Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis

Yi Cao¹, Janice A. Espinola¹, Elisa Fossale¹, Ashish C. Massey², Ana Maria Cuervo², Marcy E. MacDonald¹, and Susan L. Cotman¹

¹Molecular Neurogenetics Unit and Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA 02114
²Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461

Running Title: Disruption of autophagy in JNCL

Address correspondence to: Susan L. Cotman, Molecular Neurogenetics Unit and Center for Human Genetic Research, Massachusetts General Hospital, 185 Cambridge St., Boston, MA 02114; Tel. 617 726-9180; Fax. 617 726-5735; E-mail: cotman@helix.mgh.harvard.edu

Juvenile neuronal ceroid lipofuscinosis is caused by mutation of a novel, endosomal/lysosomal membrane protein encoded by CLN3. The observation that the mitochondrial ATPase subunit c protein accumulates in this disease suggests that autophagy, a pathway that regulates mitochondrial turnover, may be disrupted. To test this hypothesis, we examined the autophagic pathway in Cln3⁰æ⁻/⁻ knock-in mice and CbCln3³⁻⁻⁰æ⁻/⁻ cerebellar cells, accurate genetic models of juvenile neuronal ceroid lipofuscinosis. In homozygous knock-in mice, we found that the autophagy marker LC3-II was increased and mTOR was downregulated. Moreover, isolated autophagic vacuoles and lysosomes from homozygous knock-in mice were less mature in their ultrastructural morphology than the wild-type organelles, and subunit c accumulated in autophagic vacuoles. Intriguingly, we also observed subunit c accumulation in autophagic vacuoles in normal aging mice. Upon further investigation of the autophagic pathway in homozygous knock-in cerebellar cells, we found that LC3-positive vesicles were altered and overlap of endocytic and lysosomal dyes was reduced when autophagy was stimulated, compared to wild-type cells. Surprisingly, however, stimulation of autophagy did not significantly impact cell survival, but inhibition of autophagy led to cell death. Together these observations suggest that autophagy is disrupted in juvenile neuronal ceroid lipofuscinosis, likely at the level of autophagic vacuolar maturation, and that activation of autophagy may be a pro-survival feedback response in the disease process.

INTRODUCTION

Macroautophagy (herein referred to as autophagy) is a non-selective process by which cytoplasmic constituents are turned over [reviewed in (1,2)]. Elegant work in yeast has led to the genetic dissection of many of the proteins involved in this pathway (3,4), which are mostly conserved in mammals (5,6). The pathway is initiated at times of stress or nutritional deprivation to generate metabolites for cellular survival, but autophagy is also responsible for normal housekeeping. Although the regulatory control of autophagy remains to be fully elucidated, the general process from engulfment to degradation has been delineated (2). Initiation of the pathway involves de novo formation of an isolation membrane that expands to engulf cytoplasmic vacuoles and lysosomes from homozygous knock-in mice were less mature in their ultrastructural morphology than the wild-type organelles, and subunit c accumulated in autophagic vacuoles. Intriguingly, we also observed subunit c accumulation in autophagic vacuoles in normal aging mice. Upon further investigation of the autophagic pathway in homozygous knock-in cerebellar cells, we found that LC3-positive vesicles were altered and overlap of endocytic and lysosomal dyes was reduced when autophagy was stimulated, compared to wild-type cells. Surprisingly, however, stimulation of autophagy did not significantly impact cell survival, but inhibition of autophagy led to cell death. Together these observations suggest that autophagy is disrupted in juvenile neuronal ceroid lipofuscinosis, likely at the level of autophagic vacuolar maturation, and that activation of autophagy may be a pro-survival feedback response in the disease process.

