Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves

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Although Schwann cell myelin breakdown is the universal outcome of a remarkably wide range of conditions that cause disease or injury to peripheral nerves, the cellular and molecular mechanisms that make Schwann cell–mediated myelin digestion possible have not been established. We report that Schwann cells degrade myelin after injury by a novel form of selective autophagy, myelinophagy. Autophagy was up-regulated by myelinating Schwann cells after nerve injury, myelin debris was present in autophagosomes, and pharmacological and genetic inhibition of autophagy impaired myelin clearance. Myelinophagy was positively regulated by the Schwann cell JNK/c-Jun pathway, a central regulator of the Schwann cell reprogramming induced by nerve injury. We also present evidence that myelinophagy is defective in the injured central nervous system. These results reveal an important role for inductive autophagy during Wallerian degeneration, and point to potential mechanistic targets for accelerating myelin clearance and improving demyelinating disease.

Introduction

In peripheral nerves, myelin breakdown, or demyelination, is a universal outcome of a remarkably wide range of conditions that involve disturbance to Schwann cells or the nerve environment, whether due to genetic or acquired disease, toxicity, or nerve transection/crush. It has also become clear from studies on cut nerves that, perhaps surprisingly, Schwann cells themselves have the ability to turn against their own myelin and initiate myelin breakdown, in addition to being able to call on macrophages for myelin phagocytosis (Hirata and Kawabuchi, 2002). The maintenance of healthy myelin and normal nerve function depends on tight control of this intrinsic potential for myelin destruction. In contrast to Schwann cells, in dendrocytes, appear to be unable to digest myelin, a feature that has been linked to poor regenerative ability of CNS tissue (Brosius Lutz and Barres, 2014).

In spite of the central position of myelin breakdown in Schwann cell biology and pathology, the cellular and molecular mechanisms that make Schwann cell–mediated myelin digestion possible have not been established. While earlier authors were often cautious about myelin breakdown mechanisms (Holtzman and Novikoff, 1965), more recent literature frequently invokes phagocytosis as the mechanism by which Schwann cells digest their myelin after nerve transection/crush. But this notion is problematic. This is because phagocytosis is a process by which cells ingest cell-extrinsic material, but myelin is initially an intrinsic Schwann cell component, being an integral part of the Schwann cell membrane. Furthermore, there is no evidence that myelin separates from Schwann cells as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
Figure 1. Autophagy is activated during Wallerian degeneration. (A) Heat map showing qPCR analysis of changes in expression of autophagy-related genes in cut nerves in vivo at different time points (e.g., 2D Cut = 2 d after nerve cut) relative to control uncut nerves. Data are expressed as log2 fold change relative to uncut nerves (red-blue color scale). Significant changes (P < 0.05) are denoted by asterisks within the heat map. Bar graphs depicting fold changes of all the genes examined are shown in Fig. S1. (B and C) Western blotting showing expression of selected autophagy-related proteins (B),
Macroautophagy is an inducible degradation system by which cells break down their own organelles and large macromolecules. Autophagy involves the formation of an isolation membrane that extends around cytoplasmic cargo to form an autophagosome, which transfers cargo to the lysosome for degradation (Rubinstein et al., 2012). During starvation, autophagic degradation of cytoplasmic constituents provides a protective mechanism for energy release. In addition, specialized forms of autophagy mediate the delivery of specific cargo to the autophagosome, including intracellular pathogens (xenophagy; Levine et al., 2011), cellular organelles (mitophagy, ribophagy; Kiel, 2010), and storage vesicles such as lipid droplets (lipophagy; Singh et al., 2009).

While it has been understood for a long time that the first phase of myelin digestion in injured nerves is performed by Schwann cells, the cellular mechanism that enables Schwann cells to destroy their own myelin has not been established. Most nerve pathology involves breakdown of the myelin sheath, although demyelination occurs most acutely after injury, as part of the cellular reprogramming from myelin cells to repair cells. Therefore, understanding the molecular mechanisms of Schwann cell–mediated demyelination in injured nerves potentially has broader implications. Here, we present evidence that autophagy, a mechanism by which many cells digest their intrinsic cellular components, plays a central role in Schwann cell myelin breakdown. We show that nerve injury triggers strong activation of Schwann cell autophagy, find myelin debris in autophagosomes, and demonstrate a strong requirement for autophagy in myelin digestion, revealing a novel form of selective autophagy of the myelin sheath, myelinophagy.

**Results**

Autophagy is induced in Schwann cells in response to nerve injury

Transcriptional regulation of autophagy-related genes coincides with autophagy induction in several systems (Klionsky et al., 2012). To examine whether autophagy is involved in myelin degradation, we analyzed expression of autophagy-related genes broadly classified into functional subgroups comprising the core autophagy machinery and its main regulators (Feng et al., 2014). Sciatric nerves were transected and the distal stump was analyzed over the time course of demyelination. We found a strong induction of several autophagy-related genes during demyelination, notably members of the ULK complex and Atg9 cycling system, and Atg7, all essential for autophagosome formation (Fig. 1 A and Fig. S1). Concurrent with the degradative decline of myelin proteins MPZ and MBP, we observed an increase, compared with uninjured levels, of several ATG proteins including ATG7, components of the ATG16L complex, Wipi2, and Beclin1 in injured nerves, which is consistent with a role for autophagy in Wallerian degeneration (Fig. 1 B; quantified in Fig. S2).

Fluctuations in levels of lipidated LC3 (LC3 II) are frequently used to determine autophagy induction (Klionsky et al., 2012). We analyzed levels of LC3 II in response to injury in vivo and observed a clear increase in its levels, peaking at 5 d and decreasing thereafter. Conversely, levels of the autophagy substrate NBR1 (Klionsky et al., 2012) showed a clear decrease in injured nerves (Fig. 1 C).

p62, another autophagy substrate, could not be used since we found...
its levels to be transcriptionally regulated by nerve injury, unlike NBR1 (unpublished data). To confirm that this increase reflected an induction of autophagy, we examined autophagic flux in nerve segments maintained in vitro. In this established model of Schwann cell demyelination, short nerve segments are cultured in vitro, leading to progressive myelin breakdown, similar to the changes seen in vivo. Myelin breakdown is mainly performed by Schwann cells because invading macrophages are absent (Fernandez-Valle et al., 1995). We found that treatment with the lysosome inhibitor NH4Cl led to a strong accumulation of LC3 II and NBR1 in demyelinating nerve segments cultured in vitro for 5 d (5D Cut), but not in freshly isolated nerve segments (Uncut). Net LC3 II flux calculations confirmed the significantly higher autophagic flux in the demyelinating segments, compared with uncut segments (Fig. 1 D). To confirm these findings, we examined autophagy flux in dissociated Schwann cell cultures. In this model, highly pure myelinating Schwann cells, obtained from sciatic nerves of 8–10 d old rats, are plated and cultured over a period of time, leading to progressive myelin degradation in the cells. We found an accumulation of LC3 II and NBR1 in demyelinating cultures but not in freshly plated cells. These findings were confirmed by net LC3 II calculations (Fig. 1 E). Collectively, this indicates that up-regulation of LC3 II and breakdown of the autophagy substrate NBR1 after injury is a result of a strong and rapid autophagy induction, concurrent with the onset of myelin degradation.

