SHORT COMMUNICATION

Lysosomal Storage of Subunit c of Mitochondrial ATP Synthase in Brain-Specific *Atp13a2*-Deficient Mice

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Kufor-Rakeb syndrome (KRS) is an autosomal recessive form of early-onset parkinsonism linked to the *PARK9* locus. The causative gene for KRS is *Atp13a2*, which encodes a lysosomal type 5 P-type ATPase. We recently showed that KRS/*PARK9*-linked mutations lead to several lysosomal alterations, including reduced proteolytic processing of cathepsin D *in vitro*. However, it remains unknown how deficiency of *Atp13a2* is connected to lysosomal impairments. To address this issue, we analyzed brain tissues of *Atp13a2* conditional-knockout mice, which exhibited characteristic features of neuronal ceroid lipofuscinosis, including accumulation of lipofuscin positive for subunit c of mitochondrial ATP synthase, suggesting that a common pathogenic mechanism underlies both neuronal ceroid lipofuscinosis and Parkinson disease. (*Am J Pathol* 2016, 186: 3074–3082; <http://dx.doi.org/10.1016/j.ajpath.2016.08.006>)

Parkinson disease (PD) is one of the most common movement disorders caused by loss of dopaminergic neurons. Variation at the *PARK9* locus is associated with Kufor-Rakeb syndrome (KRS), which is characterized by autosomal recessive early-onset parkinsonism.¹ In 2006, the causative gene at the *PARK9* locus was identified as *Atp13a2*, which encodes a lysosomal type 5 P-type ATPase.² To date, six mutations have been identified in five probands.^{2–4} In addition, there is evidence that neuronal ceroid lipofuscinosis-12 (CLN12) is caused by homozygous mutation in *Atp13a2*.⁵ Furthermore, *Atp13a2*-deficient mice also exhibit phenotypes resembling neuronal ceroid lipofuscinosis (NCL),^{6,7} suggesting that PD/KRS and NCLs share a pathogenic mechanism.

Recent studies revealed that the protein level and enzymatic activity of cathepsin D are reduced in *Atp13a2*-deficient cells and brain tissues.^{6,8} We showed previously that cathepsin D gene-deficient mice exhibit NCL.⁹ Mutations in the cathepsin D gene are responsible for the most severe

type of NCL (CLN10),¹⁰ suggesting that one of the common pathogenic factors between PD/KRS and NCLs reduces cathepsin D activity. In many types of NCLs including cathepsin D gene-deficient mice, subunit c of the mitochondrial F₁F₀-ATPase accumulates in lysosomes^{11,12}; this protein is a specific substrate of cathepsin D.^{9,13} In the context of PD, phosphatase and tensin homolog–induced putative kinase 1 and Parkin cooperate in the clearance of dysfunctional mitochondria via mitophagy.^{14,15} This evidence strongly implicates impairment of autophagy of

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mitochondria (ie, mitophagy) in neurodegeneration associated with PD and NCLs. *In vitro* data also suggest that *Atp13a2* is involved in autophagy and mitochondrial clearance,¹⁶ although its role in mitophagy *in vivo* remains still unclear. Therefore, we generated new *Atp13a2* conditional-knockout mice and investigated the effect of brain-specific *Atp13a2* knockout on the pathology of NCLs.

Materials and Methods

Animals

Procedures were approved by the Animal Experimental Committee of the Juntendo University Graduate School of Medicine and were performed in accordance with the NIH's *Guide for the Care and Use of Laboratory Animals*¹⁷ and the Juntendo University Graduate School of Medicine.

Mice with brain-specific conditional knockout of *Atp13a2*, the sole ortholog of human *Atp13a2* in the mouse genome, were generated as follows. In the targeting vector, a 2.1-kb region of the mouse *Atp13a2* gene, including exons 2 to 3, was followed by an FRT-flanked PGK-neo expression cassette in the opposite transcriptional orientation; these two regions were flanked by *loxP* sites. The linearized targeting vector (Figure 1A) was electroporated into C57BL/6 embryonic stem cells. ES clones were isolated and confirmed by PCR (data not shown) and Southern blot analysis (Figure 1B). Chimeras were generated by injecting positive embryonic stem cells into blastocysts of BALB/c mice and mated to C57BL/6J females to generate F1 heterozygous mice, which were crossed with CAG-FLP-transgenic mice to remove the FRT-flanked selection marker. The +/Flox [neo-] FLP[-/-] (*Atp13a2*^{flox/+}) mice were intercrossed to obtain *Atp13a2*^{flox/flox} mice, which were further crossed with Nestin-Cre transgenic mice¹⁸ to produce mice deficient for *Atp13a2* specifically in the central nervous system (*Atp13a2*^{flox/flox;nestin}).