INTRODUCTION

Macroautophagy (herein referred to as autophagy) is a non-selective process by which cytoplasmic constituents are turned over [reviewed in (1,2)]. Elegant work in yeast has led to the genetic dissection of many of the proteins involved in this pathway (3,4), which are mostly conserved in mammals (5,6). The pathway is initiated at times of stress or nutritional deprivation to generate metabolites for cellular survival, but autophagy is also responsible for normal housekeeping. Although the regulatory control of autophagy remains to be fully elucidated, the general process from engulfment to degradation has been delineated (2). Initiation of the pathway involves de novo formation of an isolation membrane that expands to engulf cytoplasmic vacuoles and lysosomes from homozygous knock-in mice were less mature in their ultrastructural morphology than the wild-type organelles, and subunit c accumulated in autophagic vacuoles. Intriguingly, we also observed subunit c accumulation in autophagic vacuoles in normal aging mice. Upon further investigation of the autophagic pathway in homozygous knock-in cerebellar cells, we found that LC3-positive vesicles were altered and overlap of endocytic and lysosomal dyes was reduced when autophagy was stimulated, compared to wild-type cells. Surprisingly, however, stimulation of autophagy did not significantly impact cell survival, but inhibition of autophagy led to cell death. Together these observations suggest that autophagy is disrupted in juvenile neuronal ceroid lipofuscinosis, likely at the level of autophagic vacuolar maturation, and that activation of autophagy may be a pro-survival feedback response in the disease process.

INTRODUCTION

Macroautophagy (herein referred to as autophagy) is a non-selective process by which cytoplasmic constituents are turned over [reviewed in (1,2)]. Elegant work in yeast has led to the genetic dissection of many of the proteins involved in this pathway (3,4), which are mostly conserved in mammals (5,6). The pathway is initiated at times of stress or nutritional deprivation to generate metabolites for cellular survival, but autophagy is also responsible for normal housekeeping. Although the regulatory control of autophagy remains to be fully elucidated, the general process from engulfment to degradation has been delineated (2). Initiation of the pathway involves de novo formation of an isolation membrane that expands to engulf cytoplasmic vacuoles and lysosomes from homozygous knock-in mice were less mature in their ultrastructural morphology than the wild-type organelles, and subunit c accumulated in autophagic vacuoles. Intriguingly, we also observed subunit c accumulation in autophagic vacuoles in normal aging mice. Upon further investigation of the autophagic pathway in homozygous knock-in cerebellar cells, we found that LC3-positive vesicles were altered and overlap of endocytic and lysosomal dyes was reduced when autophagy was stimulated, compared to wild-type cells. Surprisingly, however, stimulation of autophagy did not significantly impact cell survival, but inhibition of autophagy led to cell death. Together these observations suggest that autophagy is disrupted in juvenile neuronal ceroid lipofuscinosis, likely at the level of autophagic vacuolar maturation, and that activation of autophagy may be a pro-survival feedback response in the disease process.

INTRODUCTION

Macroautophagy (herein referred to as autophagy) is a non-selective process by which cytoplasmic constituents are turned over [reviewed in (1,2)]. Elegant work in yeast has led to the genetic dissection of many of the proteins involved in this pathway (3,4), which are mostly conserved in mammals (5,6). The pathway is initiated at times of stress or nutritional deprivation to generate metabolites for cellular survival, but autophagy is also responsible for normal housekeeping. Although the regulatory control of autophagy remains to be fully elucidated, the general process from engulfment to degradation has been delineated (2). Initiation of the pathway involves de novo formation of an isolation membrane that expands to engulf cytoplasmic vacuoles and lysosomes from homozygous knock-in mice were less mature in their ultrastructural morphology than the wild-type organelles, and subunit c accumulated in autophagic vacuoles. Intriguingly, we also observed subunit c accumulation in autophagic vacuoles in normal aging mice. Upon further investigation of the autophagic pathway in homozygous knock-in cerebellar cells, we found that LC3-positive vesicles were altered and overlap of endocytic and lysosomal dyes was reduced when autophagy was stimulated, compared to wild-type cells. Surprisingly, however, stimulation of autophagy did not significantly impact cell survival, but inhibition of autophagy led to cell death. Together these observations suggest that autophagy is disrupted in juvenile neuronal ceroid lipofuscinosis, likely at the level of autophagic vacuolar maturation, and that activation of autophagy may be a pro-survival feedback response in the disease process.
diseases including Parkinson’s disease (7,8), Huntington’s disease (9-11), and Alzheimer’s disease (12,13). In each case, autophagic vacuoles accumulate, suggesting activation of autophagy is a common feature of neurodegeneration. However, the events leading to autophagic activation and how these impact any given disease process is not yet understood, and it is likely that autophagic involvement will differ in each case since the diseases are clinically and pathologically distinct.

