Neurodegeneration in Heterozygous Niemann-Pick Type C1 (NPC1) Mouse

IMPLICATION OF HETEROZYGOUS NPC1 MUTATIONS BEING A RISK FOR TAUOPATHY*

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Niemann-Pick type C1 (NPC1) disease is an autosomal recessive, fatal disorder characterized by a defect in cholesterol trafficking and progressive neurodegeneration. The disease is predominantly caused by mutations in the NPC1 gene; however, it has been assumed that heterozygous NPC1 mutations do not cause any symptoms. Here we demonstrate that cholesterol accumulation does not occur in young mouse brains; however, it does in aged (104–106-week-old) NPC1+/− mouse brains. In addition, Purkinje cell loss was observed in aged NPC1+/− mouse cerebellums. Immunoblot analysis using anti-phospho-tau antibodies (AT-8, AT-100, AT-180, AT-270, PHF-1, and SMI-31) demonstrates the site-specific phosphorylation of tau at Ser-199, Ser-202, Ser-212, Thr-214, and Thr-214 in the brains of aged NPC1+/− mice. Mitogen-activated protein kinase, a potential serine kinase known to phosphorylate tau, was activated, whereas other serine kinases, including glycogen synthase kinase 3β, cyclin-dependent kinase 5, or stress-activated protein kinase/c-Jun N-terminal kinase were not activated. Cholesterol level in the lipid raft isolated from the cerebral cortices, ATP level, and ATP synthase activity in the cerebral cortices significantly decreased in the aged NPC1+/− brains compared with those in the NPC1+/+ brains. All of these changes observed in NPC1+/− brains were determined to be associated with aging and were not observed in the age-matched NPC1+/+ brains. These results clearly demonstrate that heterozygous NPC1 mutations impair neuronal functions and causes neurodegeneration in aged mouse brains, suggesting that human heterozygous NPC1 mutations may be a risk factor for neurodegenerative disorders, such as tauopathy, in the aged population.

Niemann-Pick type C1 (NPC1) disease is an autosomal recessive disorder characterized by progressive neurodegeneration including ataxia, dystonia, seizures, and dementia (1). In NPC1-deficient cells, exogenously transported and endogenously synthesized cholesterol accumulate in late endosome/lysosomes, with delayed cholesterol transport to cellular compartments responsible for the regulation of intracellular cholesterol homeostasis (2–5). In addition to cholesterol, glyco-sphingolipids and other lipids accumulate in homozygous NPC1 brains with aging (1, 6). The gene responsible for NPC1 disease, NPC1, was cloned in both humans and mice (7, 8). It was previously demonstrated that NPC1 plays a key role in the transport of cholesterol to the trans-Golgi network, plasma membrane, and endoplasmic reticulum (9–12).

The neuropathological features of NPC1 brains are characterized by the loss of neurons such as Purkinje cells, hyperphosphorylated tau, and neurofibrillar tangle formation with the accumulation of lipid storage bodies and the presence of dendritic and axonal abnormalities (6, 13–16). These lines of evidence suggest that the intracellular accumulation of cholesterol and gangliosides correlates with the progression of NPC1 disease and induces neurodegeneration. However, there is a different viewpoint on the pathogenesis of NPC1 disease; i.e. the continuous defective use of cholesterol in NPC1 neural tissues causes tauopathy (6, 16, 17). This notion is supported by previous studies showing that there is cholesterol deficiency in cellular compartments including distal axons (18), although cholesterol accumulation occurs in the late endosome/lysosomal compartment (4, 5) due to a defect in the transport of cholesterol, the source of which is either endogenous synthesis or exogenous uptake.

With respect to neuronal gangliosides, a previous study supports the notion that the accumulation of gangliosides causes neurodegeneration (19), whereas another study indicates that the accumulation of gangliosides is not the cause of neurodegeneration in NPC1 mice (20). A recent study has demonstrated that impaired neurosteroidogenesis, due to disordered cholesterol trafficking, affects neuronal growth and differentiation and that allopregnanolone treatment delays the onset of neurological symptoms and lengthens the life of NPC1 mice (21).

