Tau Pathology Induces Intraneuronal Cholesterol Accumulation

Frauke Glöckner, MD and Thomas G. Ohm, MD, PhD

Abstract

Epidemiologic and experimental data suggest the involvement of cholesterol metabolism in the development and progression of Alzheimer disease and Niemann-Pick type C disease, but not of frontotemporal dementias. In these 3 neurodegenerative diseases, however, protein tau hyperphosphorylation and aggregation into neurofibrillary tangles are observed. To elucidate the relationship between cholesterol and tau, we compared sterol levels of neurons burdened with neurofibrillary tangles with those of their unaffected neighbors using semiquantitative filipin fluorescence microscopy in mice expressing P301L mutant human tau (a well-described model of FTDP-17) and in P301L transgenic mice lacking apolipoprotein E (the major cholesterol transporter in the brain). Cellular unesterified cholesterol was higher in neurons affected by tau pathology irrespective of apolipoprotein E deficiency. This argues for an impact of tau pathology on cellular cholesterol homeostasis. We suggest that there is a bidirectional mode of action: Disturbances in cellular cholesterol metabolism may promote tau pathology, but tau pathology may also alter neuronal cholesterol homeostasis; once it is established, a vicious cycle may promote neurofibrillary tangle formation.

Key Words: Cholesterol, Filipin, Neurofibrillary tangle, Tau, Tauopathy.

INTRODUCTION

In the adult brain, protein tau is described as an axonal protein that stabilizes microtubules via its assembly domain (1). Tau was also found to modulate kinesin axonal transport (2); it interacts with actin via its proline-rich domain, which contains the AT8 and AT100 epitopes (3). Tau may serve as a cross-linker between microtubule and actin networks, thereby impacting remodeling; however, under pathologic conditions, this connection may also promote axonal clogging (4). The projection domain of tau also mediates its association with cellular membranes in a phosphorylation-dependent manner (5). In particular, the localization of tau within lipid rafts and its association with nonreceptor Src family protein tyrosine kinases are related to cell signaling and intracellular targeting of proteins (6).

Tau is a phosphoprotein with many phosphorylation sites, not only of serine and threonine but also of tyrosine. Hyperphosphorylation of tau (which is a common feature of tauopathies) is associated with detachment from tubulin, sorting into the somatodendritic compartment of neurons, and tau filament formation (7). Tau hyperphosphorylation also occurs during development, suggesting its involvement in cell morphogenesis and plasticity. The metabolic disturbances underlying pathologic tau hyperphosphorylation and formation of neurofibrillary tangles (NFTs) in neurodegenerative diseases are not understood.

Since the 1980s, disturbances in cholesterol metabolism have been implicated in the pathogenesis of Alzheimer disease (AD), although initially more as a cardiovascular risk factor. Dysregulation of neuronal cholesterol homeostasis, however, is now linked in its own right to neurodegenerative disorders. Indeed, impairment of the intracellular transport of cholesterol, which results in its trapping in the lysosomal compartment, is the underlying defect in Niemann-Pick type C disease (NPC), and neurofibrillary changes similar to those seen in AD occur in NPC without amyloid deposition (8, 9). The abnormalities in cholesterol metabolism in NPC suggest that intraneuronal unesterified cholesterol accumulation is the primary event that influences NFT formation by unknown mechanisms. Remarkably, tangle-bearing neurons have been shown to contain higher levels of unesterified cholesterol than tangle-free neighboring neurons in NPC (10) and in the brains of patients with AD (11). Furthermore, NFTs purified from the brains of patients with AD were found to contain cholesterol (12), but the relationship between cholesterol accumulation and development of NFTs is not understood.

In contrast to AD, hypercholesterolemia has not been directly implicated in frontotemporal dementias (FTLDs). A subset of FTLD (e.g. FTLD and parkinsonism linked to chromosome 17 [FTDP-17]) is linked to tau genetic variants (13). The major genetic risk factor for sporadic AD—polymorphism of the apolipoprotein E gene (apoE) (protein, APOE)—has been studied in FTLDs, but results have been variable. Somewhat in contrast to the findings in AD, an association with apoE2 allele has been reported (14). The AD-protective effect of the apoE2 allele has to be differentiated, however, because in an autopsy-based study, very old individuals with an apoE2 allele showed higher NFT Braak stages than apoE3 allele controls (15).