The neuronal ceroid lipofuscinoses (NCLs) are the most common cause of neurodegeneration among children. These recessively inherited disorders typically manifest with blindness, seizures, progressive cognitive and motor failure, and the NCLs are always fatal (14). Although heterogeneous in genetic origin and some disease features including age-at-onset, nearly all of the NCLs lead to lysosomal accumulation of subunit c of the mitochondrial ATPase F0 complex (15), implicating defects in the turnover of this protein via the autophagic pathway. At least 7 distinct genetic loci cause NCL with subunit c accumulation [reviewed in (16)]. Juvenile-onset NCL (JNCL; Batten disease; MIM204200), the most common form of NCL, results from recessive inheritance of CLN3 mutations (17). A major founder mutation (a 1.02 kb genomic deletion) in the CLN3 gene accounts for the majority of JNCL disease alleles (17). This mutation eliminates exons 7 and 8 and the surrounding intronic DNA, giving rise to multiple stably produced mutant CLN3 mRNA species encoding multiple mutant protein isoforms, which are presumably non-functional (17,18). The full-length CLN3-encoded protein, battenin (also called CLN3, CLN3P), is a novel multi-pass transmembrane protein that localizes to the late endosomal/lysosomal membrane where it has been implicated in pH and amino acid homeostasis (19,20) and vesicle trafficking (21,22).

We have established knock-in mouse (Cln3<sup>ex7/8</sup> mice) (18) and cerebellar culture models (CbCln3<sup>ex7/8</sup> cells) (22) that faithfully replicate the common JNCL mutation in the murine Cln3 gene. As in JNCL, central nervous system neurons and non-central nervous system cells in homozygous Cln3<sup>ex7/8</sup> mice exhibit progressive accumulation of subunit c that begins embryonically (18), and homozygous CbCln3<sup>ex7/8</sup> cerebellar cells accumulate subunit c when induced by aging at confluency (22). In the studies described herein, we test the hypothesis that autophagy is involved in the JNCL disease process and the hallmark subunit c accumulation characteristic of NCL. These results provide strong evidence supporting disruption of autophagy in JNCL and a direct role for the CLN3-encoded protein, battenin, in normal autophagy.

**EXPERIMENTAL PROCEDURES**

**Reagents** - Antibodies used in these studies were: anti-LC3 antibodies (#AP1802a, Abgent, Inc., and a generous gift from Dr. Noboru Mizushima), anti-mCln3p (23) and anti-subunit c (24) antibodies (generous gifts from Dr. Junji Ezaki and Dr. Eiki Kominami), anti-Lamp 1 (1D4B, Developmental Studies Hybridoma Bank), anti-GRP94 (C-19) and anti-cathepsin D (C-20) antibodies (Santa Cruz Biotechnology, Inc.), anti-Grp75 (SPA-825, StressGen Biotechnologies Corp.), anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-p70 S6 kinase (Thr389), and anti-p70 S6 kinase antibodies (Cell Signaling Tech.). Other reagents were: dextran-BODIPY<sup>®</sup> FL, dextran-Alexa 488 and Lysotracker<sup>®</sup> Red DND-99 (Molecular Probes), GFP-LC3 expression plasmid (pCAG-EGFP-LC3, generous gift from Dr. Noboru Mizushima), rapamycin (LC Laboratories), 3-methyladenine (Sigma).

**Immunohistochemistry** - Paraffin brain sections from 10-month Cln3<sup>ex7/8</sup> homozygotes, and heterozygote and wild-type littermates, were processed and stained as previously described (18).