To show that autophagy was induced specifically in demyelinating Schwann cells, we used mice expressing a GFP-tagged LC3 transgene, a model widely used to monitor autophagy in vivo (Mizushima et al., 2004). In cultured nerves from these mice, we observed the appearance of cleaved GFP bands, indicative of autophagic breakdown of cargo (Klionsky et al., 2012), during active demyelination (2, 5, and 7 d) but not when demyelination is essentially complete (10 d; Fig. 1 F). This confirmed our findings that autophagy is induced during active demyelination at 2–7 d after injury and down-regulated thereafter.

Uninjured myelinated fibers from these mice contained few or no GFP-LC3 punctate structures. In contrast, numerous GFP-LC3 puncta, representing autophagosomes (Klionsky et al., 2012), were associated with degenerating myelin in nerve segments maintained in vitro for 3–7 d (Fig. 1, G and H). The lysosomal marker LAMP1 has been shown to be associated with degenerating myelin fragments 3 and 7 d after nerve injury (Jung et al., 2011a). Similar results were obtained with dissociated Schwann cell cultures, where numerous GFP-LC3 puncta were observed in actively demyelinating Schwann cells (MPZ+), whereas myelin-negative cells were largely negative for GFP-LC3 puncta (Fig. 1 I). Together this indicates that in individual cells, autophagosome formation correlates with active myelin digestion.

To confirm that the Schwann cell autophagy machinery is selectively targeted to myelin during active digestion, electron microscopy of cultured nerve segments and Schwann cell cultures were performed. We found myelin debris clearly enclosed in double membrane vesicles typical of autophagosomes (Fig. 2). These observations show that autophagy is activated during Wallerian degeneration, specifically in demyelinating Schwann cells.

### Pharmacological inhibition of autophagy impairs demyelination

If Schwann cells degrade myelin by autophagy, autophagy inhibitors should reduce myelin breakdown. We therefore cultured nerve segments with the widely used autophagy inhibitors 3-methyladenine (3-MA) and Bafilomycin A1 (Klionsky et al., 2012). The lysosomal inhibitor NH4Cl was included for comparison. These inhibitors strikingly prevented the degradation of the myelin proteins MPZ and MBP in nerve segments maintained in vitro for 5 d but not in uncut nerve segments, indicating that autophagy is required for myelin protein breakdown (Fig. 3 A). This was not due to transcriptional changes, as we did not find significant differences in mRNA levels in nerve segments treated with these autophagy inhibitors (Fig. 3 B).

Histologically, we found that 3-MA reduced myelin collapse in cultured nerves compared with control nerves, in which most sheaths appeared as collapsed whorls (Fig. 3 C).

Lipids constitute 70–80% of peripheral myelin (Chrst et al., 2011), and lipid droplets can serve as autophagic cargo (Singh et al., 2009). Therefore, we examined lipid degradation in the presence of 3-MA. Nerve segments stained with FluoroMyelin, a dye for myelin lipids, showed a significantly greater fluorescent area after 3-MA treatment compared with controls, indicating a reduction in myelin lipid degradation in the absence of autophagosome formation (Fig. 3 D).

Finally, we examined the effects of pharmacological inhibition of autophagy specifically in demyelinating Schwann cells, using dissociated cell cultures. We found that inhibition of autophagy led to a notable reduction in myelin protein and lipid breakdown (Fig. 3, E–G), similar to that seen in nerve segments.

These results show that in injured nerves, Schwann cell autophagy is a major contributor to degradation of myelin proteins and lipids and to the collapse of myelin architecture.

### Genetic inactivation of autophagy

The results above (Fig. 3) suggest that autophagy inhibition leads to an impairment in myelin breakdown. However, since chemical modulators cannot be guaranteed to be entirely specific, we analyzed the role of autophagy in myelin breakdown using genetic ablation of autophagy function. For this, we generated mice that lack ATG7, an essential autophagy gene, specifically in Schwann cells. Mice carrying an Atg7fl/fl allele (Komatsu et al., 2005) were crossed with mice that express Cre recombinase in Schwann cells only (Mpz-Cre; Feltri et al., 2002) to generate Mpz-Cre+;Atg7fl/fl mice (Atg7 cKO) mice (Fig. S3, A–C).

The mutant mice were born and survived normally. Notably, the uninjured adult peripheral nerves of Atg7 conditional knockout (cKO) mice were indistinguishable from wild-type (WT) control litters. Sensitormotor function, histological appearance, G ratio, the levels of myelin proteins, and lipid composition were all normal (Fig. S3, D–G; see, however, Jang et al., 2015). This is in line with our findings above (Fig. 1), that autophagy is not significantly activated in mature uncut nerves.

Nerve homogenates from uninjured and injured ATG7 cKO mice showed strongly reduced ATG7, ATG5-ATG12, and LC3 II in uninjured and cut nerves, indicating impairment of autophagy. The residual proteins are likely to be expressed by cells other than Schwann cells present in the nerve (Fig. S4, A–C).

Striking differences were found when myelin breakdown was compared in Atg7 cKO and WT nerves after injury. A significant reduction was seen in injury-induced breakdown of myelin proteins in transected nerves in vivo (Fig. 4, A and B), in cultured nerve segments (Fig. S4 D), and in Schwann cell cultures where Atg7 cKO cells typically retained bloated “cauliflower” morphology for many days due to the persistence of undigested myelin (Fig. 4 C). In transected Atg7 cKO nerves,
there was also a striking preservation of noncollapsed myelin (Fig. 4 D), confirming previous results using the autophagy blocker 3-MA (Fig. 3 C).

