Atg9a deficiency causes axon-specific lesions including neuronal circuit dysgenesis

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ABSTRACT
Conditional knockout mice for Atg9a, specifically in brain tissue, were generated to understand the roles of ATG9A in the neural tissue cells. The mice were born normally, but half of them died within one wk, and none lived beyond 4 wk of age. SQSTM1/p62 and NBR1, receptor proteins for selective autophagy, together with ubiquitin, accumulated in Atg9a-deficient neurosoma at postnatal d 15 (P15), indicating an inhibition of autophagy, whereas these proteins were significantly decreased at P28, as evidenced by immunohistochemistry, electron microscopy and western blot. Conversely, degenerative changes such as spongiosis of nerve fiber tracts proceeded in axons and their terminals that were occupied with aberrant membrane structures and amorphous materials at P28, although no clear-cut degenerative change was detected in neuronal cell bodies. Different from autophagy, diffusion tensor magnetic resonance imaging and histological observations revealed Atg9a-deficiency-induced dysgenesis of the corpus callosum and anterior commissure. As for the neurite extensions of primary cultured neurons, the neurite outgrowth after 3 d culturing was significantly impaired in primary neurons from atg9a-KO mouse brains, but not in those from atg7-KO and atg16l1-KO brains. Moreover, this tendency was also confirmed in Atg9a-knockdown neurons under an atg7-KO background, indicating the role of ATG9A in the regulation of neurite outgrowth that is independent of autophagy. These results suggest that Atg9a deficiency causes progressive degeneration in the axons and their terminals, but not in neuronal cell bodies, where the degradations of SQSTM1/p62 and NBR1 were insufficiently suppressed. Moreover, the deletion of Atg9a impaired nerve fiber tract formation.

INTRODUCTION
Macroautophagy (hereafter referred to as autophagy) is a lysosome-mediated intracellular bulk or selective degradation system to remove macromolecules and organelles. Autophagy has an essential role in the maintenance of cellular metabolism. The Atg proteins involved in autophagosome formation consist of several functional units: Atg1 kinase and its regulators, the PtdIns3K complex, Atg9, the Atg2-Atg18 complex, and 2 ubiquitin-like conjugation systems. In these functional units, the 2 ubiquitin-like conjugation systems (E1 and E2) of Atg12 and Atg8 work at the later or maturation stages of autophagosome formation. Atg12 is activated by Atg7 (E1) and conjugated to Atg5 by Atg10 (E2), while Atg7 (E1), Atg3 (E2) and Atg5 that forms a complex together with Atg12 and Atg16 (E3) are essential for the Atg8–phosphatidyethanolamine conjugation system. Knockout mouse models for Atg7, Atg3, Atg5 and Atg16l1 die soon after birth because of the neonatal energy depletion. Moreover, deficiency of Atg7 specifically in the liver accumulates SQSTM1/p62 (hereafter referred to as SQSTM1) in the cytoplasm of hepatocytes that has binding sites for MAP1LC3 (hereafter referred to as LC3) and ubiquitin, indicating that SQSTM1 itself is a substrate of autophagy and is taken up into autophagosomes by linking both ubiquitin conjugating with target molecules and LC3 localizing on the phagophore. Like SQSTM1, many other proteins have binding regions with ubiquitin and LC3 and work as receptor proteins of selective autophagy. When Atg7 or Atg5 is deleted specifically in mouse central nervous system (CNS) tissue, SQSTM1 and ubiquitin accumulate in neuronal soma and abnormal membranous structures also accumulate in axon terminals, resulting in neuron death, and abnormal limb reflexes. These manifestations indicate that the suppression of autophagy in CNS tissue causes neurodegeneration.

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Atg9 is the only transmembrane protein among autophagy-related proteins, and it is thought to work at the initial stage of autophagosome formation. There are 2 genes, Atg9a and Atg9b, that are mammalian orthologs of yeast ATG9, and the protein products of these genes are functional. ATG9A is generally expressed in mammalian tissue cells, but expression of ATG9B is limited to the placenta and pituitary gland. Like knockout mice for Atg7 and Atg5, those for Atg9a die within one d after birth, although atg9a-conventional knockout mice become embryonic-lethal after crossing with C57B6 mice. It has been shown that the degradation of SQSTM1 and long-lived proteins are impaired in mouse embryonic fibroblasts deficient in Atg9a. Moreover, ATG9A localizes on the trans-Golgi network and on early, late or recycling endosomes under the basal conditions, while ATG9A vesicles translocate to autophagosome-forming sites under starvation conditions, which play an important role in the early stage of autophagosome formation.

Our previous study reveals that ATG9A protein is localized throughout neurons including somatodendrites, axons and axonal terminals. However, the precise roles of ATG9A in neurons remain largely unknown. To further investigate the function of ATG9A, we generated Atg9a-flox mice (Atg9a^{F/F}) and crossed them with transgenic mice expressing Cre recombinase under the control of the Nes promoter (Atg9a^{F/F}: Nes-Cre, CKO) to produce a CNS-specific, conditional knockout model. We found that ATG9A is involved in the maintenance of homeostasis in the axons and in their terminals in CNS neurons.