**Cellular subfractionation and analysis of autophagic vacuoles** - Autophagic vacuoles were isolated from freshly dissected mouse livers by centrifugation in discontinuous density gradients, following an isolation protocol modified from Marzella et al (25), as previously described (13). Livers from 4 individual littermates were pooled by genotype for the fractionation procedure, for each preparation. Genotyping was as previously described (22). The total volume of the starting homogenate, and the protein concentrations in the homogenate and each fraction, were tracked for...
subsequent determination of the total protein in the fractions and normalization of densitometric data. For ultrastructural analyses, an aliquot of the purified fractions was fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2), supplemented with 0.25M sucrose for maintenance of osmolarity. Fractions were subsequently processed for transmission electron microscopy, as previously described (18). For immunoblot analyses, 10 μg of each fraction were run on standard Tris-glycine SDS-PAGE, except for immunoblots detecting subunit c and LC3, which were run on Tris-tricine SDS-PAGE. For battenin immunoblot analyses, 10 μg of each fraction were run on the 10% Bis-tris NuPAGE gel system (Invitrogen), according to manufacturer’s recommendations. All blots were processed by horseradish peroxidase-conjugated secondary antibody incubation (Amersham), followed by chemiluminescence development (Western Lightning, Perkin Elmer). X-ray film detected the chemiluminescent signal over increasing exposure times ranging from 10 seconds to 10 minutes. For densitometric analyses, immunoblots were scanned using a GS-800 Calibrated Densitometer and Quantity One software (BioRad). The intensity of bands was quantified with the volume rectangle tool, using local background determination. Multiple exposure times were scanned and values were plotted to determine linearity. Densitometric values used for further analysis were taken from within the linear range. Notably, subunit c migrates in monomeric and SDS-resistant multimeric forms on SDS-PAGE, in a concentration dependent manner (26); therefore, the signal from both forms was used for determination of ‘total subunit c’ in the fractions. ‘Total subunit c’ is defined as the densitometric measure of multimeric and monomeric subunit c, determined from immunoblots, normalized to the total protein content in the fraction.

**EM morphometric analyses -** Four to five images from random fields were digitally captured at a magnification of 6800x on a JEOL 1200EX-80kV electron microscope. Field size at this magnification was approximately 46 mm². Digital images were scored for the number of vesicles of the following subtypes: double membrane, multivesicular, multilamellar, and multidense body. Double membrane structures were defined as those vesicles with a discernable double outer membrane. Multivesicular structures were defined as vesicles of heterogeneous contents, with two or more vesicles inside of a double outer membrane. Multilamellar structures were defined as heterogeneous vesicles containing stacks of membrane contained within a single or double outer membrane. Multidense bodies were defined as those vesicles containing heterogeneous contents including electron dense spots contained within a single membrane. For each category, an unpaired, two-tailed Student’s t-test was performed to determine whether the means per frame for wild-type versus homozygous Cln³Δex7/8 were significantly different.

**Lymphoblastoid and cerebellar cell culture -** Control and JNCL patient (homozygous 1.02kb deletion) lymphoblastoid cell lines were previously collected (17) and were grown for these studies as previously described (27). CbCln³Δex7/8 cerebellar precursor cell lines have also been previously described (22). CbCln³Δex7/8 cerebellar cells were transiently transfected with the pCAG-EGFP-LC3 expression plasmid using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s recommendations. For drug studies, cells were plated on coverslips in 12-well plates or 60 mm dishes at the appropriate seeding density and allowed to attach to the dish overnight. The following day, media was exchanged for fresh media with DMSO only, 20 nM-1 μM rapamycin/DMSO or 0.5-10 mM 3-MA. The fluid-phase endocytosis, Lysotracker, and cell survival assays were as previously described (22). Fluid-phase endocytic dye was the lysine-fixable version of dextran-BODIPY FL or dextran-Alexa 488, and incubation times were from 20-50 minutes. Fluorescence imaging was performed on a BioRad Radiance 2000 scanning laser confocal microscope or a Leica SP5 AOBS scanning laser confocal microscope, and images were manipulated using Adobe Photoshop CS (v8.0). Image-capture and manipulation were always performed with identical parameters for wild-type and homozygous CbCln³Δex7/8 cells. For quantitative analysis of co-localization in endocytosis-Lysotracker co-labeling studies, Leica Advanced Fluorescence Application Suite software’s co-localization analysis tool was used.
Co-localization rate, defined as co-localization area/area of the signal, was determined for 10 randomly captured images for each sample (40X magnification). Statistical significance was determined using a 2-tailed Student’s t-test. For cell survival assays following drug treatments, the following statistical analyses were performed: Significance of cell loss at the different drug doses, compared to the no drug treatment control, for each cell line was determined using a paired, two-tailed Student’s t-test. Significance of genotypic differences in cell survival at the individual doses was determined using an unpaired, two-tailed Student’s t-test.