Next, we analyzed whether myelin lipid degradation was also affected in cut nerves from the Atg7 cKO mice. Using FluoroMyelin histochemistry, we found that degradation of myelin lipid was reduced in Atg7 cKO nerves (Fig. 4 E). Second, using TLC-based screening, we examined the lipid composition of uncut and 5 d cut nerves from Atg7 cKO and WT mice. This revealed that up-regulation of cholesteryl esters, which are by-products of myelin lipid breakdown (Goodrum et al., 1994), was markedly lower in Atg7 cKO nerves than in WT ones, indicating slower myelin breakdown (Fig. 4 F). Finally, to differentiate between myelinophagy and the generic lipophagy that likely takes place in whole nerve extracts, we purified myelin from uncut and 5 d cut nerves, and used UPLC to reveal how the lipid composition of myelin changes during the 5 d degradation period. This showed that the typical down-regulation or up-regulation of a large proportion of lipid species seen in WT myelin was notably impaired in Atg7 cKO myelin and confirmed the difference in cholesteryl esters seen previously (Fig. 4 G and Table S2). These results demonstrate that inhibition of autophagy results in aberrant breakdown of the lipid component of the myelin sheath.

After injury, myelin and nonmyelin (Remak) Schwann cells undergo a c-Jun–dependent reprogramming to form repair Schwann cells (Arthur-Farraj et al., 2012). We examined whether this important cell type conversion was altered in Atg7 cKO nerves using global proteomic analysis. Injury (5 d cut nerves) resulted in substantial changes in the proteomic profile, involving both up- and down-regulation (Fig. S4 E), as described previously (Jiménez et al., 2005). Notably, we found that 12 of the 25 most strongly down-regulated proteins after nerve cut in WT nerves were down-regulated to a significantly lesser degree in Atg7 cKO mice (Fig. 5 A). Conversely, 11 of the 25 most strongly up-regulated proteins in WT nerves were up-regulated to a significantly lesser degree in Atg7 cKO mice (Fig. 5 B). To further confirm that generation of repair Schwann cells was affected in the Atg7 cKO mice, we examined expression of markers associated with these cells after injury (Arthur-Farraj et al., 2012). We found that the expression of p75NTR protein, and of the Shh,
Figure 3. **Pharmacological block of autophagy prevents myelin degradation.** (A) Western blot showing a block in degradation of the myelin proteins MPZ and MBP in nerve segments maintained in vitro for 5 d and treated with different pharmacological inhibitors: untreated, lysosomal inhibitor, NH₄Cl, and autophagy inhibitors 3-MA and bafilomycin A1 (Baf). No such effect is seen in freshly isolated nerve segments treated with these inhibitors for 3 h. The black line indicates that intervening lanes have been spliced out. Graphs show densitometric quantification of Western blots. Data are presented as mean ± SEM (error bars) from three independent experiments. n.s., nonsignificant; *, P < 0.05. (B) qPCR analysis showing no significant differences (n.s.) in mRNA levels of Mpz and Mbp in nerve segments maintained in vitro for 5 d and treated with 3-MA and Baf, compared with untreated segments. Data are expressed as log₂ fold change in 5D cultured nerve segments relative to uncut nerves. n = 3 for each condition. Data are presented as mean ± SEM (error bars). (C) Electron micrographs showing abundant intact myelin sheaths in nerve segments cultured in vitro for 4 d in the presence of 3-MA, compared
showing a block in degradation of MPZ in dissociated Schwann cells (S100+) cultured for 5 d in the presence of 3-MA. The graph shows MPZ+ area in different conditions. *, P < 0.05; **, P < 0.01 (treated cells relative to untreated controls).

Discussion

Myelinophagy is mTOR independent, driven by JNK/c-Jun, and reduced in the injured CNS

Because the control of myelin breakdown is a central issue in nerve injury and pathology, we studied the regulation of myelinophagy, examining the mTOR and JNK/c-Jun pathways, a mouse model of demyelinating neuropathy, and autophagy in the injured CNS.

In starvation autophagy, the best-studied model of autophagy regulation, autophagy is activated by reduction in the activity of the autophagy inhibitor mTOR (Ravikumar et al., 2010). Therefore, we examined levels of pmTOR, and pAKT and pS6, which are upstream and downstream markers for mTOR, respectively. While uninjured nerves showed low levels of pmTOR, pS6, and pAKT, these were significantly up-regulated at 5 and 7 d after transection (Fig. 6 A). In addition, neither mTOR activation by starvation (serum deprivation) nor mTOR inhibition by rapamycin altered the rate of myelin breakdown in Schwann cell cultures (Fig. 6, B and C). This was confirmed by analysis of myelin sheath collapse and measurements of autophagy flux in cultured nerve segments treated with rapamycin (Fig. S5, B and C). Treatment with insulin or neuregulin, stimulators of mTOR, also failed to affect myelin protein breakdown (Fig. 6 C). Together this shows that down-regulation of mTOR does not provide the signal for activation of Schwann cell autophagy.

In contrast, two alternative autophagy inducers reported to function through mTOR-independent pathways, lithium (LiCl) and ceramide (Ravikumar et al., 2010), accelerated the rate of myelin protein breakdown both in dissociated Schwann cells (Fig. 6, B and C) and in cultured nerve segments (Fig. 6 D). Notably, we found that LiCl and ceramide significantly increased autophagy flux in nerve segments maintained in vitro (Fig. 6 E). Strikingly, we found that the acceleration of myelin degradation in Schwann cell cultures treated by these two compounds was effectively blocked when autophagy was impaired by using Atg7 cKO Schwann cells (Fig. 6 F) or by using 3-MA (Fig. S5 D; quantification not shown). Collectively, these results show that Schwann cell autophagy is regulated by mTOR-independent pathways.

Ceramide has been proposed to act via the JNK/c-Jun pathway to promote autophagy (Ravikumar et al., 2010). We reported previously that c-Jun is a master regulator of the Schwann cell response to injury, and that myelin protein and lipid degradation are delayed in c-Jun cKO mice (Arthur-Farrar et al., 2012). Accordingly, treatment with JNK inhibitor SP600125 strongly inhibited myelin protein breakdown in Schwann cell cultures (Fig. 6, B and C). To test whether the JNK/c-Jun pathway regulated myelinophagy, we examined autophagic flux in nerves from WT mice treated with SP600125 and nerves from WT and c-Jun cKO mice. Autophagic flux was significantly reduced in SP600125-treated and c-Jun cKO nerves (Fig. 7, A and B). This indicates that the JNK/c-Jun pathway is involved in driving Schwann cell autophagy in injured nerves.