To further investigate the relationship between cholesterol metabolism and tau pathology, we compared sterol levels of neurons burdened with neurofibrillary changes with those of their unaffected neighbor neurons in an established mouse line expressing P301L mutant human tau (htau-apoe2) and in...
FIGURE 1. Illustration of perikarya identification, outlining, and fluorescence intensity measurement methods. (A) AT8-IR (secondary system Alexa Fluor 594, 560 nm excitation, BA590 filter) reveals a tangle-bearing neuron. (B) Filipin staining of the same field (360 nm excitation, MBA510 filter) shows weakly fluorescent perikarya surrounded by a strongly fluorescent neuropil. (C) The AT8-positive neuron (AT8pos) was marked in the filipin picture (Photoshop; Adobe Systems Inc, San Jose, CA). (D) The adjacent AT8-negative neuron (AT8neg1) and the next adjacent neuron (AT8neg2) were then marked in the filipin picture. (E) A second investigator who was unaware of the AT8 immunohistochemical status of the marked perikarya outlined them on the original filipin picture files with ImageJ, a Java-based image processing program (free NIH software; National Institutes of Health, Bethesda, MD). The mean intensity of outlined areas was determined in counts per pixel. (F) In the last step, the marked neurons were reidentified as AT8pos, AT8neg1, or AT8neg2, respectively, from the merged picture. The same approach was chosen for confocal analysis. Scale bar = 100 μm.
P301L apoe knockout mice (htau-apoe<sup>−/−</sup>). The P301L mutation renders tau more susceptible to hyperphosphorylation and aggregation. The P301L htau-apoe<sup>1/+</sup> mouse develops age-dependent NFTs that are structurally identical to those found in AD and FTLDs (16, 17). Tau pathology in these animals is also exacerbated by APOE deficiency or administration of a cholesterol-enriched diet (18). APOE deficiency and/or administration of a cholesterol-enriched diet increased peripheral cholesterol levels in these animals without changes in total brain cholesterol (i.e. frontal lobe total cholesterol determined by gas chromatography–flame ionization detection [18] and unpublished data). In the present study, using semiquantitative filipin fluorescence microscopy, we found higher levels of cellular unesterified cholesterol in neurons afflicted with NFTs in P301L htau-apoe<sup>1/+</sup> mice and that this effect was independent of APOE deficiency.

MATERIALS AND METHODS

Animals

Transgenic mice expressing the longest human 4-repeat tau isoform with the human pathogenic mutation P301L (kindly provided by Götz et al [16]) and apoe knockout mice (B6.129P2-Apoe<sup>−/−</sup>Unc, JAX mice; Jackson Laboratory, Bar Harbor, ME) were cross-bred as described previously (18). Two experimental lines (i.e. P301Lhtau apoe wild type [htau-apoe<sup>+/+</sup>] and P301Lhtau apoe knockout [htau-apoe<sup>−/−</sup>]) were established with their respective controls without the transgene tau. Mice were kept on a standard rodent chow with 12-hour light/dark cycle. Twelve 12-month-old male mice (7 htau-apoe<sup>+/+</sup> and 5 htau-apoe<sup>−/−</sup>) and four 6-month-old male htau-apoe<sup>+/+</sup> mice were used in this study. 12-month-old apoe knockout mouse and one 12-month-old wild-type mouse without P301L human tau were used as controls. By age 6 months, neurofibrillary pathology is about to begin in P301L htau mice; by 12 months, Gallyas-positive NFTs are consistently detectable (16). The experimental protocol followed international guidelines and the German animal protection law (Deutsches Tierschutzgesetz).

Histology and Immunohistochemistry

Brains were formalin-fixed for at least 3 months. Coronal sections 40 and 80 μm thick were consecutively cut, using a vibratome, from 7 regions as follows: 1) frontal/orbital cortex; 2) anterior commissure; 3) ventral hippocampus (Hc); 4) intermediate part (level of posterior commissure); 5) dorsal Hc; 6) entorhinal cortex; and 7) brainstem/cerebellum. The Paxinos-Franklin mouse atlas was used for topographic orientation (19).

