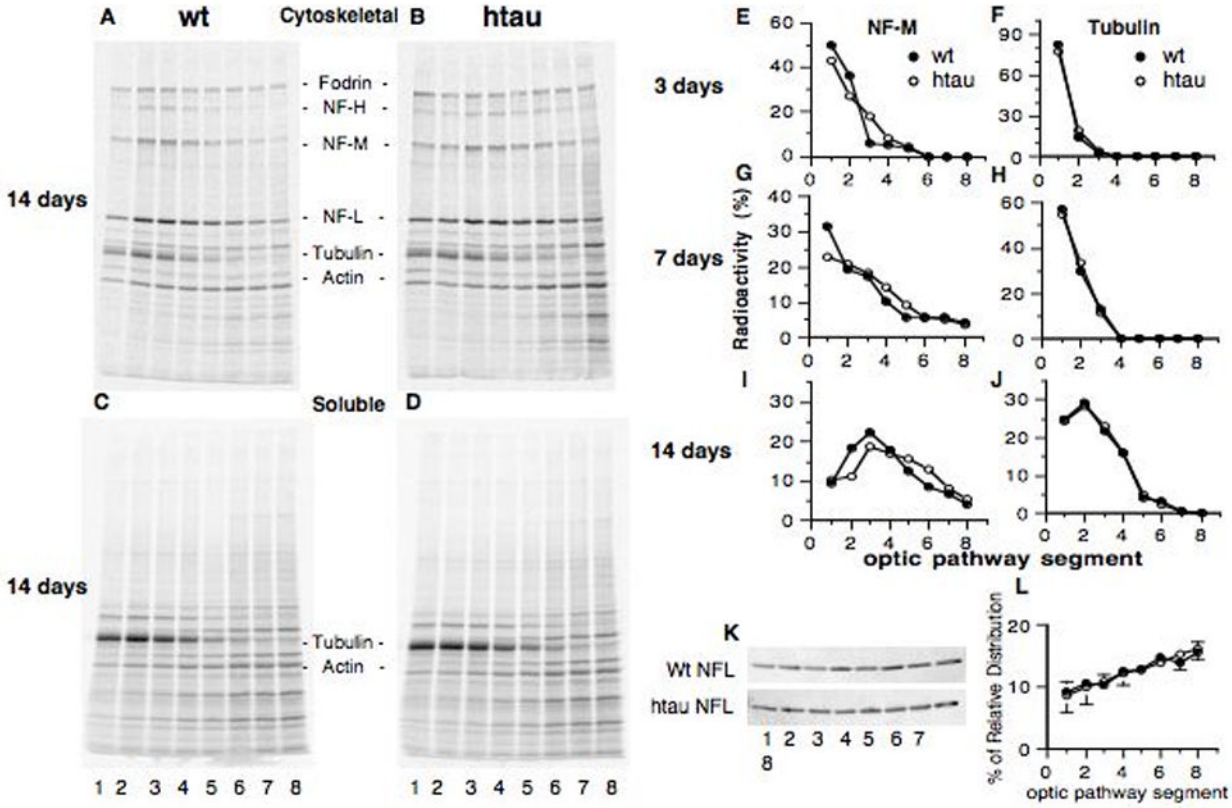

Figure 2.

Slow transport rates are not impaired in htau mice. At 3, 7 or 14 days after intravitreal injection of ^{35}S-methionine into wild-type (A, C) and htau mice (B, D), the optic pathways cut into 1-mm segments were fractionated into cytoskeleton (A, B) and soluble fractions (C, D), which were subject to 5–15% SDS-PAGE gels, transfer to nitrocellulose and autoradiography (A–D, at 14 days). Transport patterns were unaltered for either cytoskeletal or soluble proteins in htau mice. Redistribution of NF-M and tubulin along axons over time was quantified by densitometry scanning of autoradiographs. Graphs showing relative radioactivity of the labeled protein (vertical axis) plotted against distance from the eye displayed by optic segment number (horizontal axis) indicate that transport rates of labeled NF-M and tubulin are not altered in htau mice (E–J). Immunoblot analysis of the steady-state levels of NF-L along optic pathway reveals identical distribution in htau and wild-type mice (K, L, mean ± SD, n=4).