Our recent study has shown a novel mechanism underlying neurodegeneration in NPC1 disease, whereby an increased cholesterol level in mitochondrial membranes adversely affects mitochondrial membrane potential, the synthesis of ATP, and the level of cellular ATP in NPC1 mouse brains and neurons (22). Since mitochondria are a key organelle for steroidogene-sis, mitochondrial dysfunction may responsible for impaired neurosteroidogenesis. In addition, this also indicates that not whole-cell cholesterol level but rather compartment- or organelle-associated cholesterol level is critical for maintaining neuronal functions.
Note that NPC1 disease is an autosomal recessive disease, and heterozygous carriers of NPC1 mutations are not assumed to develop any neurological symptoms during their entire life span, and note that the effect of heterozygous NPC1 mutations on the development of neurological disorders has not been extensively investigated. This is also the case for NPC1+/− mice. However, intermediate abnormalities in terms of cholesterol metabolism have been shown in nonneuronal cells from NPC1 patients (23–25) and in nonneuronal cells or tissues from NPC1+/− mice (26–28). In addition, our recent study has demonstrated that NPC1+/− exhibits intermediate dysfunction in the mitochondria of the brains of NPC1+/− mice at 9 weeks of age (22). These lines of evidence have led us to examine whether there occurs neuronal dysfunction or damage in NPC1+/− mouse brains under certain conditions such as aging.

In this study, morphological and biochemical studies were performed on brains of aged heterozygous NPC1+/− mice. In contrast to what has been assumed, neurodegeneration as demonstrated by Purkinje cell loss, enhanced phosphorylation of tau, and activated MAPK/ERK1/2 were clearly observed in NPC1+/− mouse brains. It has been assumed that mutant NPC1 heterozygotes do not develop any clinical symptoms; however, these results suggest that heterozygous NPC1 mutations may be a risk factor for tauopathy. Since it is estimated that the incidence of NPC1 disease is as high as 1:150,000 (1), the percentage of the total population with the heterozygous NPC1 mutation is estimated to be around 0.5%. Thus, our present results show that heterozygous NPC1 mutations unexpectedly affect brains with aging and thus may lead to the investigation of the link between heterozygous NPC1 mutations and neurodegenerative diseases, such as Alzheimer disease.

EXPERIMENTAL PROCEDURES

BALB/c NPC1/NH Mice—The animal care and experiments using animals performed in this study were carried out in accordance with institutional guidelines. BALB/c mice carrying the genetic mutation for NPC1 were obtained from The Jackson Laboratory (Bar Harbor, ME). These heterozygous mice were bred to acquire NPC1+/+ and NPC1+/− mice used in this study. The genotypes of the mice were determined from genomic DNA isolated from tail snip DNA using a PCR-based technology, Inc. (Santa Cruz, CA). The polyclonal anti-calbindin D28K antibody was purchased from BD Transduction Laboratories (Lexington, KY). The rabbit anti-cholera toxin B and the filipin complex were purchased from Sigma. The monoclonal anti-flotillin-1 antibody and anti-β-tubulin antibody were purchased from Covance (Richmond, CA) and BD Transduction Laboratories (San Jose, CA), respectively. Horseradish peroxidase-conjugated cholera toxin B and the filipin complex were purchased from Sigma.

Histological Analysis—The mice were sacrificed by CO2 inhalation and perfused intracardially with 0.1 M phosphate-buffered saline (PBS) containing heparin (50 units/ml). The brains were removed and fixed in 4% paraformaldehyde in PBS overnight at 4 °C and then rinsed with PBS and cryoprotected in a solution of 30% sucrose in PBS. Fixed tissues were sectioned on a seminmotorized rotary microtome (LEICA RM2145, Wetzlar, Germany) at 30 μm and processed for immunohistochemistry with the anti-calbindin D28K (1:500) and AT8 (1:500). Immunoreactivity was detected with diaminobenzidine using the ABC Elite kit according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). For cresyl violet staining, slides were immersed in 70, 80, and 95%, ethanol for 5 min each and in 100% ethanol for 5 min and then in xylene for 15 min. The slides were then sequentially immersed in 100% xylene, 95, and 70% ethanol solutions and then in distilled water for 5 min each. The slides were stained for 1 min in filtered cresyl violet solution and then briefly rinsed twice in distilled water. They were then sequentially dehydrated again in 70, 80, 95, and 100% ethanol solutions for 2 min each. The slides were placed in xylene for another 10 min and then mounted with Permount. For double fluorescence staining, the sections were treated overnight with a rabbit anti-calbindin D28K antibody (1:500) and AT-8 antibody (1:500) at 4 °C overnight, followed by incubation with secondary antibodies, rhodamine-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA) diluted at 1:500, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (American Qualex, San Clemente, CA) diluted at 1:50, for 1 h at room temperature. The slides were then washed with PBS and mounted with Vectashield (Vector). Fluorescent images were obtained using a model LSM 510 laser-scanning confocal microscope (Zeiss) equipped with a × 63 Plan Apochromat numerical aperture and a 1.4 oil immersion objective.