Antibodies

Polyclonal antibodies against the mitochondrial subunit c of the F₁F₀-ATPase, cathepsin D, and tripeptidyl peptidase 1 (TPP1) were produced as described previously.^{11,13,19} Antibodies against *Atp13a2* (5879S; Cell Signaling Technology, Danvers, MA), glial fibrillary acidic protein (GFAP) (G3893; Sigma-Aldrich, St. Louis, MO), light chain 3 of microtubule-associated protein (LC3; L7543; Sigma-Aldrich), mannose 6 phosphate receptor (M6PR; ab124767; Abcam, Cambridge, United Kingdom), and actin (MAB1501; Millipore, Billerica, MA) were obtained from the indicated suppliers.

Immunoblot Analysis

Anesthetized mice ($n = 3$) were euthanized by decapitation, and lysates for SDS-PAGE were prepared from brain tissue

as described previously.⁸ After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham Biosciences, Little Chalfont, United Kingdom), which were incubated overnight at 4°C with the indicated primary antibodies and visualized using the Enhanced Chemiluminescence Kit (GE Healthcare, Little Chalfont, United Kingdom). Signals were detected on an ImageQuant LAS4000 (GE Healthcare).

Histologic Analysis

Mice ($n = 3$) were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2). Brain tissues were processed for paraffin sections or cryosections, as previously described.²⁰ Luxol fast blue and periodic acid–Schiff (PAS) staining were performed as previously described.²¹ For the detection of auto-fluorescence, sections stained with DAPI were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). For immunohistochemistry, sections were incubated overnight at 4°C with anti-GFAP (dilution 1:200) or anti-subunit c (dilution 1:100). The reaction was visualized with 3-3' diaminobenzidine tetrahydrochloride, as described previously.^{9,20}

Electron Microscopy and Immunoelectron Microscopy

For conventional electron microscopy,^{9,20} mice ($n = 3$) were fixed by cardiac perfusion with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2). Brain slices were embedded in epoxy resin, and ultrathin sections (70 nm thick) were cut and observed with a Hitachi HT7700 electron microscope (Hitachi, Tokyo, Japan). Quantitation was performed using a previously described method²⁰: 30 images were taken per animal, and then, using a double lattice with a spacing of 1 cm, the ratio of points falling on lipofuscins to those falling within the whole neuronal perikarya was determined. Immunoelectron microscopy was performed as previously described.^{9,19} Briefly, mice ($n = 3$) were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2). The hippocampal region was immersed overnight at 4°C in 2.3 mol/L sucrose in 0.1 mol/L phosphate buffer (pH 7.2) and frozen in liquid nitrogen. Ultrathin cryosections (60 nm) prepared with a Leica UC7/FC7 were incubated with rabbit anti-subunit c (dilution 1:10) and further with anti-rabbit IgG conjugated with 10-nm colloidal gold (dilution 1:40; British Biocell International, Cardiff, United Kingdom). The sections were observed on a H-7100 electron microscope (Hitachi, Tokyo, Japan).

Accelerating Rotarod Test

Tests were performed on a rotarod machine with automatic timers and falling sensors (MK-660D; Muromachi Kikai,

Tokyo, Japan). Male *Atp13a2*^{fllox/flox} and *Atp13a2*^{fllox/flox}:nestin mice ($n = 10$ for each genotype) were placed on a 3-cm diameter rotating rod covered with rubber, and rotation was accelerated from 3 to 35 rpm over 5 minutes. Fall latency was recorded, and the first fall latency of the third trial was used for analysis.