Results

**Generation of Atg9a^{F/F}:Nes-Cre (atg9a-CKO) mice and their symptoms**

To further examine the roles of ATG9A in neurons, we generated knockout mice for Atg9a specifically in CNS tissue (Atg9a^{F/F}:Nes-Cre mice, atg9a-CKO) (Fig. 1A). We confirmed that 2 distinguishable bands of wild-type (WT) (404 bp) and flox (554 bp) alleles were detected by PCR analyses, while protein bands immunopositive for ATG9A largely disappeared in atg9a-CKO brains (Fig. 1B and Fig. 2D).
Figure 2. Accumulation of ubiquitin, SQSTM1 and NBR1 in atg9a-CKO mouse brains. (A) Immunofluorescence for SQSTM1, ubiquitin and NBR1 in neurons of DCN in atg9a-CKO mice at P15. White arrows indicate colocalization of positive signals for these proteins. Scale bars: 20 μm. (B) Electron micrographs of a DCN neuron in an atg9a-CKO mouse brain at P15. The square in the upper panel is enlarged in the lower panel. Asterisks in both figures indicate an ubiquitin aggregate that is encircled by ER. Scale bars: 1 μm. (C) Immunostaining of ubiquitin, SQSTM1 and NBR1 in the cerebrum cortexes of floxed control, atg7-CKO and atg9a-CKO mice at 2 and 4 wk of age. Scale bar: 20 μm. (D) Western blot analyses of ubiquitin, SQSTM1, NBR1, ATG7, ATG9A, and MAP1LC3A/B in brain lysates from floxed control, atg7-CKO and atg9a-CKO mice at 2 and 4 wk of age. Immunostaining for actin is used for an internal control. (E) Quantification of each protein band of SQSTM1 and NBR1. Results are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, ns, not significant, n = 3 for each. Statistical analyses were performed using the Student t test.
Impairment of autophagy in neurons

Immunohistochemical analyses revealed that dot-like immunoreactivity for ubiquitin, SQSTM1 and NBR1 accumulated in neurons of the deep cerebellar nucleus (DCN) in atg9a-CKO mice at P15, and they colocalized with each other in the neurons (Fig. 2A). Results from electron microscopy showed that numerous large inclusions containing fine fibrous structures were tightly surrounded by a partially rough-surfaced endoplasmic reticulum (ER) with a cytoplasmic surface attached by ribosomes. Such ER-membranes often extended into fibrous inclusions and, in some cases, formed vesicles (Fig. 2B). Fibrous inclusions without ER-association were similar to those detected in atg7-CKO mouse neurons, and so-called ubiquitin aggregates. In fact, immunoelectron microscopic observations using cryo-thin sections revealed that gold particles indicating ubiquitin were deposited on the fibrous inclusion (Fig. S1A).

Dot-like immunoreactivity for ubiquitin, SQSTM1 and NBR1 appeared abundant in atg9a-CKO cerebral cortical neurons at 2 wk of age, but were decreased in both size and number at 4 wk of age (Fig. 2C). Accumulated inclusions did not change in atg7-CKO neurons. Drastic changes in the accumulation of ubiquitin aggregates were also detected in the neurons of hippocampus, thalamus, hypothalamus and pons at 2 and 4 wk of age (Fig. S2 to 4). These immunocytochemical data for SQSTM1 and NBR1 were confirmed by western blot analysis (Fig. 2D), which showed that protein amounts of SQSTM1 and NBR1 were significantly increased in brain lysates from atg9a-CKO mice at 2 wk of age compared with those from floxed control mice (Fig. 2E), which was similar to those of atg7-CKO mice. Protein amounts of both SQSTM1 and NBR1 were decreased in brain lysates from atg9a-CKO mice at 4 wk of age, but such a reduction was not found in brain lysates from atg7-CKO mice. Moreover, a reduction in ubiquitinated proteins was also found in atg9a-CKO brain lysates of mice between 2 and 4 wk of age, although no clear-cut difference was detected in atg7-CKO and floxed control brain lysates between 2 and 4 wk of age (Fig. 2D). Furthermore, the conversion of LC3-I to LC3-II occurred in atg9a-CKO brains, as evidenced by western blot (Fig. 2D). Interestingly, immunoreactivity for LC3 in atg9a-CKO DCN neurons was detected in puncta immunopositive for SQSTM1 and NBR1 (Fig. S1B). Ubiquitin aggregates with an ER-association were detected, but both the size and incidence were much smaller in atg9a-CKO neurons than in atg7-CKO neurons (Fig. S2 and S5A to D). In addition, atg9a-CKO neurons in DCN, in some cases, contained autophagosome-like structures at 4 wk of age (Fig. S5E and F). Although degenerative changes were not clearly detected in neuronal cell bodies of atg9a-CKO mouse brains, the issue of whether neuronal cell death occurs in the cerebral, thalamic and hippocampal regions of floxed control and atg9a-CKO brains were examined at P15 and also at P28 with staining of TUNEL. The result showed that the number of neurons positively stained with TUNEL was very small in brain sections of cerebral cortical, thalamic and hippocampal regions, and it did not significantly differ between floxed control and atg9a-CKO mouse brains at P15 and also at P28 (Fig. S6A and B).

These data suggest that although ATG9A is essential for the execution of autophagy and the loss of ATG9A caused the accumulation of ubiquitin aggregates until 2 wk of age, it is possible to assume that the autophagic degradation pathway for ubiquitin, SQSTM1 and NBR1 may be executed, to some extent, in the perikaryal region of atg9a-CKO neurons after 2 wk of age.