Protein Preparation—The mice were sacrificed by CO2 inhalation, and their brains were harvested, rinsed in PBS, and immediately frozen in liquid nitrogen. The cerebral cortex and cerebellum were separated and homogenized in 10 volumes of Tris-saline (50 mM Tris-HCl (pH 7.4) and 150 mM NaCl), containing protease inhibitors (CompleteED) and phosphatase inhibitors (CompleteED) and phosphatase inhibitors (CompleteED). The homogenates were centrifuged at 10,000 rpm for 2 min at 4 °C, and the supernatants were stored for biochemical analyses. Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce).

Determination of ATP Level—ATP level in mouse brain tissues was determined as previously reported (22). In brief, the homogenates of cerebral cortex and cerebellum were centrifuged at 10,000 rpm for 10 min at 4 °C and then rinsed three times with PBS. ATP level in these samples was determined using the ATP Bioluminescence Assay kit CLS II (Roche Applied Science). In brief, the pellet was resuspended in 50 μl of ice-cold ATP lysis buffer (100 mM Tris and 4 mM EDTA, pH 7.5), to which 150 μl of boiling ATP lysis buffer was added, and the samples were incubated for 2 min at 98 °C. The samples were cooled at 10,000 rpm for 1 min at 4 °C, and the supernatants were collected. Finally, the ATP level was determined by combining 50 μl of the supernatant with 50 μl of the luciferase reagent. After a 20-s delay, chemiluminescence was measured with a 2.6-s integration time using a microplate luminescence meter (EG&G Berthold, Bad Wildbad, Germany). Luciferase activity was expressed in fluorescence units/μg of protein.

Determination of ATP Synthase Activity—Brain mitochondria were isolated as previously reported (22). For the determination of ATP synthase activity, 100 μg of mitochondrial protein from each sample was used to measure the rate of ATP synthesized. The reaction was initiated by adding 100 μg of mitochondrial protein into 100 μl of reaction buffer (10 mM K-HPO4 (pH 7.4) 300 mM K-mannitol, 10 mM KCl, and 5 mM MgCl2) at 37 °C. After 1 min, 10 μl of ADP (50 μM) was added, and the intensity of bioluminescence was recorded at 37 °C, where peak height was proportional to the amount of ATP synthesized. Inmunoblot Analysis—Equal amounts of proteins separated using 4–20% gradient Tris/Tricine SDS−PAGE (Dai-iichi Pure Chemical Co., Ltd., Tokyo, Japan) were electrophoretically transferred onto a polyvinilidene difluoride membrane (Millipore Corp., Bedford, MA). Nonspecific binding was blocked with 5% nonfat dry milk and washed with PBS containing 0.1% Tween 20. The blots were then incubated with primary antibodies overnight at 4 °C. For the detection of both monoclonal and polyclonal antibodies, appropriate peroxidase-conjugated secondary antibodies were used in conjunction with SuperSignal chemiluminescence (Pierce) to obtain images on the film.

Preparation of Raft Fractions—Detergent-insoluble membrane raft fractions were obtained according to the established method previously validated for Triton X-100 insoluble membrane fractions. The final product was dissolved in a solution of Triton X-100 (2% in PBS) and used for biochemical analysis. The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce).

Biochemical Analyses—Brain mitochondria were isolated as previously reported (22). For the determination of ATP synthase activity, 100 μg of mitochondrial protein from each sample was used to measure the rate of ATP synthesized. The reaction was initiated by adding 100 μg of mitochondrial protein into 100 μl of reaction buffer (10 mM K-HPO4 (pH 7.4) 300 mM K-mannitol, 10 mM KCl, and 5 mM MgCl2) at 37 °C. After 1 min, 10 μl of ADP (50 μM) was added, and the intensity of bioluminescence was recorded at 37 °C, where peak height was proportional to the amount of ATP synthesized.
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RESULTS