Wire Hang Test

Tests began with the animal hanging from an elevated wire cage top. Male *Atp13a2*^{fllox/flox} and *Atp13a2*^{fllox/flox}:nestin mice ($n = 10$ for each genotype) were placed on a cage top, which was then inverted and suspended above the home cage; the latency until the animal fell was recorded. This test was performed 3 days per week with three trials per session.

Statistical Analysis

Statistical significance was determined by *t*-test (STATVIEW version 3.0; SAS Institute, Cary, NC). Data are expressed as means $\pm$ SEM. $P < 0.05$ was considered significant.

Results

Generation of Brain-Specific *Atp13a2* Conditional-Knockout Mice

We generated brain-specific conditional *Atp13a2* knockout mice (*Atp13a2*^{fllox/flox}:nestin). Western blot analysis in 18-month-old animals confirmed that total levels of Atp13a2 protein were reduced >90% in *Atp13a2*^{fllox/flox}:nestin brain relative to control (*Atp13a2*^{fllox/flox}) brain (Figure 1C).

Behavioral and Neuropathologic Analysis of *Atp13a2*-Deficient Mice

Atp13a2^{fllox/flox}:nestin mice were viable at birth and indistinguishable in appearance from their littermates, and their survival rate was not diminished. Motor and behavioral deficits did not appear during the first 12 months of life, although fall latency in the accelerating rotarod and wire hang tests were reduced in 18-month-old mice (Figure 1D). Therefore, we mainly analyzed neuronal pathology in 18-month-old mice. *Atp13a2*^{fllox/flox}:nestin mice exhibited accumulation of autofluorescent, PAS-, and Luxol fast blue-positive granules in the perikarya of cortical or hippocampal neurons (Figure 1, E–L). All forms of NCL share unifying pathomorphologic characteristics, that is, autofluorescent, PAS-, and Luxol fast blue-positive granules in the cytoplasm of most nerve cells.²² Thus, *Atp13a2*^{fllox/flox}:nestin mice exhibited typical neuropathologic phenotypes of NCL. Furthermore, immunoreactivity for GFAP was elevated in the cerebral cortex of *Atp13a2*^{fllox/flox}:nestin mice (Figure 1, M and N); higher magnification revealed that the GFAP-immunoreactive processes of astrocytes were thicker in *Atp13a2*^{fllox/flox}:nestin mice than in wild-type mice (Figure 1, O and P), suggesting activation of astrocytes in this region.

Accumulation of Electron-Dense Lamellated Inclusions in *Atp13a2*^{fllox/flox}:nestin Mice

Typical electron dense lipofuscins, some of which contained lipids,²³ were observed by electron microscopy in the perikarya of control neurons of the substantia nigra in 18-month-old mice (Figure 2, A and B). By contrast, in various brain regions of *Atp13a2*^{fllox/flox}:nestin mice,