Gallyas silver impregnation was performed on 80-μm sections from each region, as previously described, to ensure neurofibrillary pathology (9). Filipin staining was performed on 40-μm-thick sections from dorsal Hc, as described previously (11). Filipin complex (Sigma, Taufkirchen, Germany) was solubilized in dimethylformamide (1 mg of filipin per 100 μL). Free-floating slices were incubated for 3 hours in the dark at room temperature with 50 μg of solubilized filipin per milliliter of phosphate buffer (pH 7.4) and 3% normal goat serum as blocking reagent for subsequent immunohistochemistry.

Filipin-stained sections were incubated overnight with AT8 or AT100 antibody (both 1:1000 phosphate buffer/1% normal goat serum, mouse monoclonal; Pierce Biotechnology, Rockford, IL) (20). Alexa 568- or Alexa 594–conjugated goat anti-mouse antibody (1:250 phosphate buffer; Molecular Probes, Leiden, the Netherlands) was used as secondary antibody. All steps were performed under strict protection from light. Phosphate buffer was used for all washing steps. AT8/AT100 immunohistochemistry was also performed without prior filipin staining and with primary antibody omitted as controls. Animals and samples of different groups were always handled together to minimize technical bias.

Confocal Microscopy

Three 12-month-old htau-apoe<sup>1/+</sup> mice and four 6-month-old htau-apoe<sup>−/−</sup> mice were subjected to confocal evaluation of filipin intensities. Forty-micrometer-thick sections were evaluated in a Zeiss confocal microscope (LSM 5 Exciter). Imaging of AT8- or AT100-immunonegative neurons, respectively, including the surrounding area, was performed sequentially using a 543 laser line (Alexa 568; red) and a 405 laser line (filipin, UV). Negative controls did not show any confounding fluorescence at either channel. Intensities of confocal fluorescent signals were scored across each profile using ZEN2008 software (Zeiss).

Fluorescence Microscopy

Five 12-month-old male mice of each genotype were subjected to quantitative fluorescence measurement. An inverted fluorescence microscope (Olympus IX70; Olympus Corporation, Tokyo, Japan) attached to a monochromator setup (Till Photonics, Gräfelfing, Germany) was used. Micrographs were taken with a charge-coupled device camera and adjusted with Fucal software (Till Photonics) as previously described (11). For Alexa 594, 560 nm excitation and a BA590 filter were selected (AT8 picture; Fig. 1A); for filipin, 360 nm excitation and an MBA510 filter were selected (filipin; Fig. 1B). Pictures of AT8-immunonegative (AT8pos) neurons, including the surrounding area, were taken using both wavelengths. Negative controls did not show any confounding fluorescence at either channel. A detailed description of the analysis is given in Figure 1.

Analysis

Neuronal perikarya were easily identified in filipin images (Fig. 1B). Neurons were selected from 3 hippocampal subfields: dentate gyrus (Dg), CA3, and CA1 from dorsal Hc. No absolute fluorescence intensity quantification was attempted because filipin fluorescence in microscopy is dependent on too many variables; this also prevented a direct comparison between the genotype groups. By comparing only closely associated neurons within the same image, we minimized potential microenvironmental differences. Therefore, 2 ratios were computed from the measured filipin fluorescence (area × intensity) and compared as follows: AT8pos/AT8neg1 and AT8neg2/AT8neg1 (AT100pos/AT100neg1 and AT100neg2/AT100neg1, respectively). Ratios were computed from the respective areas only, and pairs with an area difference of more than 20% were excluded. Care was taken to compare neurons with the same morphology because a smaller neuron might have represented
FIGURE 2. Confocal evaluation. Histograms of test (gray) and reference (white) ratios from hippocampal area CA1 of 4 younger htau-apoe<sup>+/+</sup> mice (sixth month of life [MOL]) (A) and 3 older htau-apoe<sup>+/+</sup> mice (12 MOL) (B, C) on filipin fluorescence in relation to AT8-IR (A, B) and AT100-IR (C). Confocal micrographs of AT8 (D) or AT100 (E) fluorescence, corresponding filipin images (F, G), and merged signals (H, I). Scale bar = 100 μm. AT8/AT100neg/pos1, AT8/AT100neg/pos2, AT8/AT100-negative/positive neurons.
a perikaryon outside of the plane, therefore biasing the fluorescence measurement.