Cholesterol Accumulation in Brains of Aged NPC1 Heterozygous Mouse—The brains of NPC1+/+ and NPC1+/- mice at 24, 40, and 104–106 weeks of age were isolated, and the levels of cholesterol and phospholipids were determined. The cholesterol levels in the cerebrum and cerebellum of the NPC1+/- mice were significantly elevated compared with those in the same brain areas of the NPC1+/+ mice at 104–106 weeks of age (Fig. 1a), whereas the cholesterol levels were similar in the two groups at 24 and 40 weeks of age (Fig. 1a). The phospholipid levels were similar in the two genotypes at every age examined (Fig. 1b). The filipin and calbindin staining of brain samples (the third lobe of each cerebellum) prepared from the NPC1+/- and NPC1+/- mice at 104 weeks of age shows that cells exhibiting a strong filipin-positive signal in the NPC1+/- cerebellar section (from the third lobe) are also calbindin-positive, whereas the calbindin-positive cells in the NPC1+/- cerebellar section exhibit a very weak signal for filipin staining (Fig. 1c). These results indicate that cholesterol accumulation occurs in Purkinje cells of aged NPC1+/- mouse cerebellum.

Purkinje Cell Loss in Aged NPC1 Heterozygous Mice—The cerebellums of NPC1+/- and NPC1+/- mice at 104–106 weeks of age were immunohistochemically analyzed using the anti-calbindin antibody, which specifically recognizes Purkinje cells. The number of anti-calbindin antibody-positive cells decreased in the NPC1+/- cerebrum (Fig. 2, d-f) compared with that in the NPC1+/+ cerebellum (Fig. 2, a-c), suggesting that Purkinje cell loss occurred in the aged NPC1+/- cerebellum. However, this was not the case for the NPC1+/- cerebellum at 24 and 40 weeks of age (data not shown). Next, the number of anti-calbindin antibody-positive cells in each lobe of the cerebellums of the NPC1+/- and NPC1+/- mice at 104–106 weeks of age was determined. The number of Purkinje cells was significantly decreased in first + second, third, seventh, and eighth lobes of NPC1+/- cerebellum compared with those of NPC1+/- cerebellum (Fig. 2g).

Hyperphosphorylation of Tau in NPC1+/- Mouse Brain—Since neurodegeneration found in the NPC homozygous mouse brain is accompanied by hyperphosphorylated tau (6, 16), the sagittal sections of the brains were immunohistochemically stained using the anti-phospho-tau antibody AT-8. AT-8 stained neurons strongly in the cerebral cortices and hippocampus of the NPC1+/- mice (Fig. 3, b and d) compared with those of the NPC1+/+ mice (Fig. 3, a and c). For cerebellar sections, double staining using anti-calbindin antibody was performed. The Purkinje cells demonstrated as being calbindin-positive were very faintly stained with AT-8 in the aged NPC1+/- mouse cerebellum, whereas calbindin-positive Purkinje cells were also AT-8-positive in the aged NPC1+/- mouse cerebellum (Fig. 3, e–h). The ratio of AT-8-positive Purkinje cells to total Purkinje cells was very high in NPC1+/- mouse cerebellum compared with that of NPC1+/- mouse cerebellum (Fig. 3i).

To verify that the histologic abnormalities are a result of enhanced tau phosphorylation, immunoblot analysis was conducted using lysates of the NPC1+/- and NPC1+/- cerebella and cerebellums. The antibodies used were T-46, which recognizes phospho-independent tau; AT-8, AT-100, AT-180, AT-270, PHF-1, and SMI-31, which recognize site-specific phosphorylation of tau; and the anti-β-tubulin antibody as an internal standard. The intensities of the signals corresponding to phosphorylated tau demonstrated by AT-8 and AT-100 increased in the cerebellum and cerebellum of the NPC1+/- mice at 104–106 weeks of age compared with those for the NPC1+/- mice of at the same age (Fig. 4a). However, the intensities of the signals...
FIG. 2. Cerebellar histology in aged NPC1−/− mouse brains. Brain sections (cerebellum) from the NPC1+/+ and NPC1−/− mice at 104–106 weeks old were subjected to immunohistochemistry using the anti-calbindin antibody D28, as described under "Experimental Procedures." Calbindin-immunoreactive neurons in the third lobes of the cerebellum of NPC1−/+ (a–c) and NPC1−/− (d–f) are shown on different scales. Scale bars, 100 μm. g, the number of calbindin-immunopositive neurons was determined in all of the cerebellar lobes of the NPC1+/+ and NPC1−/− mice (n = 5 for each genotype). *p < 0.05.

Determined by AT-180, AT-270, PHF-1, and SMI-31 did not differ between samples from the NPC1+/+ and NPC1−/− brains (Fig. 4a). The phosphorylation site of tau in the cerebrums and cerebellums of the NPC1+/+ and NPC1−/− mice at 24 and 40 weeks of age was also determined (Fig. 4, b and c, respectively). The phosphorylation state of tau and the total tau level in both samples demonstrated by AT-8 and T-46 did not differ between the NPC1+/+ and NPC1−/− mice (Fig. 4, b and c).