Figure 1 Generation of *Atp13a2*^{fllox/flox} mice and behavioral and neuropathologic examination of *Atp13a2*^{fllox/flox}:nestin mice. **A:** Schematic representation of the targeting vector and targeted allele of the *Atp13a2* gene. A 2.1-kb region of the mouse *Atp13a2* gene including exons 2 to 3 was followed by an FRT-flanked PGK-neo expression cassette in the opposite transcriptional orientation, and these two regions were flanked with *loxP* sites. In the targeting construct, a 5.0-kb 5' fragment and a 2.8-kb 3' fragment were used as the long and short homologous arms, respectively. **Arrows** indicate primers used to check 3' homologous recombination (f1, r1). **B:** Southern blot analysis of genomic DNA from ES cells that underwent homologous recombination. Genomic DNA was digested with EcoRV and hybridized with the 5' probe (internal) or 3' probe (external). In addition, a Neo probe was used to screen for random insertion events. **Left panel:** The 5' probe detected a 13.0-kb band from the WT allele and 5.5-kb band from the targeted allele. **Middle panel:** The 3' probe detected a 13.0-kb band from the WT allele and 9.2-kb band from the targeted allele. **Right panel:** Neo probe. **C:** Immunoblot of Atp13a2 in mice brain. Lysates of whole brains of the indicated genotypes were immunoblotted for Atp13a2 and actin. **D:** Movement disorder in 18-month-old male *Atp13a2*^{fllox/flox}:nestin mice. Motor coordination and neuromuscular strength were tested by accelerating rotarod assay and wire hang test, respectively. In the accelerating rotarod assay, rotation was accelerated from 3 rpm to 35 rpm over 5 minutes, and fall latency was recorded. In the wire hang test mice were placed on the cage top, which was then inverted and suspended above the home cage. The latency of falling onto the bedding was recorded. **E and F:** AF storage material in the cortical neurons of a 16-month-old *Atp13a2*^{fllox/flox} control mouse (**E**) and an age-matched *Atp13a2*^{fllox/flox}:nestin mouse (**F**). Nuclei were counterstained with DAPI (magenta). **Arrowheads** indicate neurons possessing granular AF materials (green) in an *Atp13a2*^{fllox/flox}:nestin mouse. **G–P:** Histologic analyses of a 16-month-old *Atp13a2*^{fllox/flox}:nestin mouse (**H, J, L, N, and P**) and an age-matched *Atp13a2*^{fllox/flox} control (**G, I, K, M, and O**). Cryosections were stained with periodic PAS (**G and H**) and LFB (**I–L**), and immunostained for GFAP (**M–P**). Square areas in **M** and **N** are enlarged and shown in **O** and **P**, respectively. Sections from the Cx (**E–J, M, and N**) and the CA3 region of the hippocampus (**K and L**) are shown. **Arrowheads** in **H and J** indicate cortical neurons accumulating PAS- or LFB-positive granules in an *Atp13a2*^{fllox/flox}:nestin mouse. **M–P:** Immunoreactivity for GFAP was slightly increased in the cerebral cortex of an *Atp13a2*^{fllox/flox}:nestin mouse (**M and N**). **Boxed areas** in **M and N** are shown at higher magnification in **O and P**. The GFAP-immunoreactive astrocytes of *Atp13a2*^{fllox/flox}:nestin mice had thicker processes than those of *Atp13a2*^{fllox/flox} mice (**O and P**). Data are expressed as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ (*t*-test). Scale bars: 20 μ m (**E–L, O, and P**); 100 μ m (**M and N**). AF, autofluorescent; CC, corpus callosum; Cx, cortex; ES, embryonic stem; GFAP, glial fibrillary acidic protein; LFB, Luxol fast blue; P, positive; PAS, periodic acid–Schiff; TA, targeted allele; WA, wild allele; WT, wild-type.

Figure 2 Accumulation of electron-dense lamellated inclusions in neurons of the substantia nigra of *Atp13a2*-deficient mice. **A–I:** Electron micrographs of neurons in the SN (**A**, **B**, **D–F**), granular layer of the cerebral cortex (**G**), and CA3 of the hippocampus (**H** and **I**) of 18-month-old *Atp13a2^{flox/flox}* (**A** and **B**) and *Atp13a2^{flox/flox};nestin* (**D–I**) mice. **Boxed areas** in **A** and **D** are shown at higher magnification in **B** and **E**, respectively. **Boxed areas** in **E**, **F**, **G**, and **I** are shown at higher magnification in the **insets**. **B:** In an *Atp13a2^{flox/flox}* mouse, typical lipofuscin accumulated in the perikaryal region (**arrowheads**). **C:** Volume densities (%) of typical lipofuscins and electron-dense lamellated inclusions in the perikarya of neurons in CA3 of the hippocampus from *Atp13a2^{flox/flox}* and *Atp13a2^{flox/flox};nestin* mice, respectively. **E** and **H:** In an *Atp13a2^{flox/flox};nestin* mouse, electron-dense lamellated inclusions (**black arrows**) accumulated instead of lipofuscins. A **white asterisk** indicates typical lamellated membrane structures within an inclusion. A **white arrow** indicates mitochondria surrounded with autophagosome-like multilayered membranes (a **black asterisk** in the **inset**). **F:** A degenerating neuron with electron-dense lamellated inclusions (**black arrows**) in an *Atp13a2^{flox/flox};nestin* mouse. **G:** An electron-dense lamellated inclusion in the perikarya of a granular neuron of the cerebral cortex of an *Atp13a2^{flox/flox};nestin* mouse. High-power view reveals that each concentric lamellated membrane is composed of tightly associated double membranes (**arrowheads**). **H** and **I:** In addition to electron-dense lamellated inclusions (**arrow**), membrane-bound structures with loosely arranged lamellated membrane structures, which were not arranged concentrically but instead unevenly distributed, were observed around mitochondria (**white asterisks**). **Boxed area** in **I** is shown at higher magnification in the **inset**. **I, inset:** Lamellated membrane composed of tightly associated double membranes (**arrowheads**). Data are expressed as means $\pm$ SEM. ****P* < 0.001 (*t*-test). Scale bars: 2 μ m (**A** and **D**); 1 μ m (**B** and **E**, **F**, and **inset** in **F**); 500 nm (**G** and **H** and **insets** in **E**); 200 nm (**I**); 50 nm (**insets** in **G** and **I**). SN, substantia nigra.