Axonal degeneration of Purkinje cells in the cerebellum

Since autophagy defects by deletion of the Atg5, Atg7, Rb1cc1/Fip200 or Wdr45/Wipi4 gene cause axonal swelling with an accumulation of abnormal membranes in Purkinje cells, the present study investigated changes in the axons and their terminals of Purkinje cells in atg9a-CKO mice. When examined by immunostaining of CALB (calbindin), a marker for Purkinje cells, and SLC32A1/VGAT, a marker for the axon terminals of GABAergic neurons, CALB-positive axonal spheroids appeared, to some extent, and SLC32A1/VGAT-positive axon terminals became slightly larger in the DCN of atg9a-CKO mice at 2 wk of age (Fig. S7A and B). This tendency was also confirmed by electron microscopy (Fig. S7C and D), but when the mean length of contact sites with neurons in the DCN was measured, it did not differ from the mean length of floxed control axon terminals (Fig. 3A). At 4 wk of age, CALB-positive axonal spheroids appeared abundant and became larger in size, while SLC32A1/VGAT-positive axon terminals became larger than those at 2 wk of age (Fig. 3B). Enlarged spheroids or axons frequently appeared in the DCN and contained numerous aberrant membrane or amorphous structures with double-membrane vesicles and mitochondria that sometimes became dense in the matrix (Fig. 3C), while similar aberrant membrane structures and amorphous structures often occupied the axon terminals (Fig. 3D). The mean contact length of atg9a-CKO axon terminals with DCN neurons was much larger than that of the floxed control axon terminals (Fig. 3A).

As for immunoreactivity for ubiquitin, it was diffuse and weak in these swollen axons and axon terminals of Purkinje cells, but aggregate-like intense signals were not detected in these axonal regions (Fig. S7E and F). By electron microscopy
Figure 3. Morphological changes in Purkinje cell axons and their terminals. (A) The bar graph shows the lengths of the contacting zones of the axon terminals of Purkinje cells with DCN neurons in floxed control and atg9a-CKO mice at P15 and P28. Results are expressed as mean ± SEM. (n = 10 to 15 axon terminals of each). **P < 0.01. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test. (B) Immunoreactivity for CALB and SLC32A1/VGAT in the DCN of atg9a-CKO mice at P28. Insets show enlarged axon terminals with positive staining of SLC32A1/VGAT (arrowheads). Scale bar: 50 μm. (C, D) Electron micrographs of a spheroid in the DCN of an atg9a-CKO mouse (C) and axon terminals of the floxed control and atg9a-CKO mice (D) at P28. A spheroid with thin layers of myelin is occupied with various profiles of vesicles and vacuoles (C), while a synaptic region in the floxed control DCN and a large synaptic region with an amorphous structure or degenerating mitochondria in the atg9a-CKO DCN are shown (D). Degenerating mitochondria with electron dense materials are demonstrated and aberrant membrane structures are detected near these mitochondria (white arrowheads). Some structures are encircled by double membranes (white arrows). Insets show the squared portions, in which mitochondria with low contrast are demonstrated. Scale bar: 1 μm. (E, F) Axonal spongiform changes in the white matter (E) and in the DCN (F) of the cerebellum in an atg9a-CKO mouse. HE staining of the white matter of a floxed control and an atg9a-CKO mouse cerebellum (E) and that of the DCN of floxed control and atg9a-CKO mouse cerebellum (F). Electron micrographs showing large swollen axons with high electron dense materials in the white matter (E, right) and DCN (F, right). Scale bars: 50 μm for light microscopy in (E and F) and 5 μm for electron microscopy in (E and F).
observations, ubiquitin aggregate-like structures as shown in Fig. 2B were not detected in axons and their terminals of atg9a-CKO brains.

Because axons and their terminals in the DCN of atg9a-CKO mice at 4 wk of age were degenerative, we further examined Purkinje cell bodies and found changes in the Golgi apparatus. When stained with GOLGA2/GM130, a marker for cis-Golgi apparatus, GOLGA2/GM130-positive immunoreactivity took a round form at the perinuclear region of the Purkinje cells at 4 wk of age, but it appeared flat in Purkinje cells from floxed control littermate brains of atg9a-CKO mice and atg7-CKO brains at 4 wk of age, as well as floxed control littermate brains of atg9a-CKO mice, and atg7-CKO and atg9a-CKO brains at 2 wk of age (Fig. S8). Although clear-cut degenerative changes in Purkinje cell bodies were not detected at this stage, axonal spongiform changes were also detected in the white matter and DCN of the cerebellum (Fig. 3E and F). By electron microscopy, large swollen axons with thin myelin were detected in the white matter and DCN, along with slightly high electron dense materials (Fig. 3E and F). Moreover, axonal spongiform changes were also detected in the thalamus, cerebral cortex and spinal cord of atg9a-CKO neuronal tissues at 4 wk of age (Fig. S9A to C). Electron microscopy also showed large swollen axons occupied by amorphous substances in the thalamic region (Fig. S9D). As stated above, progressive structural alterations were detected in axons and their terminals of atg9a-CKO mouse brains, no clear-cut degenerative changes were observed in the neuronal cell bodies.

These results indicate that severe degenerative changes in axons proceeded in atg9a-CKO brains, although accumulation of ubiquitin, SQSTM1 and NBR1 decreased in the neuronal cell bodies of these brains.

**Dysgenesis of the corpus callosum and anterior commissure**

Histological observations of brains of atg9a-CKO mice at 2 and 4 wk of age showed callosal dysgenesis. We therefore analyzed various white matter tracts in atg9a-CKO brains at 3 wk of age by diffusion tensor magnetic resonance (DTM) imaging. As shown in Figure 4A and B, crossing nerve fibers were detected in the midline of the corpus callosum in the floxed control mouse brains, but such nerve fibers were massively decreased in the anterior portion of the corpus callosum of atg9a-CKO brains and completely lost in the posterior portion. Beneath the anterior portion of the corpus callosum, the crossing nerve fibers of the anterior commissure were observed in the floxed control brains, but no crossing fibers were detected in the corresponding regions of atg9a-CKO brains (Fig. 4A and B). Unlike the anterior commissures, abundant nerve fibers were observed as the posterior commissures in both floxed control and atg9a-CKO mouse brains (Fig. 4A and B). To confirm the impairment of nerve fiber extension, histological examinations of floxed control, atg7-CKO and atg9a-CKO brains were performed using coronal sections stained with hematoxylin-eosin (HE). Crossing nerve fibers in the middle portion of the corpus callosum were distinctly detected in coronal sections from the anterior to the posterior portions of the cerebrum in the floxed control brains, although the middle portion of the corpus callosum was not formed and the third ventricular space was continuous to the subdural space of the cerebral longitudinal fissure (Fig. 4C and D). Moreover, the place where the anterior commissure passed in the floxed control brains showed no nerve fibers in atg9a-CKO brains (Fig. 4E and F). By immunohistochemistry for myelin basic protein (MBP) and neurofilaments (SMI31), similar defects in nerve fiber tracts were observed in atg9a-CKO brains (Fig. 4D and F). Crossing nerve fibers in the corpus callosum and anterior commissure were discerned in the atg7-CKO brains at P28 (Fig. S10A to D). These results suggest that ATG9A plays important roles not only in the autophagosome formation but also in neuronal circuit formation.