MAPK Was Activated in NPC1−/− Mouse Brains—Since our previous reports demonstrated that tau phosphorylation in NPC1-deficient cells is caused by enhanced MAPK activity (6, 17), the activity of tau kinases including MAPK was determined. Among the kinases examined, including MAPK/ERK1/2, GSK-3β, JNK, and Cdk5/p25, the levels of phospho-MAPK/ERK1/2 in the cerebrum and cerebellum of the NPC1−/− mice at 104–106 weeks of age increased compared with those for the NPC1+/+ mice (Fig. 5a, whereas the levels of phospho-GSK-3β, JNK, and Cdk5/p25 remained unchanged (Fig. 5, b, c, and d). The activities of MAPK/ERK1/2 in the cerebrums and cerebellums of the NPC1+/+ and NPC1−/− mice at 24 and 40 weeks of age were also determined. The activities of MAPK/ERK1/2 in both samples did not differ between the NPC1+/+ and NPC1−/− mice (Fig. 5, e and f).

Cholesterol Level in Lipid Rafts Decreased in NPC1−/− Mouse Brains—Because the enhanced MAPK/ERK1/2 activity due to the decreased cholesterol level in the lipid rafts is suggested to induce tau phosphorylation in NPC1−/− mouse brains and NPC1−/− cells (6, 17), lipid compositions in the raft fractions isolated from the cerebral cortices of the NPC1+/+ and NPC1−/− mice were analyzed. The raft marker GM1 ganglioside was recovered in fractions 4 and 5, and another raft marker, flotillin-1, was recovered in fraction 5 (Fig. 6c). The cholesterol levels in fractions 4 and 5 isolated from the NPC1−/− brain were significantly lower than those in the same fractions isolated from the NPC1+/+ brain (Fig. 6a), whereas the levels of phospholipids in these fractions showed no significant difference between the two genotypes (Fig. 6b).

Level of ATP in NPC1+/+ and NPC1−/− Brains—Our previous study demonstrated that ATP levels in NPC1−/− organs including the brain, liver, and muscles as well as in NPC1−/− cultured neurons decrease compared with those in NPC1+/+ organs and neurons (22); thus, ATP level and ATP synthase activity in the aged NPC1+/+ and NPC1−/− brains were determined. As shown in Fig. 7, ATP levels in the cerebrum and cerebellum of the NPC1+/+ mice at 104–106 weeks of age significantly decreased compared with those in the same brain areas of the NPC1+/+ mice (Fig. 7a). ATP synthase activity in the cerebellum of the aged NPC1+/− mice also decreased compared with that of the aged NPC1+/+ mice (Fig. 7b).

DISCUSSION

Here we demonstrate for the first time the unexpected phenomenon that neurodegeneration occurs in aged NPC1−/− mouse brains. This neuronal damage is accompanied by tau hyperphosphorylation and enhanced MAPK/ERK1/2 activity, which are observed in young NPC1−/− mouse brains and NPC1−/− cells (6, 17). The significant accumulation of cholesterol in neurons in the brain and the significant reduction in cholesterol level in raft fractions isolated from aged NPC1−/− cerebellums were also observed. These changes are found to depend on aging in NPC1−/− mice.
NPC1 disease is a hereditary disorder that develops in an autosomal recessive manner; thus, it has been assumed that heterozygous carriers of NPC1 mutations do not develop any neurological symptoms during their entire life span (1). It has also been presumed that this is the case for NPC1/H11001/H11002 model mice. Therefore, little attention has been paid to whether NPC1/H11001/H11002 mice develop any symptoms with aging. To our surprise, however, the present study has clearly shown that neurodegeneration demonstrated as Purkinje cell loss accompanied by intracellular cholesterol accumulation and its deficiency in lipid rafts occurs in the aged NPC1/H11001/H11002 mouse brain. A few studies have investigated NPC1 heterozygotes and have shown that NPC1 heterozygotes have “intermediate” abnormalities in cholesterol metabolism at the nonneuronal cell level (23, 26, 27); however, it has been shown that there is no significant abnormality in terms of cholesterol metabolism in cultured NPC1/H11001/H11002 neurons (30) and young NPC1/H11001/H11002 brains (6). These lines of evidence suggest that cholesterol metabolism in the central nervous system changes and neurodegeneration accompanied by abnormal tau phosphorylation occurs only in aged NPC1/H11001/H11002 mice. The reason for the change in cholesterol metabolism found only in the aged NPC1/H11001/H11002 brains may be the expression of the NPC1 protein in NPC1/H11001/H11002 brains being approximately half that in NPC1/H11001/H11001 brains (6), which may...