Previously, we showed aberrant up-regulation of Schwann cell c-Jun in human demyelinating neuropathies and in the C3 mouse model of the most common hereditary demyelinating neuropathy, Charcot-Marie-Tooth disease (CMT1A; Hutton et al., 2011; Verhamme et al., 2011; Hantke et al., 2014). We therefore postulated that C3 nerves might also show aberrant up-regulation of autophagy even in the absence of nerve injury. We found that compared with control nerves of the same age (8 mo), C3 nerves showed significant up-regulation of LC3 II (Fig. 7 C). This finding awaits further investigation, but it points to aberrant autophagy induction in response to myelin instability and suggests a common mechanism for Schwann cell–mediated demyelination in injury and disease.

Poor axonal regeneration in the CNS is in large part caused by the inability of oligodendrocytes to break down myelin effectively (Brosius Lutz and Barres, 2014). We therefore examined autophagy in the injured CNS. In the optic nerve, we found that LC3 II levels were not significantly elevated after injury, in contrast to the sciatic nerve (Fig. 7 D). This suggests that the failure of the CNS to clear myelin is associated with reduced activation of autophagy.
Figure 4. Genetic inactivation of autophagy retards myelin degradation in vivo. (A) Western blot showing higher levels of the myelin proteins MPZ, MBP, and Periaxin in 5 d cut nerves from Atg7 cKO mice compared with WT controls. (B) Densitometric analysis of Western blots showing higher levels of myelin proteins in 5 and 7 d cut nerves from Atg7 cKO mice compared with WT controls. For each comparison, the value for cKO is normalized to that seen in WT. n = a minimum of three mice for the genotype/time point. Data are presented as mean ± SEM (error bars). *, P < 0.05; **, P < 0.01 (Atg7 cKO relative to WT). (C) Immunolabeling showing MPZ+ myelin inclusions in cultured WT Schwann cells and cells from Atg7 cKO nerves that often show bloated “cauliflower” morphology. The graph shows quantification of MPZ+ area. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 60 cells analyzed per condition. **, P < 0.01 (Atg7 cKO relative to WT). (D) Electron micrographs showing several intact myelin sheath profiles in 5 d cut nerves from Atg7 cKO mice. The graph shows a quantification of the number of intact myelin sheaths. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 18 picture frames analyzed per condition/experiment. **, P < 0.01.
dication of a cell-autonomous mechanism for reduction of autophagy once myelin is digested. Collectively, these observations indicate that after injury of peripheral nerves, myelin proteins and lipids are selectively targeted for degradation by activation of myelin autophagy in Schwann cells.

This injury-induced Schwann cell myelinophagy is mTOR-independent. Namely, it is not activated by suppression of mTOR, the classical mechanism for autophagy activation, as we found that mTOR was activated, rather than suppressed, in transected nerves (Kroemer et al., 2010; Ravikumar et al., 2010). mTOR-independent autophagy has been described in other systems, and can be stimulated by lithium and ceramide (Ravikumar et al., 2010), both of which also promoted myelin autophagy in Schwann cells. The mechanism for activation of autophagy by mTOR suppression also exists in Schwann cells, however, because mTOR inhibition by starvation or rapamycin activates autophagy in Trembler J mice, where it functions to clear PMP22 myelin protein aggregates and improve myelination (Ranganarju et al., 2010).

Our data show that activation of autophagy is an important component of the reprogramming of Schwann cells that is triggered by nerve cut (Jessen et al., 2015). In injured nerves, we see activation of mTOR, a pathway that drives major biosynthetic processes and accelerates catabolism and growth. These functions are likely to be required during the dynamic Schwann cell response to injury, which, in addition to myelin breakdown, involves major reorganization of the molecular and morphological profile of myelin and nonmyelin (Remak) cells to generate repair (Bungner) Schwann cells (Arthur-Farraj et al., 2012). The coexistence of autophagy to promote removal of redundant myelin and mTOR activation therefore makes biological sense.

The JNK1/c-Jun pathway stimulates autophagy in several cell types, and ceramide stimulates autophagy in part by activating this pathway (Pattingre et al., 2009). In line with this, we find that pharmacological or genetic inhibition of JNK1/c-Jun reduces Schwann cell autophagic flux. Because nerve injury rapidly activates JNK1/c-Jun in Schwann cells (Parkinson et al., 2008), it is likely that the delay in myelin clearance seen when the JNK1/c-Jun pathway is inhibited pharmacologically or genetically is in part mediated by defective autophagy (Mirsy et al., 2008; Arthur-Farraj et al., 2012). Another potential link between JNK1/c-Jun and autophagy is seen in uninjured nerves of C3 mice that model CMT1A demyelinating neuropathy. We found that in these nerves, high Schwann cell c-Jun expression (Hantke et al., 2014) is accompanied by elevated LC3 II, which is consistent with autophagy activation.

Myelin breakdown in the CNS is ineffective. In line with this, we found a marked difference between the injured optic and sciatic nerves, as LC3 II levels, a key indicator of autophagy, were not significantly elevated in the injured optic nerve in contrast to the sciatic. It is intriguing that elevation of c-Jun in cut optic nerves is also minimal relative to that seen in Schwann cells (Vaudano et al., 1996), although the relevance of this to myelin breakdown and regeneration remains to be established.

In conclusion, we have identified myelinophagy as a major mechanism for Schwann cell-mediated myelin breakdown in injured nerves. We also provide evidence that myelin autophagy is aberrantly regulated in demyelinating peripheral neuropathy, and defective in CNS glia after injury. Identifying an endogenous Schwann cell mechanism for the myelin breakdown that takes place when nerves are cut has broader implications, since activation of autophagy can now be considered to be a plausible candidate mechanism for myelin breakdown in other situations. This includes acquired and inherited demyelinating disease and the age-related degeneration of myelin. Therefore, the recognition of this mechanism in Schwann cells potentially provides novel targets for manipulating demyelination in injury and disease. It also throws new light on the remarkable absence of myelin breakdown by oligodendrocytes. It would be important in future studies to test whether modulation of myelinophagy improves repair in both the injured peripheral nervous system and CNS, and prevents myelin breakdown in demyelinating neuropathies.

Materials and methods

Animals

Animal experiments conformed to UK Home Office guidelines under the supervision of UCL Biological Services, and to the Spanish/European regulations on animal use under the supervision of the institutional committee at CIC bioGUNE (AAALAC-accredited facility) and the Instituto de Neurociencias de Alicante.