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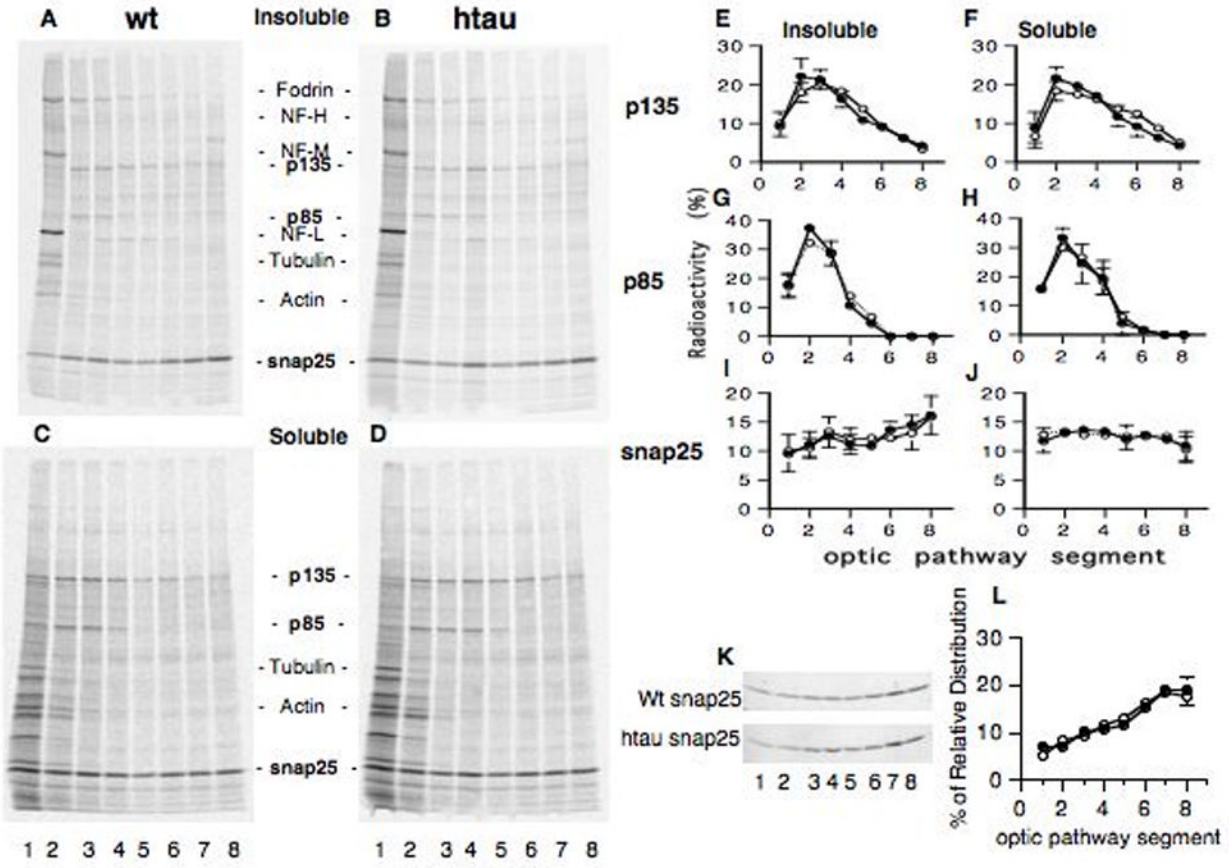

Figure 3.

Fast transport rates in htau mice. Fast axonal transport was measured as described in Figure 2, except the post-injection time was 5 hours (A–D). Fast transport rates determined for protein p135, p85 and snap25 in Triton-soluble and insoluble fractions of optic axons were unaltered in htau mice (E–J). Fast transport marker snap25 determined by quantitative immunoblot analysis showed the same distribution in htau and wild-type mice (K, L). Wild-type and htau mice are represented by filled and unfilled circles, respectively.