**Fig. 4.** Immunoblot analysis of tau in cerebrum and cerebellum of 104–106-week-old NPC1+/+ and NPC1+/− mice. *a*, equal amounts of postnuclear supernatant protein from the cerebrum and cerebellum were subjected to immunoblot analysis using the site-specific phospho-tau antibodies AT-8, AT-100, AT-180, AT-270, PHF-1, and SMI-31 in addition to an antibody that recognizes phospho-independent tau, T46, and the anti-β-tubulin antibody as an internal standard. Tau in the cerebrum and cerebellum of 24-week-old (*b*) and 40-week-old (*c*) NPC1+/+ and NPC1+/− mice was immunohistochemically analyzed using AT-8 and AT-100 antibodies.

**Fig. 5.** Immunoblot analysis of tau-directed kinases in cerebrum and cerebellum of 104–106-week-old NPC1+/+ and NPC1+/− mice. Equal amounts of postnuclear supernatant protein from the cerebrum and cerebellum were subjected to immunoblot analysis using the monoclonal antibodies specific for pan-ERK1/2 and phospho-ERK (p-ERK) (*a*), pan-GSK-3β and phospho-GSK-3β (p-GSK-3β) (*b*), pan-SAPK/JNK and phospho-SAPK/JNK (p-SAPK/JNK) (*c*), and Cdk5 (*d*). Immunoblot analysis of ERK1/2 in the cerebrum and cerebellum of 24-week-old (*e*) and 40-week-old (*f*) NPC1+/+ and NPC1+/− mice was performed using anti-pan-ERK and anti-phospho-ERK antibodies.
very slightly affect cellular cholesterol trafficking. As a result, it takes a longer time for neurons to show any abnormalities in terms of cholesterol accumulation in the late endosome/lysosome compartment and the subsequent shortage of cholesterol in other compartments.

Since a decreased cellular cholesterol level stimulates MAPK activity (17, 31) and tau phosphorylation (32), it is reasonable to postulate that a reduced cholesterol level in lipid rafts, due to impaired cholesterol trafficking, increases MAPK activity and enhances tau phosphorylation in aged NPC1+/− brains. This is the case for younger NPC1+/− brains, i.e., neurodegeneration in NPC1+/− brains is linked with an increased MAPK activity and enhanced tau phosphorylation induced by a sustained cholesterol shortage due to the absence of its trafficking (6, 17).

In addition, it is likely that impaired cholesterol trafficking and the resultant intracellular cholesterol accumulation may result in an increased cholesterol level in mitochondria, which induces mitochondrial dysfunction, thereby affecting ATP synthase activity and reducing cellular ATP level, because, as we have reported, both increased and decreased cholesterol level in the mitochondrial membrane cause mitochondrial dysfunction and reduce cellular ATP level, which causes neurodegeneration in the younger NPC1+/− mice (22). With these results taken together, it is possible that the altered cellular cholesterol metabolism enhances tau phosphorylation and decreases cellular ATP level, both of which synergistically or independently induce neuronal neurodegeneration. One may raise the questions of what causes neurodegeneration, enhanced tau phosphorylation or low ATP level, and whether these two pathways work independently or dependently. The present study cannot answer these questions. Previous studies demonstrated that mitochondrial dysfunction with energy depletion causes cell death (33, 34) and that tau abnormalities alone can cause neurodegenerative diseases (35, 36), indicating that each pathway can independently cause neuronal death. However, previous studies showed that there is a correlation between ATP level and the activity of tau kinases including MAPK/ERK1/2 (37, 38). These results enable us to assume that mitochondrial dysfunction with a decreased ATP level and the enhancement of tau phosphorylation synergistically contribute to neurodegeneration in aged NPC1+/− mouse brains.

Finally, the results of our present study, contrary to what has been assumed, suggest that heterozygous NPC1 mutations are a risk for neuronal impairment. Since the ratio of the population with heterozygous NPC1 mutations is estimated to be as high as 0.5% (1), our findings lead us to further examine whether heterozygous NPC1 mutations are a risk for tauopathy, including Alzheimer disease. These issues should be addressed in future studies.

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