Atg7$$^{+/+}$$ mice were made by insertion of loxP sequences and a stop codon within introns 13 and 14 of the atg7 gene (Komatsu et al., 2005). N. Mizushima (The University of Tokyo, Tokyo, Japan) allowed us to use the Atg7$$^{+/+}$$ mice and GFP-LC3 transgenic mice for these studies, and S. Toso (Cancer Research UK, London, England, UK) and A.K. Simon (University of Oxford, Headington, Oxford, England, UK) provided these mice.

Mpz-Cre mice express Cre recombinase under the control of a mouse mpz promoter and regulatory sequences on chromosome 15. This transgene contains the complete mouse mpz gene with 6 kb of promoter, in which the ATG start of translation has been mutated and substituted with Cre, and is expressed in Schwann cells, but not in other cell types (Feltiri et al., 2002). Mpz-Cre mice were provided by L. Feltiri and L. Wrabetz (State University of New York at Buffalo, Buffalo, NY).

These mice were crossed with Atg7$$^{+/+}$$ mice to generate the Mpz-Cre$$^{+/-}$$;Atg7$$^{+/+}$$ mice to obtain Schwann cells deficient in atg7.

Genotyping

DNA for genotyping was extracted from ear or tail samples using the Hot Sodium Hydroxide and Tris method (HotSHot; Truett et al., 2000).

(Atg7 cKO relative to WT). [E] Teased fibers of 5 d cut nerves from WT and Atg7 cKO mice stained with FluoroMyelin red to show myelin. The graph shows a quantification of the myelin fluorescent area. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 10 picture frames analyzed per condition/experiment. ***, P < 0.01 (Atg7 cKO relative to WT). [F] The lipid composition of whole sciatic nerves from 5 d cut WT and Atg7 cKO mice expressed as log2 fold change compared with uncut nerves. n = a minimum of four mice for each genotype. Data are presented as mean ± SEM (error bars). ***, P < 0.01. (G) The lipid composition of purified myelin obtained from sciatic nerves from 5 d cut WT and Atg7 cKO mice, expressed as log2 fold change compared with uncut nerves. The individual lipid species detected by UPLC were grouped in distinct lipid classes as shown in the graph. “Membrane lipids” refers to all lipid species detected that are the major structural lipids in the eukaryotic membrane, including phosphatidylethanolamines, phosphatidylcholines, and phosphatidylinositol species, and “storage lipids” include triacylglycerides and cholesteryl esters. See Table S2 for changes in levels of individual lipid species in WT and Atg7 cKO mice. n = 3 mice for each genotype. Data are presented as mean ± SEM (error bars). *, P < 0.05; **, P < 0.01 (Atg7 cKO relative to WT).
Figure 5. Genetic inactivation of autophagy retards the generation of repair cells after injury in vivo. (A and B) Graph showing fold change of the top 25 down-regulated (A) and up-regulated (B) proteins in 5 d cut nerves relative to uninjured nerves in WT and Atg7 cKO mice from proteomics analysis (Fig. S4 E). n = 5 mice for each genotype. Data are presented as mean ± SEM (error bars). *, P < 0.05 (Atg7 cKO relative to WT). (C) Western blot showing lower levels of the repair Schwann cell marker p75NTR in 5 d cut nerves from Atg7 cKO mice compared with WT controls. The graph shows densitometric analysis of Western blots. n = 3 mice for each genotype. Data are presented as mean ± SEM (error bars). **, P < 0.01. (D) qPCR analysis showing significantly lower levels of the mRNA levels of the repair Schwann cell markers Shh, GDNF, and Olig1 in 2, 5, and 7 d cut nerves from Atg7 cKO mice compared with WT controls. Data are expressed as fold change in cut nerves relative to uncut nerves. n = 3 mice for each genotype/time point. Data are presented as mean ± SEM (error bars). **, P < 0.01 [Atg7 cKO relative to WT].
The ear or tail samples were incubated in alkaline solution (25 mM NaOH and 0.2 mM disodium EDTA, pH 12) for 1 h at 95°C, cooled, and then mixed with neutralizing buffer (40 mM Tris-HCl, pH 5). 1 or 2 μl of this reaction mixture was used for a 25-μl PCR reaction.

Primers for genotyping Atg7 flox/flox were 5′-TGGCTGC-TACTTCTGCAATGATG-3′ and 5′-CAGGACAGAGACCAT-CAGCTCCAC-3′ (1,500-bp band for WT allele and 550-bp band for flox allele; Komatsu et al., 2005). The primers for the mpz-cre transgene are 5′-GCTGGCCCAAAATGTTGCTGG-3′ and 5′-CCACCACCTCTC-CATTGCAC-3′ (480-bp band; Feltrì et al., 2002).

Models to study Schwann cell myelin breakdown
We used three models to study Schwann cell myelin breakdown in response to nerve injury. (1) After nerve transaction (6–8 wk old mice), which results in axonal degeneration, the distal stump was most commonly cut into further 2–3-mm segments and left in situ, most commonly for 3–5 d, before examination. (2) After nerve transaction (6–8 wk old mice), the distal stump was removed, cut into 3–5 mm segments, and maintained in tissue culture most commonly for 3–5 d before analysis (nerve segment experiments). (3) Schwann cell cultures were generated by enzymatic dissociation of postnatal day 10 (P10) nerves (that are already extensively myelinated).

Surgical nerve injury
Sciatic nerve surgeries were done as described previously (Woodhoo et al., 2009). The right sciatic nerve was exposed and transected at the sciatic notch (distal stump). Skin was closed using veterinary autoclips. The transected nerve was excised for analysis at various time points. Contralateral uninjured sciatic nerves were used as controls for Western blotting, immunohistochemistry, or electron microscopy analysis.

Optic nerve surgeries were done as described previously (Parrilla-Reverter et al., 2009). The left optic nerve transection was performed intracranially, 3 mm from the posterior pole of the eye. Contralateral uninjured optic nerves were used as controls for Western blotting. Schwann cell autophagy

Surgical nerve segments and Schwann cell culture
Sciatic nerves from adult mice were dissected and placed in L15 medium on ice. The epineurium and superficial connective tissue were removed using forceps. Desheathed nerves were then cut into 5-mm segments, transferred to DMEM supplemented with 5% FBS, and incubated at 37°C/5% CO2. Segments were removed from culture for 2 h at RT in 4% paraformaldehyde for 1 h. The epineurium and superfluous connective tissue were dissected, and the epineurium was trimmed with neutralizing buffer (40 mM Tris-HCl, pH 5). 1 or 2 μl of this reaction mixture was used for a 25-μl PCR reaction.