membrane-bound inclusions containing lamellated membranes within electron-dense amorphous materials accumulated in the perikarya of neurons (Figure 2, D–H). The volume density of electron-dense lamellated inclusions in *Atp13a2*-deficient neurons was significantly higher than that of lipofuscins in control neurons (Figure 2C). Some neurons with these inclusions underwent degeneration

(Figure 2F). These lamellated membrane structures contained concentric membranes composed of tightly associated double membranes (Figure 2, G and I), indicating that they were distinct from so-called fingerprint profiles, one of the typical ultrastructural hallmarks of NCLs.^{9,24} Furthermore, in neurons of the *Atp13a2^{flox/flox};nestin* mice we often observed membrane-bound structures around

mitochondria, whose lamellated membranes were loosely arranged and unevenly distributed (Figure 2, H and I). We consider these structures to represent immature forms of the electron-dense lamellated inclusions.

Accumulation of Subunit c of Mitochondrial ATP Synthase and Delayed Maturation of Cathepsin D in *Atp13a2*^{flox/flox}:nestin Mice

Immunoblot analysis revealed a significant increase in the level of mitochondrial ATP synthase subunit c in *Atp13a2*^{flox/flox}:nestin brain (Figure 3A), consistent with immunohistochemistry showing elevated levels of subunit c immunoreactivity in the cortical neurons of *Atp13a2*^{flox/flox}:nestin mice (Figure 3, B and C). Immunoelectron microscopy confirmed that gold particles indicating subunit c were detected only in mitochondrial inner

membranes in control neurons (Figure 3D), whereas in the *Atp13a2*-deficient neurons they were localized within the electron-dense lamellated inclusions as well as in mitochondria (Figure 3, E and F), indicating accumulation of subunit c in abnormal lysosomes in *Atp13a2*^{flox/flox}:nestin mice. We also compared the protein levels of cathepsin D and TPP1, which are associated with CLN10 and CLN2, respectively, and are essential for the degradation of subunit c in lysosomes.^{9,13} Immunoblotting for TPP1 revealed no significant changes in the levels of either protein (Figure 3A). The reduction in cathepsin D activity in *Atp13a2*^{flox/flox}:nestin brain was quite mild (data not shown) relative to those in stable *Atp13a2*-knockdown cells and medaka.⁸ However, we observed elevated levels of the single-chain form (42 kDa) and down-regulation of the light chain (15 kDa)²⁵ in whole brains of *Atp13a2*^{flox/flox}:nestin mice (Figure 3, G and H), as well as elevated levels of the