To further examine the impairment of nerve fiber extensions in the developing cerebrum, histological approaches were applied to brain tissues from atg9a-CKO mice and floxed control littermate mice at embryonic d 18 (E18). As shown in Figure 5A and B, nerve fibers formed the corpus callosum by crossing in the middle portion of floxed control mouse brains, as evidenced by HE staining and immunostaining for neurofilaments. However, crossing nerve fibers were not detected in the middle portion of the expected corpus callosum in the atg9a-CKO brains, but the longitudinal callosal fascicles (Probst bundles) were detected, and these presented a failure of the callosally-projecting neurons to extend axons across the midline (Fig. 5A and B). Instead of crossing fibers in the midline, GFAP-positive astrocytes occupied the middle portion (Fig. 5C). Posterior to this portion of the brains, the developing corpus callosum was not formed and the third ventricular space was continuous to the subdural space of the longitudinal fissure in the atg9a-CKO brains (Fig. 5D). Moreover, the anterior commissures were formed in the middle portion of the third ventricle of the floxed control mouse brains at E18, whereas no communication was detected in the expected portions where the third ventricular space was continuous to the basal portion of the atg9a-CKO brains (Fig. 5D). Although a dysgenesis of neuronal tract formation was detected in atg9a-CKO developing brains at E18, cerebral cortical layers were distinctly formed in these brains (Fig. 5E). These results indicate that dysgenesis of the corpus callosum and anterior commissure occur in atg9a-CKO brains even at E18.

**Impairment of the neurite extension of primary cultured cortical neurons**

Since the dysgenesis of neuronal tract formation occurred in atg9a-CKO brains, axonal extension was examined in the cultures of primary cerebral cortical neurons (PCN) obtained from control littermate brains of each KO mouse, and atg7-, atg16l1-, and atg9a-knockout (KO) mouse brains at E14.5. PCNs transfected with the GFP expression vector were cultured for 3 d (DIV) and compared with PCNs obtained from each of control littermate brains, and atg9a-, atg7- and atg16l1-KO mouse brains for degrees of neurite elongation. Neurites from each of control littermate PCNs extended straight, while those from atg7- and atg16l1-KO PCNs were similar to those from control littermate neurites. Different from control littermate neurons, neurites from atg9a-KO PCNs did not elongate and extend straight (Fig. 6A), while they often coiled in one place at
Neurite growth was compared between control littermate and knockout PCNs of each pair at 3 DIV; the longest neurite in \( \text{atg9a} \)-KO PCNs was significantly shorter than that from control littermate PCNs, but the longest neurites in other pairs of PCNs did not differ between control littermate and KO-PCNs (Fig. 6C).

To examine whether suppression of neurite elongations are attributed to the inhibition of autophagy or an ATG9A-proper role independent of autophagy, the neurite length of \( \text{atg9a} \)-knockdown PCNs that were obtained from the cerebral cortex of \( \text{atg7} \)-KO mouse embryos at E14.5 was measured at 4 DIV. The suppression ratio of ATG9A expression by 3 kinds of siRNA probes was examined by using mouse embryonic fibroblasts (MEFs), and the ratio of each siRNA probe to the control siRNA probe was similar between each probes and about 40% (Fig. 6D). One of these siRNA probes (\( \text{Atg9a} \#1 \) to 3) was introduced to PCNs obtained from WT or \( \text{atg7} \)-KO mice at 1 DIV, and the length of the longest neurites was measured at 4 DIV. Similar to the result of \( \text{atg9a} \)-KO PCNs, the neurite outgrowth was impaired in \( \text{Atg9a} \)-knockdown PCNs (Fig. 6E). Moreover, the length of the longest neurites was significantly shorter in \( \text{Atg9a} \)-knockdown PCNs than in WT and \( \text{atg7} \)-KO PCNs, both of which showed similar length (Fig. 6F). These results suggest that roles of ATG9A are deeply associated not only with autophagy but also with neurite outgrowth independent of autophagy.

**Discussion**

\( \text{atg9a} \)-KO mice die within 24 h of birth because of impaired autophagy, or the mice are embryonic lethal.\(^{19,20}\) The \( \text{atg9a} \)-KO mice used in the present study were backcrossed with C57B6J over several generations and all of backcrossed mice became embryonic lethal and died around E15. We therefore generated \( \text{Atg9a}^{\text{F/F}} \) mice and produced its KO mice specifically in CNS tissue. Using these \( \text{atg9a} \)-CKO mice, we demonstrated...
SQSTM1 is known to be involved in autophagy defects that include not only starvation but also various pathological stresses such as neurodegenerative diseases and numerous other disorders. SQSTM1 and NBR1. These proteins are used for indicators of autophagy defects that include not only starvation but also various pathological stresses such as neurodegenerative diseases and numerous other disorders.