For dissociation, nerves were transferred to a dissociating enzyme solution containing 10 μg/ml collagenase in Kreb’s Ca2+ and Mg2+ free medium plus 0.25% trypsin and placed at 37°C/5% CO2 for 30 min. Nerves were triturated gently before the addition of 10% FBS/DMEM and centrifuged at 1,000 rpm for 10 min. The cells were plated as drops on coverslips coated with poly-l-lysine and laminin. Cells were incubated at 37°C/5% CO2, and dissociated Schwann cell cultures were fixed with 1% OsO4, embedded in Agar 100 epoxy resin, and sectioned osmicated in 1% osmium tetroxide and 1.5% potassium ferrocyanide.

Electron microscopy
Samples were fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C. Samples were post-fixed with 1% OsO4, embedded in Agar 100 epoxy resin, and sectioned at 70 nm. Transverse ultrathin sections of tibial nerve at 3 mm from the cut site or uncut nerve were mounted on film and 18–20 photographs were taken at 2,500x using an electron microscope (1010; Jeol) with Gatan software and analyzed with ImageJ software.

For detection of autophagosomes, a modified version of the electron microscopy protocol was used. In brief, cells or nerve segments were fixed in fixative solution (2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C), and then osmicated in 1% osmium tetroxide and 1.5% potassium ferrocyanide.
Figure 6. Myelinophagy is mTOR independent and promoted by lithium and ceramide. (A) Western blot showing increased expression of p-mTOR, p-S6, and p-AKT in cut WT nerves. Quantification of Western blots is shown in Fig. S5A. (B) Immunolabeling showing regulation of MPZ breakdown by rapamycin, ceramide, or JNK inhibitor SP600125 in Schwann cell cultures (from P8 mice animals, treated 3 d in vitro). Myelin breakdown is unchanged by rapamycin, reduced by ceramide, and increased by JNK inhibitor compared with untreated cultures. (C) Graph showing MPZ+ myelin area in Schwann cell cultures (as in Fig. 6B) after different treatments. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 200 cells analyzed per condition/experiment. *, P < 0.05; **, P < 0.01 (treated cells relative to untreated controls). (D) Electron micrographs showing fewer intact myelin sheaths in nerve segments maintained in vitro for 4 d in the presence of ceramide and lithium, compared with control cultures. The graph shows quantification of the number of intact myelin sheaths in control segments and segments treated with ceramide and lithium. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 10 picture frames analyzed per condition/experiment. *, P < 0.05; **, P < 0.01 (treated cells relative to untreated controls). (E) Immunoblot showing increased LC3II expression and decreased LC3I expression in lithium and ceramide cultures. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 200 cells analyzed per condition/experiment. *, P < 0.05; **, P < 0.01 (treated cells relative to untreated controls). (F) Graph showing percentage of MPZ+ area in untreated, ceramide, and lithium cultures. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 10 picture frames analyzed per condition/experiment. *, P < 0.05; **, P < 0.01 (treated cells relative to untreated controls).
Figure 7. Regulation of myelinophagy. (A and B) Graph showing reduced LC3 II accumulation in nerve segments (A) maintained in vitro for 5 d and treated with JNK inhibitor in the presence and absence of NH4Cl (3 h treatment) and in nerve segments (B) maintained in vitro for 5 d from cjun cKO mice compared with WT mice, in the presence and absence of NH4Cl (3 h treatment). Graphs show reduced net LC3 II flux. Data are presented as mean ± SEM (error bars) from three independent experiments. **, P < 0.01 (treated cells relative to untreated cells; cjun cKO relative to WT). (C) Western blots showing elevated LC3 II levels in uninjured C3 nerves compared with WT nerves. The graph shows densitometric quantification of blots. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05 (sciatic nerves relative to optic nerves); **, P < 0.01 (cut sciatic nerves relative to uncut nerves). (D) Western blots showing that LC3 II levels in optic nerves are substantially lower than in sciatic nerve 3 and 5 d after cut. The graph shows densitometric quantification of blots. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05 (C3 mice relative to WT). (E) Western blots showing elevated LC3 II levels in uninjured C3 nerves compared with WT nerves. The graph shows densitometric quantification of blots. Data are presented as mean ± SEM (error bars) from three independent experiments. **, P < 0.01 (treated cells relative to untreated cells; cJun cKO relative to WT). (F) Graph showing quantification of MPZ+ area in control Schwann cell cultures (WT) and cultures in which autophagy was blocked (Atg7 cKO cultures). The increased myelin degradation of MPZ seen after treatment with ceramide and lithium in WT dissociated Schwann cell cultures is blocked in Atg7 cKO cultures. See Fig. S5 D for pictures of immunolabeling. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of 10 picture frames analyzed per condition/experiment. *, P < 0.05; ns, not significant (treated cells relative to untreated controls).
proteomic analysis

Protein from five WT and five cKO samples (both uncut and cut nerves) was extracted in a buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS. After in-solution tryptic digestion, peptide mixtures obtained from the digestions were separated by on-line NanoLC (nLC) and analyzed using electrospray tandem mass spectrometry. Peptide separation was performed on a nanoACQUITY UPLC system (Waters) connected to a mass spectrometer (LTQ Orbitrap XL ETD; Thermo Fisher Scientific).

Progenesis LC-MS software (Nonlinear Dynamics) was used for differential protein expression analysis. Raw files were imported into the program, and one of the samples was selected as a reference run to which the precursor masses in all the other samples were aligned. Abundance ratios between the run to be aligned and the reference run were calculated for all features at given retention times. These values were then logarithimized and the software, based on the analysis of the distribution of all ratios, automatically calculated a global scaling factor. Once normalized, samples were grouped in the corresponding experimental category and compared. Differences between groups were only considered for peptide abundances with an ANOVA p-value <0.05 and a ratio >1.5 in either direction were considered as significantly deregulated. To calculate total protein volumes, searches with unfiltered data were also performed following the same approach. The fold change between cut nerves and their respective control uncut nerves were then calculated for each replicate.

Western blotting

Nerves from surgical transection or cultured nerve segments were snap-frozen in liquid nitrogen and transferred to tubes containing 10–12 10B lysing matrix beads and 100 µl of lysis buffer (5 M urea, 2.5% SDS, 50 mM Tris, and 30 mM NaCl). Samples were placed in a Fastprep homogenizer for 45 s at level 6, chilled on ice, and then subjected to a repeat homogenization at the same setting. Lysates were then centrifuged at 4°C at 10,000 rpm for 2 min. Supernatants were transferred to new tubes and centrifuged again at the same settings. New supernatants were collected and stored at −80°C.