Figure 3 Accumulation of subunit c of mitochondrial F_1F_0 -ATPase and delay in cathepsin D maturation in the brains of 18-month-old *Atp13a2*^{flox/flox}:nestin mice. **A:** Immunoblot for Sc, TPP1, and actin. Lanes 1–3: *Atp13a2*^{flox/flox} mice; lanes 4–6: *Atp13a2*^{flox/flox}:nestin mice. **B and C:** Immunohistologic analyses of subunit c in the cortex of *Atp13a2*^{flox/flox} (**B**) and *Atp13a2*^{flox/flox}:nestin mice (**C**). **Arrowheads** indicate cortical neurons accumulating subunit c—immunopositive granules in an *Atp13a2*^{flox/flox}:nestin mouse. **Inset** in **C:** A neuron with massive accumulation of subunit c—immunopositive granules and pyknotic nuclei is shown. **D–F:** Immunoelectron microscopy of subunit c of mitochondrial F_1F_0 -ATPase (indicated by 10-nm colloidal gold) using ultrathin cryosections of the CA3 region of the hippocampus of *Atp13a2*^{flox/flox} (**D**) and *Atp13a2*^{flox/flox}:nestin mice (**E** and **F**). The boxed area in **E** is shown at higher magnification in the **inset**. **Arrowheads** indicate mitochondria positive for subunit c in the inner membranes. A **black asterisk** indicates an electron-lucent membrane-bound lysosome in an *Atp13a2*^{flox/flox} mouse. **White asterisks** indicate lamellated inclusions positive for subunit c in an *Atp13a2*^{flox/flox}:nestin mouse. **Arrows** in the **inset** indicate lamellated structures. **G:** Immunoblot for CD, M6PR, LC3, and actin using whole brain tissues from *Atp13a2*^{flox/flox} and *Atp13a2*^{flox/flox}:nestin mice (lanes 1–3 and 4–6, respectively). **H:** Quantitation of the immunoblot for CD. Ratio of CD single chain to actin (**upper left panel**). CD single chain (42 kDa) accumulated in the brains of *Atp13a2*^{flox/flox}:nestin mice. Ratio of CD heavy chain (35 kDa) to actin (**upper right panel**). Ratio of CD light chain (15 kDa) to actin (**lower panel**). **I:** Immunoblot CD using Cx and Hp from *Atp13a2*^{flox/flox} and *Atp13a2*^{flox/flox}:nestin mice. *P* < 0.05, *P* < 0.01 (t-test). Scale bars: 20 μm (**B** and **C**, main image and inset); 500 nm (**D–F**, main image and inset). CD, cathepsin D; Cx, cerebral cortex; Hp, hippocampus; LC3, light chain 3 of microtubule-associated protein; M6PR, mannose 6-phosphate receptor; Sc, subunit c of mitochondrial F_1F_0 -ATPase; TPP1, tripeptidyl peptidase 1.

single-chain form in the cortical and hippocampal tissues of the *Atp13a2*^{fllox/fllox}:nestin mice (Figure 3I), indicating a delay of cathepsin D maturation in the brains of the *Atp13a2*^{fllox/fllox}:nestin mice. To further investigate the mechanisms underlying lysosomal accumulation of subunit c and delayed maturation of cathepsin D in *Atp13a2*^{fllox/fllox}:nestin mice, we performed immunoblotting for LC3 and M6PR, which are essential for autophagy/mitophagy²⁶ and sorting of cathepsin D to lysosomes,²⁷ respectively. However, we observed no differences in the amounts of the two forms of LC3 and M6PR between *Atp13a2*^{fllox/fllox} and *Atp13a2*^{fllox/fllox}:nestin mice (Figure 3G).

Discussion

This study showed that even without complete *Atp13a2* knockdown, the model animals exhibited morphologic abnormalities in the lysosome. Thus, the 80% decrease in *Atp13a2* levels in medaka⁸ and the 90% decrease in our *Atp13a2*^{fllox/fllox}:nestin mice were sufficient to confer a marked phenotype similar to those observed in other lines of *Atp13a2*-deficient mice.^{6,7}

Two lines of *Atp13a2*-deficient mice generated independently by other groups exhibited various age-related neuropathologic changes, although phenotypes directly related to PD were not prominent.^{6,7} Neither line exhibited specific degeneration of dopaminergic neurons in the substantia nigra or a decrease in striatal dopamine levels.^{6,7} The relationship between *Atp13a2* deficiency and α -synuclein accumulation in mouse brains *in vivo* is not significant⁷; instead, neurotoxicity due to *Atp13a2* deficiency has been suggested to be independent of α -synuclein.⁶