ATG7, ATG3, ATG5 and ATG16L1 are involved in the C-terminal lipidation of MAP1LC3, while the loss of each of these genes suppresses autophagy at the later or maturation stage of autophagosome formation, which causes a scarcity of amino acids that results in individual death within 24 h. To further understand the precise roles of gene products of ATG7 and ATG5 in CNS tissue, CKO mice with deletion of the corresponding genes were specifically generated in CNS tissue and caused growth retardation. Different from atg9a-CKO mice, these CKO-mice showed mild neurological phenotypes including a longer life span. Rb1cc1/Fip200, a mammalian ortholog of yeast ATG17, is involved in autophagosome formation at the initial stage by forming a complex with ULK1, ATG13 and ATG16L1 in mammalian cells. rb1cc1-CKO mice have shown more severe phenotypes than those of atg5- or atg7-CKO mice and much similar phenotypes to those of atg9a-CKO mice. Although the life span of rb1cc1-CKO mice is longer than atg9a-CKO mice, neurological phenotypes are very similar. In particular, severe spongiform degeneration is discerned in the white matter of these 2 types of CKO mice, although large axon terminals with amorphous materials in atg9a-CKO mice were not detected in rb1cc1-CKO mice. These results indicate that like Rb1cc1, deletion of Atg9A in CNS tissue, that works in the upper stream of autophagosome formation will show neurological phenotypes that are more severe than those of Atg7 or Atg5, which are involved in autophagosome formation at a later or maturation stage. Although neurological phenotypes of atg9a-CKO mice were degenerative and similar to those of rb1cc1-CKO mice, those of atg9a-CKO mice were distinctly different from those of rb1cc1-CKO mice in the following points; in atg9a-CKO brains, 1) massive neuronal cell death examined with TUNEL staining was not discerned in various brain regions at P15 and also at P28, and 2) dysgenesis of the corpus callosum and anterior commissure was detected. In particular, it remains largely unknown how impairment of nerve fiber connections affects severe degenerative changes in axons and their terminals of atg9a-CKO neurons.

Until recently, deletion of essential genes for autophagy resulted in accumulation of ubiquitin aggregates, which contained adaptor or receptor proteins for selective autophagy, SQSTM1 and NBR1. These proteins are used for indicators of autophagy defects that include not only starvation but also various pathological stresses such as neurodegenerative diseases and numerous other disorders. SQSTM1 is known to accumulate progressively in neurons of CNS-specific CKO mice of various Atg’s that work in either the early or late stages of autophagosome formation. In the present study, SQSTM1 and NBR1 accumulated in atg9a-CKO neurons at 2 wk of age, but drastically decreased in them at 4 wk of age, as evidenced by analyses of immunostaining, electron microscopy and western blot. Since cell death occurred in a very small in number in atg9a-CKO mouse brains at P15 and also at P28 when examined with TUNEL staining, the decreases in these
receptor proteins were not attributed to massive loss of neurons in atg9a-CKO brains after 2 wk of age. Moreover, ubiquitin was present in axons and their terminals of atg9a-CKO brains, but no aggregate was discerned in them when examined by immunocytochemistry and electron microscopy. Taken together, the fact that the amount of ubiquitin aggregates decreased in atg9a-CKO neuronal cell bodies after 2 wk of age indicates that the autophagic degradation pathway for ubiquitin, SQSTM1 and NBR1 may be executed, to some extent, in the perikaryal region of atg9a-CKO neurons after 2 wk of age. However, since SQSTM1 is also a target substrate of the ubiquitin proteasome pathway, we cannot deny the possibility that the decrease in amounts of the receptor proteins is attributed to degradation by the proteasome. Moreover, it remains largely unknown whether a deep association of ER with ubiquitin aggregates, autophagosome-like structures, and positive puncta for

Figure 6. Impaired neurite extension of primary cultured cortical neurons. (A) Representative pictures of primary cultured cortical neurons obtained from control littermates of each knockout mouse embryo (control), atg9a-, atg7- and atg16l1-conventional KO mouse embryos at E14.5. Each PCN was transfected with a GFP expression vector at 1 DIV and observed at 3 DIV. (B) Profiles of extending axons of PCNs obtained from each of control littermate embryos and atg9a-KO mouse embryos at E14.5. Each PCN was transfected with a GFP expression vector at 3 DIV and observed at 7 DIV. Scale bar; 50 μm. (C) Quantification of the longest neurites of PCNs in A. Results are expressed as mean ± SEM n = 20 to 74 neurons of each. * P < 0.05, ** P < 0.01, ns, not significant. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test. (D) Western blot analyses of ATG9A in lysates of WT MEFs transfected with si-RNA probes (control and Atg9a [1 to 3]). Immunostaining for actin is used for an internal control (left). Quantification of a protein band of ATG9A. Results are expressed as mean ± SEM. ** P < 0.01, (n = 3 for each) (right). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test. (E) Representative pictures of primary cultured cortical neurons transfected with GFP expression vector and control or Atg9a si-RNA probe (control and Atg9a [3]) obtained from WT embryos and atg7-conventional KO mouse embryos at E14.5. Each PCN was transfected at 1 DIV, and fixed, and measured at 4 DIV after immunostaining for GFP. (F) Quantification of the longest neurites of PCNs in E. Results are expressed as mean ± SEM n = 20 to 24 neurons of each. ** P < 0.01, ns, not significant. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test.
MAP1LC3 that were colocalized with those for ubiquitin, SQSTM1 and NBR1 are correlated with the degradation processes of ubiquitin aggregates.