Statistics

Data were analyzed with BiAS for Windows version 8.3 (21). Test ratios (AT8pos/AT8neg1 or AT100pos/AT100neg1) and corresponding control ratios (AT8neg2/AT8neg1 or AT100neg2/AT100neg1) were compared with Wilcoxon signed rank test. Distributions of the 2 ratios were also analyzed. Mann-Whitney-Wilcoxon U test was used to compare differences between the 2 genotype groups (htau-apoe +/+ vs htau-apoe −/−). Level of significance was set p = 0.05 for all statistical tests.

RESULTS

Gallyas Silver Impregnation

Gallyas staining was performed on 7 brain regions to ensure the presence of neurofibrillary pathology in the mice used. In 6-month-old mice, no Gallyas-positive structures were detectable. All 12-month-old htau mice (7 htau-apoe +/+ and 5 htau-apoe −/−) showed some neurofibrillary changes on Gallyas silver impregnation, but the htau mice deficient in APOE had more intense staining, with numerous positive neurons in the amygdala-piriform transition area and in the Hc, and occasional positive neurons in isocortical areas. Htau-apoe −/− mice showed higher total numbers of Gallyas-positive neurons (median, 181; range, 115–468) than htau-apoe +/+ mice (median, 75; range, 0–185; Mann-Whitney-Wilcoxon U test, not significant).

The most intense Gallyas staining was observed in the amygdalae, particularly in the basolateral complex, also in htau-apoe +/+ mice. The 12-month-old control mice (apoe knockout and wild type) without the P301L transgene construct did not show any silver impregnation. Gallyas staining mirrored the distribution and intensity of immunoreactivity (IR) of the phosphorylation-dependent anti-tau antibodies AT8 and the conformation-dependent anti-tau antibody MC1, as previously described in these mouse lines (18). Significance (htau-apoe −/− vs htau-apoe +/+ ) was only reached for the isocortical areas in this study but not within the hippocampal subdivisions, probably owing to sample size (one slice per region for Gallyas staining).

Confocal Evaluation

The distribution of AT8-IR also followed the same pattern as previously described (18). AT100-IR also mirrored that distribution, but fewer neurons were AT100-positive in 12-month-old htau-apoe +/+ mice (n = 3). In 6-month-old mice (n = 4), AT8-IR was much less pronounced, and AT8-positive neurons were only consistently found in CA1. About twice as many slices as in 12-month-old mice were used for analysis to find a sufficient number of positive neurons for statistical evaluation. One mouse rarely showed any AT8-positive neurons. In the mouse with the most pronounced AT8-IR, a few AT8-positive granule cells were found in the Dg. Only a few AT100-positive neurons were found in 2 of the 6-month-old htau-apoe +/+ mice and were not sufficiently numerous for statistical analysis. Detailed analysis was carried out in the dorsal Hc. The Dg and CA3 and CA1 subdivisions of the Hc proper were chosen as distinct divisions; neurons were selected from the fascia dentata (granule cells) and from the pyramidal cell layer of the Hc proper (CA3 and CA1 pyramidal neurons, respectively). All AT8- and AT100-immunoreactive neurons found within one slide were included in the analysis. To identify the relationship between AT8-IR and filipin staining, we analyzed 32 sets of pyramidal cells from CA1, 11 sets of pyramidal cells from CA3, and 21 sets of granule cells from the Dg in 12-month-old mice. In 6-month-old mice, we analyzed 53 sets of pyramidal cells from CA1. For evaluation of filipin intensity in AT100-positive neurons in 12-month-old mice, 23 sets of pyramidal cells were analyzed from CA1, 16 sets of pyramidal cells were analyzed from CA3, and 27 sets of granule cells were analyzed from the Dg. Chi-squared test showed deviations from a parametric distribution in some groups. Therefore, nonparametric tests and median descriptions were used for further analysis.