Our neuropathologic analyses presented here, as well as previous studies, indicate that *Atp13a2*-deficient mice exhibit phenotypes resembling NCL rather than PD.^{6,7} In mouse models for many types of NCLs, massive activation of glial cells is the common prominent phenotype.^{19,28} However, in this study we could not clearly demonstrate that activation of astrocytes in the *Atp13a2*^{fllox/fllox}:nestin mice was due to a cell-autonomous process, a secondary effect of neurodegeneration, or both. In contrast to the conventional *Atp13a2*-deficient mice generated by two other groups,^{6,7} our *Atp13a2*^{fllox/fllox} mice can be used to generate animals with neuron- and astrocyte-specific deficiency in *Atp13a2*, allowing further clarification of the cause of astrocyte activation under *Atp13a2* deficiency.

Lipofuscins are considered to be a hallmark of aging.²⁹ In 18-month-old control brains, we observed typical lipofuscins in neurons, whereas in neurons of *Atp13a2*^{fllox/fllox}:nestin mice, membrane-bound electron-dense inclusions accumulated. Such inclusions were similar to those observed in stable *Atp13a2*-knockdown cells and medaka⁸ and patients with KRS.³⁰ These lamellated membrane structures in *Atp13a2*^{fllox/fllox}:nestin mice resembled so-called fingerprint

profiles, one of the ultrastructural hallmarks of NCLs.^{9,24} However, apart from fingerprint profiles composed of densely packed single unit membranes, each concentric lamellated membrane within the inclusions was composed of tightly associated double membranes. Therefore, we considered these lamellated membrane structures to be distinct from fingerprint profiles. Judging from the ultrastructural changes in various types of neurons in our mice, it is reasonable to assume that the electron-dense lamellated inclusions are a specific ultrastructural hallmark of the *Atp13a2*-deficient neurons.

In addition to the electron-dense lamellated inclusions, we often observed membrane-bound structures with loosely arranged lamellated membranes in *Atp13a2*^{fllox/fllox}:nestin mice. We consider these to be immature forms for the electron-dense lamellated inclusions. Interestingly, these immature inclusions were usually surrounded with mitochondria, and the electron density of their amorphous materials was similar to those of mitochondrial matrix. Damaged mitochondria are eliminated via mitophagy.³¹ Recent studies suggested that loss of *Atp13a2* is associated with mitochondrial damage or oxidative stress.^{32,33} Thus, it would be reasonable to assume that chronic oxidative stress, in excess of the levels associated with normal aging, occurred in the brain of the *Atp13a2*^{fllox/fllox}:nestin mice. Nevertheless, no evidence for the acceleration of autophagy or mitophagy in *Atp13a2*-deficient neurons was obtained in this or previous studies.⁶ However, it is possible that damaged mitochondria in *Atp13a2*-deficient neurons underwent other morphologic changes, such as mitochondrial spheroid formation.^{34,35} Further study is required to reveal the fate of the mitochondria damaged by *Atp13a2* deficiency.

Accumulation of subunit c of mitochondrial ATP synthase in abnormal lysosomes is a characteristic feature of NCLs. In the study, we showed that subunit c accumulates abnormal inclusions in *Atp13a2*-deficient neurons, reinforcing the NCL-related features of KRS/PARK9. Although we did not detect accumulation of subunit c in lysosomes of mice heterozygous for cathepsin D gene deficiency until at least 1 month of age, a recent study revealed several neurologic phenotypes in mice heterozygous for cathepsin D deficiency.³⁶ Thus, in *Atp13a2*-deficient neurons, the decrease in cathepsin D activity would have pathologic effects in combination with aging. A previous study of conventional *Atp13a2*-knockout mice suggested that the aberrant processing of cathepsin D occurs in lysosomes and late endosomes but not in Golgi or transporting vesicles.⁶ This idea was partially confirmed by our results on M6PR.

In this study, we observed lysosomal storage with specific ultrastructural features and accumulation of subunit c of mitochondrial ATP synthase in neuronal lysosomes of *Atp13a2*-deficient mice. These phenotypes were the result of lysosomal dysfunction and possibly impaired clearance of damaged mitochondria. Our results provide conclusive evidence that NCL and KRS/PARK9 share etiologic features and implicate the lysosomal pathway in the pathogenesis of PD.

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