The present study showed contradictory results concerning the decreased accumulation of SQSTM1 and NBR1 in neuronal cell bodies and progressive degeneration of axons and their terminals. At some point, the defects in autophagy-related genes show the manifestations of phenotypes such as axonal degeneration and neuron death.\textsuperscript{13,15,30,31} Deficiency of Atg5 or Atg7 induces axonal degeneration and accumulation of ubiquitin aggregates in the neurosoma, resulting in neuron death, as shown by positive TUNEL staining. In addition to spongiform axonal degeneration, the deletion of Rb1cc1 has induced the neuronal loss of Purkinje cells and granule cells. In that study, degenerative changes in the cytoplasm were detected in SQSTM1-positive inclusions and abnormal mitochondria in Purkinje cells. These studies indicate that degenerative changes occur in parallel in both cell bodies, and axons and their terminals. As stated above, clear-cut degenerative changes were not detected in neuronal cell bodies of atg9a-KO mouse brains when analyzed with light and electron microscopy, and TUNEL staining. These findings were distinct from those of axons and their terminals, in which severe degeneration such as spongiosis and spheroids were discerned. From these results, it seems likely that severe axonal degeneration impairs neuronal function, leading to individual death. In other words, Atg9a deficiency caused axon-specific lesions.

Probst bundles that appear in embryonic brains with the agenesis of the corpus callosum were detected in the embryonic brains of atg9a-KO mice at E18. Until recently, there has been no report of the dysgenesis of nerve fiber tracts such as the corpus callosum and anterior commissures due to a deficiency in autophagy-related genes. Different from autophagy-related genes, Mapk8ip3/JIP3- or Map1b-deficient mice show an agenesis of the corpus callosum and severe growth retardation.\textsuperscript{36–38} It is, therefore, reasonable to assume that this phenotype of dysgenesis of the corpus callosum and anterior commissure is not the role of ATG9A concerning autophagosome formation. In fact, PCNs obtained from atg9a-KO mouse brains at E14.5 could not extend their axons straight and coiled them in one place, while neurite extension of atg7-KO PCNs were also impaired when Atg9a was knocked down by siRNA, indicating that the regulation of neurite outgrowth was independent of autophagy. Knockdown of ULK1/2, mammalian protein orthologs of yeast Atg1, in primary cultured dorsal ganglion neurons obtained from E12.5 mouse embryos causes impairment of axonal extension due to the suppression of the endocytosis of NGF.\textsuperscript{39} Since no trophic factors were used for the culturing of PCNs, axonal extension of PCNs is not attributed to the endocytosis of trophic factors. Moreover, it remains unknown whether ATG9A-positive vesicles are essential for membrane traffic to supply membranes for extending axons. Further studies are required to understand the relationship between the impairment of axonal extension and the dysgenesis of the corpus callosum and anterior commissures.

Collectively, our present study demonstrated contradictory results concerning decreases in the accumulated receptor proteins in neuronal cell bodies and the severe degeneration of axons and their terminals at 4 wk of age. Although dysgenesis of the nerve fiber tracts may differ from the role of ATG9A in autophagosome formation, ATG9A plays an essential role in the maintenance of homeostasis in axons. That is, Atg9a deficiency causes axon-specific lesions.

Materials and methods

Animals

Atg9a targeting vector was constructed by flanking exons 6 to 7 of Atg9a by 2 loxP sequences. Atg9a\textsuperscript{Flox/Flox} (Atg9a\textsuperscript{Flox/Flox}) mice were crossed with Nes-Cre transgenic mice (Jackson Laboratories) to produce Atg9a\textsuperscript{Flox/Flox}; Nes-Cre (atg9a-CKO) mice. Atg9a\textsuperscript{Flox/Flox} mice were produced by homologous recombinants using the C57BL/6N ES cell line RENKA. Atg7\textsuperscript{Flox/Flox} (Atg7\textsuperscript{Flox/Flox}) mice were bred with Nes-Cre transgenic mice to produce Atg7\textsuperscript{Flox/Flox}; Nes-Cre (atg7-CKO) mice, and Atg9a, Atg7 and Atg16l1 heterozygous knockout (KO) mice were intercrossed to produce atg9a\textsuperscript{−/−}, atg7\textsuperscript{−/−} and atg16l1\textsuperscript{−/−} KO mice, as described previously.\textsuperscript{7,8,19} All of these conventional KO mice were backcrossed onto a genetic background of C57BL6, and used in the present study.

The procedures involving animal care and sample preparation were approved by the Animal Experimental Committee of the Juntendo University Graduate School of Medicine, and were performed in accordance with the NIH guidelines and regulations and guidelines for the care and use of laboratory animals at the Juntendo University Graduate School of Medicine.

The present study used mice expressing Atg7\textsuperscript{Flox/Flox}, Atg9a\textsuperscript{Flox/Flox} or Atg9a\textsuperscript{Flox/Flox}, Atg9a\textsuperscript{Flox/Flox} without expressing Cre recombinase as floxed controls.

Genotype analyses by PCR

To determine the genotypes of these lines, genomic DNA was prepared from mice tails and analyzed by Thermal Cycler for polymerase chain reaction (C1000 Touch, BIO-RAD, CA, USA). The following primer sets were used for genotyping to distinguish between wild-type and Atg9a flox alleles. These primers amplify 404 bp and 554 bp fragments from wild-type and Atg9a flox allele, respectively.

\begin{align*}
\text{Fw: 5'}-\text{GGATGATATGTATTCCTGAG} & \text{ –3’;} \\
\text{Rv: 5’-TCCTGACCTGCTTCCAGTTCAG-3’}
\end{align*}

Survival periods

Survival periods of atg9a-CKO mice, atg7-CKO mice and their littermate floxed control (for each of atg9a-CKO and atg7-CKO) mice were monitored until all of them died.