In 12-month-old mice, the median AT8pos/AT8neg1 ratio was 1.12, and the median AT100pos/AT100neg1 ratio was 1.3 in CA1, 1.21 (AT8) and 1.22 (AT100) in CA3, and 1.17 (AT8) and 1.15 (AT100) in the Dg. In 6-month-old mice, AT8-positive neurons were found only in CA1 (median AT8pos/AT8neg1 ratio, 1.13). The median AT8pos/AT8neg1 or AT100pos/AT100neg1 ratios were significantly higher than the respective AT8neg2/AT8neg1 or AT100neg2/AT100neg1 control ratios (Wilcoxon signed rank test, p < 0.01 for all regions and both antibodies). The test ratios tended to be higher in the AT100 study compared with the findings with the AT8 antibody (Wilcoxon-Mann-Whitney U test, p < 0.01 in CA1, not significant in CA3 and Dg), whereas no differences were found in the distribution of control ratios. The distribution of test ratios seemed to be rather narrow in younger mice compared with old mice, but this was not statistically significant (Wilcoxon-Mann-Whitney U test). Distribution of test ratios and corresponding control ratios from the CA1 region and representative images of both stainings are shown in Figure 2.

Filipin Staining and AT8 Immunohistochemistry

To ensure comparability of the results with our previous work (10, 11), we used traditional fluorescence microscopy in a more extended study covering both APOE wild-type mice and APOE-deficient mice. As for Gallyas silver impregnation, AT8-IR was much more intense in htau-apoe +/+ than in htau-apoe −/− mice. The distribution of AT8-IR followed the same pattern as previously described (18). No AT8-IR was seen in apoe knockout and wild-type mice without P301L human tau. As with confocal evaluation, detailed analysis was performed in the dorsal Hc with the same subdivisions (CA1, CA3, and Dg). If possible, all AT8-immunoreactive neurons found within a single slide were included in the analysis. In htau-apoe −/− mice, however, there was such intense AT8-IR, particularly in CA1, that sometimes no reference neurons could be defined. Altogether, 63 sets (htau-apoe +/+ ) and 37 sets (htau-apoe −/−) of pyramidal cells were analyzed from CA1, 44 sets (htau-apoe +/+ ) and 79 sets (htau-apoe −/−) of pyramidal cells were analyzed from CA3, and 60 sets (htau-apoe +/+ ) and 44 sets (htau-apoe −/−) of granule cells were analyzed from the Dg.
FIGURE 3. Histograms of test (gray) and reference (white) ratios of 5 htau-apoe\textsuperscript{+/-} mice (A, C, E) and 5 htau-apoe\textsuperscript{-/-} mice (B, D, F) from 3 hippocampal areas: CA1 pyramidal layer (A, B), CA3 pyramidal layer (C, D), and Dg granule cell layer (E, F). The number of perikarya pairs reflects the total number of AT8-positive neurons, except in area CA1 in htau-apoe\textsuperscript{-/-} mice (B), where there were too many AT8-positive neurons to define reference neurons.
The 3 regions were also analyzed in control animals. The filipin fluorescence ratios of 2 neighboring neurons fluctuated around 1, irrespective of apoe genotype. The ranges were as follows: CA1, 0.88 to 1.15 (n = 209); CA3, 0.89 to 1.12 (n = 97); Dg, 0.88 to 1.17 (n = 240).

In P301Lhtau mice, the ratios between 2 AT8-negative neurons within the same region and layer also fluctuated around 1 in both genotype groups. In contrast, in every single case, the mean test ratio (AT8pos/AT8neg1) was higher than 1, irrespective of hippocampal subdivision and APOE deficiency. Deviations from a parametric distribution of ratios were found. Thus, nonparametric tests and median descriptions were used for further analysis. The median AT8pos/AT8neg1 ratios were 1.06 (htau-apoe+/+) and 1.07 (htau-apoe−/−) in CA1, 1.055 (htau-apoe+/+) and 1.05 (htau-apoe−/−) in CA3, and 1.06 (htau-apoe+/+) and 1.065 (htau-apoe−/−) in the Dg. The median AT8pos/AT8neg1 ratios were significantly higher than the respective AT8neg2/AT8neg1 control ratios (Wilcoxon signed rank test, p < 0.0001 for all regions and genotype groups). Distribution of test ratios and corresponding control ratios are shown in Figure 3. Comparison between the 2 genotype groups revealed no significant differences in the distribution of test or control ratios.