Cultured Schwann cells were extracted by cell scraping. Cells on 35-mm tissue culture dishes were washed twice with PBS before addition of 100 µl of lysis buffer (T-PER Tissue Protein Extraction Reagent; Thermo Fisher Scientific). Cells were scraped with a plastic cell lifter and the lysate was collected. Lysates were then centrifuged at 4°C at 10,000 rpm for 2 min. Supernatants were transferred to new tubes and centrifuged again at the same settings. New supernatants were collected and stored at −80°C.

Between 5 µg and 40 µg of protein samples were loaded onto acrylamide gels and run alongside prestained standard molecular weight markers (PageRuler prestained protein ladder; Thermo Fisher Scientific) to enable identification of band sizes. Gels were run using the mini Protein II gel electrophoresis apparatus (Bio-Rad Laboratories). Gels were transferred to nitrocellulose (Hybond ECL; Amersham Biosciences) or PVDF (Immobilon-P; EMD Millipore) membranes using the Bio-Rad Laboratories semi-dry system.

Membranes were blocked in 5% fat-free milk powder (Sigma-Aldrich) in PBS for 1 h (at room temperature) and subsequently incubated overnight at 4°C in primary antibodies diluted in 5% milk/PBS on a slow rotator (Gallenkamp). HRP-conjugated secondary antibodies (1:2,000) were used (Jackson ImmunoResearch Laboratories) and membranes were developed with ECL reagent (GE Healthcare). Experiments were repeated at least three times with fresh samples and representative pictures are shown. Densitometric quantification of Western blots was performed using ImageJ analysis software or Image Lab 4.1 (Bio-Rad Laboratories). The measurements were normalized to loading controls (GAPDH or β-Actin), and for each experiment, at least three different biological replicates were used.
Behavioral tests

All experiments were performed according to UK Home Office guidelines. Four mice from each genotype were tested and at least three measurements from each mouse were taken to obtain mean values.

Walking beam. Mice walked along a wooden beam 1 m long, 20 cm high, and 0.5 cm wide to assess sensorimotor function. Foot slips and beam falls were counted over the middle distance of 70 cm. A performance score was given as follows: 0 and 1 foot slips = a score of 1; 2–5 foot slips = a score of 2; >5 foot slips or at least 1 beam fall = a score of 3. Mice were first accustomed to the beam in training sessions on two days that included walking on wider beams of 2 cm and 1.2 cm. At the end of the second day, mice were scored on the 0.5 cm beam.

Sciatic function index (SFI). Paw prints were taken from 6-week-old mice to calculate the SFI. The SFI formula correlates mouse paw prints with nerve function, as described previously (Inserra et al., 1998). The hind limb paws of the mice were painted on the plantar surface with black paint. Mice then walked down a 54 × 5-cm wooden corridor lined with 200 gsm highly absorbent paper (Goldline). Prints were subsequently scanned (Perfection 3100; Epson) to generate digital paw prints. Toe spread (the distance between the first and fifth toes) and print length (the distance between the third toe and the heel) were measured using ImageJ, and Excel (Microsoft) was used to convert the measurements into SFI values using an established formula (Inserra et al., 1998). The SFI formula is: SFI = 118.9 (ETS − NTS/NTS) − 51.2 (EPL − NPL/NPL) − 7.5, where ETS is experimental toe spread, NTS is normal toe spread, and EPL is experimental paw length, and NPL is normal paw length. An SFI value of −100 represents complete loss of function.

Hanging wire test. Masking tape was placed around the perimeter of a cage lid. Mice were placed individually on top of the lid, which was then slowly turned around with the mouse hanging on upside down by all four legs. Mice were allowed to hang for 2 min. Latency until fall was noted, unless mice remained on the lid for the full 2 min.

Net LC3 II flux

Net LC3 II flux was calculated by analyzing LC3 II turnover by Western blotting in the presence or absence of lysosomal inhibitors. In brief, net LC3 II flux was calculated by subtracting the densitometry value of normalized (relative to GAPDH) LC3 II in the sample treated with lysosomal inhibitor by the value in the control sample (untreated). A minimum of three biological replicate experiments were performed for net LC3 II flux calculations.

Statistics

Values are given as means ± SEM (n = 3 unless otherwise stated). Statistical significance was analyzed with the Student’s t test and p-values <0.05 were considered significant.

Online supplemental material

Fig. S1 shows qPCR analysis of autophagy-related genes from Fig. 1A. Fig. S2 shows densitometric quantification of Western blots from Fig. 1. Fig. S3 shows that uninjured Atg7 cKO nerves are normal using biochemical and biochemical analyses. Fig. S4 shows that Atg7 cKO nerves have reduced autophagy activation after nerve injury, and that this retards myelin degradation. Fig. S5 shows that myelination is mTOR-independent, and is regulated by ceramide and lithium. Table S1 shows the list of primers used for qPCR analysis. Table S2 shows changes in levels of individual lipid species after nerve injury in Atg7 WT and cKO mice as determined by UPLC analysis of purified myelin. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201503019/DC1.

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References


Supplemental material

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Figure S1. qPCR analysis of autophagy-related genes. Bar graphs depicting changes in expression of autophagy-related genes after nerve injury, as shown in Fig. 1 A. n = 3 mice for each time point. Data are presented as mean ± SEM (error bars). *, P < 0.05.
Figure S2. 