Rotarod performance test

Motor coordination of floxed control and atg9a-CKO mice was determined by a rotarod performance test, as reported previously with some modifications.\textsuperscript{40} Briefly, the mice were placed on a rotating rod that was constantly accelerated to 50 r.p.m. for 5 min, and the time spent on the rod was measured. If mice
were stayed on the rod over 5 min, the time of 300 sec was recorded. Moreover, normal and abnormal movements or reflexes such as convulsion, locomotor ataxia, and limb clasp-ing reflexes of floxed control and atg9a-CKO mice were recorded at P15 and also at P28 by movies (Movies S1–3).

**Antibodies**

The following antibodies were used in the present study: rabbit anti-ATG9A,28 guinea pig anti-SQSTM1 (C-terminal; Progen, GP62-C), rabbit anti-SQSTM1 (MBL, PM045), mouse anti-NBR1 (Abcam, ab55474), rabbit anti-LC3A/B (Cell Signaling Technology, 4108), mouse anti-GOLGA2/GM130 (BD Biosciences, 610822), mouse anti-ACTB/b-actin (Sigma-Aldrich, A5441), rabbit anti-ubiquitin (Dako, Z0450), mouse anti-ubiquitin (P4D1; Santa Cruz Biotechnology, sc-8017), mouse anti-GFAP (Sigma-Aldrich, G3893), rabbit anti-AIF1/iba1 (Wako Pure Chemical Industries, 019–19741), goat anti-VGAT (Frontier Institute, VGAT-Go-Af620), goat anti-CALB/calbindin (Frontier Institute, Calbindin-Go-Af1040), rat anti-myelin basic protein (AbD Serotec, MCA409), mouse anti-NEFH/Neurofilament heavy (clone SMI31; BioLegend, 80163), rabbit anti-ATG7 (Cell Signaling Technology, 8535), and goat anti-GFP (Frontier Institute, GFP-Go-Af1480–1).

**Sample preparations for biochemical analyses**

Deeply anesthetized mice were transcardially perfused with solu-lact (Terumo, TP-AB05NR) for blood removal. The whole brains were rapidly dissected and frozen in liquid nitrogen, and stored at −80°C until used. Each brain was homogenized in 5-volumes of 1 × RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS [Nacalai Tesque, 08933–05] and 1% NP40 [Nacalai Tesque, 23640–94]) with protease inhibitor cocktail (Nacalai Tesque, 25955) and phos-phatase inhibitor cocktail (Nacalai Tesque, 07574–61). Homogenates were centrifuged at 15,000 g for 15 min at 4°C, and the resultant supernatants were collected. The protein concentrations were measured using the BCA protein assay system (Thermo Fisher Scientific, 23225).

**Immunoblot analysis**

Immunoblots were performed as described previously.11 Equal amount of the lysates was subjected to SDS-PAGE, and transferred to a PVDF membrane (Immobilon-P; Millipore, IPVH07850) and then blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6 [Nacalai Tesque, 35434–21], 137 mM NaCl [Nacalai Tesque, 31320–05], 0.05% Tween 20 [Nacalai Tesque, 35624–15]) for 30 min at room temperature. Membranes were incubated with primary antibodies at 4°C overnight. After washing with TBST, the membranes were incubated with HRP-labeled secondary antibodies (Jackson ImmunoResearch, 111–035–144 [rabbit], 315–035–045 [mouse], 106–035–003 [guinea pig] and 305–035–003 [goat]) for 1 h at room temperature. Immunoreactivity was determined using an Enhanced Chemiluminescent kit (Millipore, WBKLS0500). The intensity of each protein band was scanned using a LAS-4000 scanner (Fuji Photo Film., Tokyo, Japan) and quantified using Multi Gauge V3.0 software (Fuji Photo Film).

**Sampling procedures for morphological analyses**

Sample preparation for light and electron microscopy was performed as described previously.42 Deeply anesthetized mice were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (0.1M NaH2PO4 [Nacalai Tesque, 31718–15], 0.1M Na2HPO4 [Nacalai Tesque, 31723–35], pH 7.4) for light microscopy. Tissues were postfixed with the same fixative for 2 h, and embedded in paraffin or OCT-compound (Tissue-Tek, 4583). For electron microscopy, deeply anesthetized mice were transcardially perfused with 2% glutaraldehyde (Nacalai Tesque, 17003–05), −2% paraformaldehyde (Millipore, 1.04005.1000) in 0.1 M phosphate buffer, and postfixed in the same fixative at 4°C overnight.

**Immunohistochemistry for light microscopy**

Deparaffinized or frozen sections, or cultured cells were immuno-nostained according to a method described previously.42 For immunofluorescence analyses, tissue sections were subjected to antigen retrieval using the microwave method (in 0.01M citrate buffer [Nacalai Tesque, 09109–85] for 10 min at 105°C). After blocking with TNB buffer (PerkinElmer, FP1012), sections were incubated with primary antibodies at 4°C overnight, followed by 1 h of incubation with fluorescently labeled secondary antibodies (Alexa Fluor 488;– Jackson ImmunoResearch, 706–545–148 [guinea pig], Molecular Probes, A-31573 [rabbit], A-21208 [rat], A-21202 [mouse], Cy3; Jackson ImmunoResearch, 711–165–152 [rabbit], 715–165–151 [mouse], 705–165–147 [goat], Cy5; Jackson ImmunoResearch, 715–175–151 [mouse]). Samples were viewed with a confocal laser scanning microscope (FV1000; Olympus., Tokyo, Japan) after counterstaining with 4′–6′-diamindino-2-phenylindole (DAPI; Invitrogen, D21490). For immunohistochemical analyses, the sections were treated with 0.3% H2O2 (Wako Pure Chemical Industries, 801–04215) in methanol for 20 min, and blocked with TNB buffer for 30 min, followed by incubation with primary antibodies at 4°C overnight. Then, sections were incubated with anti-rabbit (Vector laboratory, MP-7401), -mouse (Vector laboratory, MP-7422) or -goat (Vector laboratory, MP-7405) IgG secondary antibody reagent containing microparticles coupled with either active peroxidase (ImmPRESS) or anti-guinea pig biotinylated secondary antibody (Vectastain ABC kit; Vector laboratory, PK-4007) for 1 h at room temperature. Staining for peroxidase was performed with 0.0125% 3,3′-diamobenzidine tetrahydrochloride (DAB) (Wako Pure Chemical Industries, 34900903) and 0.003% H2O2 in 0.05 M Tris-HCl buffer (pH 7.6) for 5 min. Digital Images of immunostained sections were exposed using a standard light microscope (BX50 Olympus, Tokyo, Japan).