Care was taken to compare neurons of the same morphology and size situated close together within the same region. Occasionally, AT8-positive ballooned neurons were found. These cells were located in the amygdala-piriform transition area and in the entorhinal cortex and, in some cases, also along the border of stratum oriens and the pyramidal cell layer. These AT8-positive ballooned neurons always showed intense filipin staining. Because no reference neurons could be defined, swollen neurons were not included in the analysis.

Some AT8-positive neurons (particularly at the border of stratum oriens and, in CA3, next to the fimbria of the He) showed very intense filipin staining and were hardly distinguishable from the neuropil in the filipin picture. Those neurons were not included. The presence of these cells points to a second distribution with considerably higher filipin ratios, as described by Distl et al (11) in human brains.

**DISCUSSION**

This study demonstrates a direct impact of tau pathology on cellular cholesterol homeostasis in vivo. In the past, our group provided evidence that, in 2 distinct tauopathies, tangle-bearing neurons contain more unesterified cholesterol than their tangle-free neighbors (10, 11). Both AD and NPC are epidemiologically and/or genetically linked to cholesterol metabolism, but with different etiologies. In NPC, cellular handling of cholesterol is a primary disturbance. Although it is not a component of NPC, hypercholesterolemia is discussed as a risk factor for AD. The major genetic risk factor linked to sporadic AD—possession of an apolipoprotein E4 allele—also points to the pathogenic involvement of cholesterol at the systemic and cellular levels. Interestingly, the apolipoprotein E4 allele has also been suggested to play a promoting role in the pathogenesis of tau pathology in NPC (22, 23). Together, these findings fit well into current concepts suggesting a direct relationship between disturbed cholesterol metabolism and tau pathology.

Here, we provide evidence that neurons hampered by neurofibrillary changes similarly contain more unesterified cholesterol than their unaffected neighbors but in a tauopathy mouse model without any alterations in cholesterol homeostasis. We found an increase in intracellular unesterified sterols in all AT8- and AT100-positive neurons irrespective of hippocampal area and cell type. The AT100 epitope is highly specific for phosphorylated tau in AD paired helical filaments and seemed to depend on prior phosphorylation of the AT8 epitope (24). The difference between test and control filipin ratios seemed to be higher in the AT100 study. This finding, as well as the finding of some AT8-positive neurons with a very intense filipin staining (not included in the quantitation), suggests that progress of neurofibrillary changes is accompanied by intraneuronal cholesterol accumulation. This also suggests that there is a pathogenetic relationship between tau hyperphosphorylation and altered cholesterol homeostasis.

We found a similar shift in the distribution of filipin ratios in P301Lhtau apoe knockout mice. These mice have disturbed cholesterol metabolism, with the hypercholesterolemia and atherosclerosis of apoe knockout mice. APOE is the major cholesterol transporter in the brain; however, despite the well-described profound effects on circulating sterols, data on brain cholesterol in APOE-deficient mice are less abundant and ambiguous (25–27). No direct comparison of filipin intensities between the 2 genotypes is feasible in a microscopic study, but there was no difference in the magnitude of filipin ratios between the 2 genotypes that we investigated.

Biosynthesis and receptor-mediated uptake are alternative sources of cellular cholesterol supply. Import of glia-derived cholesterol via lipoproteins has been shown to be essential for synaptogenesis (28), although neurons synthesize cholesterol and also traffic sterols along their neurites. Cholesterol trafficking within the cell involves transfer between different subcellular compartments (29), and cholesterol uptake and de novo synthesis interact. Actin filaments and microtubules are involved in this process, particularly in endosome movement (30). Although no pathologic phenotype is observed in tau knockout mice, double-mutant NPC-deficient mice lacking tau show a more severe phenotype (31), suggesting a role of tau in vesicular shuttling (32). Much attention has been paid recently to the binding of tau to cellular membranes, especially its association with lipid rafts (33). Plasma membrane rafts are involved in cholesterol biosynthesis (34). An exchange of precursor sterols, de novo synthesized cholesterol, and endocytosed sterols seems likely, and rafts may be involved as cholesterol-enriched membrane domains. On the other hand, disruption of membrane rafts caused by cholesterol depletion led to perturbations of membrane raft–associated cytoskeleton proteins in neurons; tau was particularly sensitive to this (35). Also, reduced raft cholesterol has been found to promote tau hyperphosphorylation in NPC-deficient cells (36) and may thus promote microtubule disassembling. Taken together, there are numerous interaction points where tau may be involved in intraneuronal sterol shuttling beyond its regulating role in kinesin transport along microtubules. Under pathologic conditions of tau filament formation, missorting into the somatodendritic...
compartments. Because tau filaments are still very dynamic (37), new interactions may also occur.