Western blot quantification. [A] Densitometric quantification of Western blots from Fig. 1 B. For each comparison, the value for cut nerves is normalized to that seen in uncut nerves. [B] Densitometric quantification of Western blots from Fig. 1 C. For each comparison, the value for cut nerves is normalized to that seen in uncut nerves. [C] Densitometric quantification of NBR1 Western blots from Fig. 1 D. For each comparison, the values for different conditions are normalized to those seen in uncut nerves without NH\textsubscript{4}Cl treatment. [D] Densitometric quantification of NBR1 Western blots from Fig. 1 E. For each comparison, the values for different conditions are normalized to those seen in freshly plated Schwann cell cultures without NH\textsubscript{4}Cl treatment. [E] Densitometric quantification of Western blots from Fig. 1 F. For each comparison, the value for nerve segments maintained in vitro for different time points is normalized to that seen in uncut nerves. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05; n.s., not significant.
Figure S3. **Uninjured Atg7 cKO nerves are normal.** (A) RT-PCR showing the mutant allele in genomic DNA samples from P8 purified Schwann cells from Atg7 cKO mice. (B) RT-PCR showing reduced expression of Atg7 mRNA in P8 purified Schwann cells from Atg7 cKO mice. (C) Western blot showing reduced expression of ATG7 in P8 Schwann cells (~95% pure) from Atg7 cKO mice. The residual ATG7 band that remains is likely due to the presence of contamination with fibroblasts (~5% of total cell population). (D) Behavioral analyses (sciatic function index [SFI], hanging wire, and walking beam) show that P90 Atg7 cKO mice fed ad libitum have normal sensorimotor function. Sciatic function index: Atg7 cKO mice and control mouse paws were analyzed for toe spreading and footprint length, and used to calculate SFI values. Atg7 cKO mice and control mice were within a healthy range of SFI values and were not found to be significantly different. Hanging wire: Atg7 cKO and control mice were allowed to hang upside-down from a grid for 2.0 min to assess grip strength. Atg7 cKO mice and control mice all remained on the grid for the full length of time and were not found to be significantly different. Walking beam: Atg7 cKO and control mice were allowed to walk across a 0.5-cm beam and were scored on foot slips and falls (higher score = worse performance). Atg7 cKO mice and control mice all scored in the lowest range and performed as expected for healthy mice. n = 4 mice for each genotype. Data are presented as mean ± SEM (error bars); individual p-values are shown. (E) Electron micrographs showing normal myelin profile in uncut nerves from P90 Atg7 cKO mice fed ad libitum. Box plots correspond to center quartiles, with the black bar indicating the median, and whiskers extend from the 5th to the 95th percentiles. (F) Western blot analysis showing normal levels of myelin proteins MPZ, MBP, and periaxin in uncut nerves from P90 control and Atg7 cKO mice fed ad libitum. Graphs show densitometric analyses of these myelin proteins (relative to Gapdh). n = a minimum of four mice for each genotype. Data are presented as mean ± SEM (error bars); individual p-values are shown. (G) Thin-layer chromatography analysis shows no significant differences in the amount of different lipid classes in control and Atg7 cKO mice. n = a minimum of 4 mice for each genotype. Data are presented as mean ± SEM (error bars); individual p-values are shown.
Figure S4. Genetic inactivation of autophagy retards myelin degradation. (A) Western blot showing reduced levels of ATG7 and ATG5-ATG12 in uncut and 5 d cut nerves from Atg7 cKO mice, compared with WT nerves. In addition, the increase in LC3 II levels seen after nerve cut in WT nerves compared with uncut nerves is not seen after nerve transection in Atg7 cKO mice. Please note that Atg7 cKO nerves have residual expression of ATG7 and ATG5-ATG12, which is likely due to the presence of contaminating cells in the nerves, including endoneurial and perineurial fibroblasts, and resident macrophages, which also activate autophagy [C]. (B) Densitometric quantification of Western blots shows significantly lower levels of ATG7, ATG5-ATG12, and LC3 II in uncut and 5 d cut nerves from Atg7 cKO mice, compared with WT nerves. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05 (Atg7 cKO relative to WT). (C) Immunolabeling showing expression of GFP-LC3 puncta in F4/80+ macrophages found in 5 d cut nerves. (D) Western blot analysis showing reduced degradation of the myelin proteins MPZ and MBP from Atg7 cKO nerve segments maintained in vitro for 5 and 7 d. Densitometric analysis of Western blots showing higher levels of myelin proteins in nerve segments from Atg7 cKO mice compared with WT controls. For each comparison, the value for cKO is normalized to that seen in WT. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05 (Atg7 cKO relative to WT). (E) Heat map showing proteomics analysis of 5 d cut nerves from control and Atg7 cKO mice. Data are expressed as log2 fold change of cut nerves relative to control uninjured nerves (red-blue color scale) for each of five replicates (1–5) for each genotype.
Figure S5. Regulation of myelinophagy. (A) Densitometric quantification of Western blots from Fig. 6 A. For each comparison, the value for cut nerves is normalized to that seen in uncut nerves. Data are presented as mean ± SEM (error bars) from three independent experiments. *, P < 0.05 (cut nerves relative to uncut nerves). (B) Western blot showing increased LC3 II accumulation in nerve segments maintained in vitro for 5 d in the presence of NH4Cl (3 h treatment). The graph shows no significant difference in net LC3 II flux after rapamycin treatment compared with control cultures. Data are presented as mean ± SEM (error bars) from three independent experiments. (C) Electron micrographs showing similar numbers of intact myelin sheaths in nerve segments cultured in vitro for 3 d in the presence of rapamycin, compared with control cultures. The graph shows quantification of the number of intact myelin sheaths in control segments and segments treated with rapamycin. Data are presented as mean ± SEM (error bars) from three independent experiments with a minimum of picture frames analyzed per condition/experiment. *, P < 0.05. (D) Immunolabeling showing regulation of MPZ breakdown by ceramide or lithium in dissociated Schwann cell cultures from P8 WT or Atg7 cKO mice, treated 3 d in vitro.
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<td>TGCGTGGGGGCTGACGATT</td>
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<tr>
<td>Mbp</td>
<td>NM_001025251.2</td>
<td>AGATCGAGCGGCCAGGCT</td>
<td>TGCGTGGGGGCTGACGATT</td>
</tr>
</tbody>
</table>

**Table S1. List of primers used for RT-qPCR**
Table S1. List of primers used for RT-qPCR (Continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Sense sequence (5′→3′)</th>
<th>Antisense sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>NM_001289726.1</td>
<td>TGCACCACCAACTGCTTAG</td>
<td>GGATGCAGGGATGATGTTC</td>
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<tr>
<td>Shh</td>
<td>NM_009170.3</td>
<td>AAAGCTGACCCCTTTAGCCTA</td>
<td>TTCGGAGTTITCTTGTGATCTTCC</td>
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<tr>
<td>Olig1</td>
<td>NM_016968.4</td>
<td>CCGCCCCAGATGTACTATGC</td>
<td>AACCCACCAGCTCATAACGC</td>
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<tr>
<td>Gdnf</td>
<td>NM_010275.3</td>
<td>GATTCGGGCCACTTGGAGTT</td>
<td>GACAGCCACGACATCCCAT</td>
</tr>
</tbody>
</table>

Table S2 shows changes in levels of individual lipid species after nerve injury in ATG7 WT and cKO mice as determined by UPLC analysis of purified myelin and is provided as a Word file.