**TUNEL staining**

Paraffin or frozen sections of floxed control and atg9a-CKO brains were stained for TUNEL according to a modified method.43 Briefly, the sections treated with proteinase K
(Nacalai Tesque, 29442–85) were incubated with 150 U/ml terminal deoxynucleotidyl transferase (TdT; Promega, M1871) and 10 nmol/ml Biotin-16-UTP (Roche, 11093070910) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 g/ml gelatin) at 37°C for 1 h. They were further incubated with peroxidase-conjugated streptavidin (Vector Laboratories, SA-5014) for 20 min, while the DAB reaction was performed by the same manner as described above. Positively stained nuclei for TUNEL were counted in 8 fields under a light microscope at 400 x and the fields were randomly selected in each of the cerebral cortex, hippocampus and thalamus of the both floxed control and atg9a-KO brains at P15 and also at P28. The number counted was averaged in each region of brains and calculated statistically using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test.

**Transmission electron microscopy**

Fixed samples for electron microscopy were postfixed with 1% OsO₄ (Merck Millipore, 1.24505.0001) for 2 h, followed by dehydration with graded ethanol solutions and QY-1 (NISHIN EM, 310–1), and embedded in Epon812 (TAAB, T026). Ultrathin sections were cut at 70 nm using an ultramicrotome (UC6 or 7; Leica Microsystems., Wetzlar, Germany), stained with 2% uranyl acetate for 30 min and lead citrate (1% lead acetate [TAAB, L021], 1% lead citrate [TAAB, L018], 1% lead nitrate [TAAB, L019], 2% sodium citrate [Wako Pure Chemical Industries, 191–0785]) for 4 min. They were then examined with an HT7700 electron microscope (Hitachi., Tokyo, Japan).

**Cultures of primary cerebral cortical neurons and plasmid transfection**

Primary cerebral cortical neurons were prepared from the cerebral cortex at E14.5, based on a method reported previously. The transfection of GFP plasmids (pAcGFP-C1; Clontech, 632470) was performed using Lipofectamine 2000 (Invitrogen, 11668–632470) was performed using Lipofectamine RNAi-MAX (Invitrogen, 13778–150) according to the manufacturer's instructions. PCNs were transfected with these regents at 1 DIV, while at the next d, the medium was changed to the normal Neurobasal medium (Gibco, 21103–049), and then the PCNs were further cultured for 48 h (until 4 DIV). As stated above, at 3 or 4 DIV, the PCNs were fixed with 4% paraformaldehyde and stained for GFP. PCNs were randomly photographed in each sample and the longest neurite of over 20 PCNs in each sample was measured. The mean and standard errors of the mean were calculated using data from each sample.

**Sampling procedures for MRI**

Sample preparation for MRI was performed as described previously. Deeply anesthetized mice were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and brains were carefully dissected and postfixed in the same fixative for one wk at 4°C. Brains were then embedded in 1% agarose (Nacalai Tesque, 02468–95) for MRI. MRI images were acquired using a 7T animal MRI system (MRI system, Agilent Technologies Inc., CA, USA). Diffusion tensor tractography (DTT) was performed as described previously. The FA threshold for tracking was set under 0.18, and the stop length was set at 160 steps. The bending angle of the tract was not allowed to exceed 45°.

**Statistical analysis**

Statistical analyses were performed using the Student t test or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test. The data were expressed as the mean ± standard error of the mean (SEM), and P-values <0.05 were considered statistically significant.

**Abbreviations**

AC = anterior commissure  
CALB = calbindin  
CC = corpus callosum  
CKO = conditional knockout  
CNS = central nervous system  
DCN = deep cerebellar nuclei  
DIV = ds in vitro  
DTM = diffusion tensor magnetic resonance  
ER = endoplasmic reticulum  
GFAP = glial fibrillary acid protein  
GFP = green fluorescent protein  
golgin A2 = hematoxylin eosin  
LV = lateral ventricle  
mitogen-activated protein kinase 8 interacting protein 3/JNK-interacting protein 3 = mitogen-activated protein kinase 8 interacting protein 3  
microtubule-associated protein 1B = microtubule-associated protein 1B  
microtubule-associated protein 1 light chain 3 = microtubule-associated protein 1 light chain 3  
NBR1 = neighbor of BRCA1 gene 1  
Pb = Probst bundle  
PC = posterior commissure  
PCN = primary cerebral cortical neuron  
phosphatidylinositol 3-kinase = phosphatidylinositol 3-kinase  
RB1 inducible coiled-coil = RB1 inducible coiled-coil  
solute carrier family 32 member 1 = solute carrier family 32 member 1  
quiescentose 1 = quiescentose 1  
TdT-mediated dUTP nick-end labeling = TdT-mediated dUTP nick-end labeling  
unc-51 like kinase 1 = unc-51 like kinase 1  
WD repeat domain 45 = WD repeat domain 45  
WM = white matter

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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