No conclusion can be drawn as to whether cholesterol uptake via the endosomal compartments or cholesterol biosynthesis accounts more, or maybe exclusively, for the present data. With respect to the finding in P301L htau apoE knockout mice, trapping of cholesterol in the endosomal-lysosomal compartment, as in NPC, seems unlikely because receptor-mediated lipid uptake should be compromised in APOE-deficient mice. Indeed, insufficient supply of glial lipoproteins has been implicated in impaired synaptic remodeling and poor outcome in response to acute and chronic brain injuries in apoE knockout mice (38). Besides its role in receptor-mediated lipid uptake, APOE may modulate intracellular lipid metabolism (39). Changes in the transbilayer distribution of cholesterol in brain synaptic plasma membranes were found in APOE-deficient mice, with an increase in the exofacial leaflet compared with wild type (40). Thus, APOE deficiency may influence raft composition, triggering and accelerating tau pathology.

The apoE knockout mice showed more pronounced tau pathology than the apoE wild-type mice, as shown by AT8 immunohistochemistry and Gallyas staining. Why APOE deficiency promotes tau phosphorylation and aggregation is not clear, and there is evidence that mechanisms other than its pure lipid-shuttling role might be involved. An isoform-dependent impact on the activity of glycogen synthase kinase has been found in a cell culture study applying (lipid-free) human recombinant apoE isoforms (41). A biologically active APOE synthetic peptide derived from the receptor binding domain has been shown to influence several signaling pathways and to induce dephosphorylation of tau (42). Thus, APOE deficiency could result in an altered net balance of tau kinases and phosphatases that promote tau phosphorylation independently of the disturbed cholesterol metabolism found in these animals. On the other hand, because it is the major cholesterol transporter in the brain, APOE likely affects neuronal cholesterol metabolism, and it may be difficult to separate its influence on cell signaling processes in vivo from its effects on lipid uptake.

The data presented here add to our present knowledge the first experimental evidence that tau filament formation elevates intraneuronal unesterified cholesterol. With respect to the role of cholesterol in tauopathies, we propose a bidirectional mode of action (i.e., a disturbed cellular cholesterol metabolism may promote tau pathology, but tau filament formation disturbs cellular traffic and can also alter cholesterol homeostasis). Both mechanisms in one cell may trigger a vicious circle. Although the molecular mechanisms may differ under various conditions (i.e., AD, NPC, and the P301L htau- apoE<sup>−/−</sup> mouse model), the common result is that tangle-bearing neurons contain more unesterified cholesterol than their tangle-free neighbors. A mechanistic link showing how that circle may form between changes in tau and cholesterol may relate to lysosomal and autophagosomal functions. Histopathologic alterations suggesting impairment in the endosomal-lysosomal system are among the earliest alterations in the brains of patients with AD (43, 44). In NPC1, the autophagic flux is hampered at the level of lysosomal degradation (45). The impaired function of NPC1-related autophagolysosomal degradation is rectified after cholesterol removal using cyclodextrin (45, 46). This suggests that lipid accumulation may lead to lysosomal impairment, which may then impair degradation of tau aggregates. In turn, hyperphosphorylated tau and tau aggregates are known to impair transport of vesicles, and impaired tau function may promote lipid loading in NPC (31, 47). Thus, accumulating autophagosomes and lipophagosomes may contribute to lipid loading. Under a genetic condition such as the P301L htau- apoE<sup>−/−</sup> mouse model, tau aggregation is induced by the mutated tau and associated with impaired microtubule-mediated transport (48).

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