Subunit c inclusion assay - Wild-type and homozygous CbCln3<sup>x<sub>ex7/8</sub></sup> cerebellar cells were plated at a density of 3x10<sup>4</sup> cells/well into 12-well plates containing coverslips and allowed to attach overnight. This seeding density maintained subconfluent cultures for the duration of the experiment. The following day, media was exchanged for fresh media containing only DMSO or 25 nM rapamycin/DMSO. After 48 hours, cells were fixed in 4% formaldehyde and processed for subunit c immunostaining as previously described (22). Subunit c immunopositive puncta were scored in 20 random frames with a 60X objective. Significance was determined using an unpaired, one-tailed Student’s t-test.

RESULTS

Activation of autophagy in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> mice - To first test the hypothesis that autophagy is involved in the pathophysiology of JNCL, we examined the state of autophagy in Cln3<sup>x<sub>ex7/8</sub></sup> knock-in mice. LC3 is a microtubule-associated protein that is lipidated upon activation of autophagy (LC3-I=un-lipidated, LC3-II=lipidated). LC3-II tightly associates with the pre-autophagosomal and autophagosomal membrane and thus is considered a marker of autophagy (28). We first probed brain sections with an LC3 antibody that detects both forms of LC3 (Experimental Procedures). While only occasional LC3 immunopositive puncta were observed in brain sections from control mice (wild-type or heterozygous Cln3<sup>x<sub>ex7/8</sub></sup> littermates), strong LC3 immunopositive puncta were observed in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> brain, in regions known to exhibit significant mitochondrial ATPase subunit c accumulations, including the Purkinje cells of the cerebellum, CA2/3 pyramidal cell layer of the hippocampus, and periventricular cells (18) (Fig. 1A). To more specifically determine the proportion of LC3 that was in the LC3-II form, which migrates faster than LC3-I on SDS-PAGE and is the form associated with the autophagosomal structures, we performed immunoblot analyses and densitometry of LC3-I and LC3-II in wild-type (Cln3<sup>+/+</sup>) and homozygous Cln3<sup>x<sub>ex7/8</sub></sup> whole brain extracts. Elevated LC3-II/LC3-I ratios in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> knock-in brain, relative to wild-type brain, were observed at each age examined. These observations are consistent with an increased activation of autophagy in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> mice.

Battenin is highly enriched in purified autophagic vacuoles - To more closely examine the autophagic pathway, we performed a previously established fractionation procedure (13) to isolate high-purity autophagic vacuoles (AV) from wild-type and Cln3<sup>x<sub>ex7/8</sub></sup> knock-in liver for subsequent immunoblotting and structural studies. This procedure allows the isolation of autophagic vacuoles of two different densities, a light fraction (AV15) particularly enriched in autophagosomes and a heavy fraction (AV20) predominantly consisting of autophagolysosomes. Given the hypothesized role of autophagy in JNCL and the observed changes in autophagosomal-like structures in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> mice (18), we examined the two AV fractions separately.

To first determine whether the CLN3-encoded protein, battenin, was present in the different AV fractions, which might suggest a direct role for battenin in the autophagic pathway, we probed immunoblots of wild-type (Cln3<sup>+/+</sup>) and homozygous Cln3<sup>x<sub>ex7/8</sub></sup> fractions using a previously characterized anti-battenin antibody (23). On wild-type immunoblots, we observed a faint ~60-65 kDa smear (*, Fig. 2) in the homogenate and tubulovesicular fraction (E, mostly consists of ER), which was remarkably enriched in the AV15, AV20 and lysosomal fractions, and this band was missing on homozygous Cln3<sup>x<sub>ex7/8</sub></sup> blots, as expected. Consistent with stable mutant mRNAs and low abundance mutant battenin species in homozygous Cln3<sup>x<sub>ex7/8</sub></sup> mouse tissues (18), a
novel faint smear ~25-30 kDa fractionating with AVs and lysosomes was detected on homozygous Cln3<sup>ex7/8</sup> blots (arrows, Fig. 2). Additional unidentified immunopositive bands fractionating mostly with the cytosol, and a previously described cross-reactive band at ~50 kDa, fractionating with mitochondria (23), were not different on wild-type and homozygous Cln3<sup>ex7/8</sup> blots.

**Ultrastructural abnormalities of purified autophagic vacuoles from homozygous Cln3<sup>ex7/8</sup> mice** - During the fractionation of AVs from wild-type and homozygous Cln3<sup>ex7/8</sup> liver, we noted an intriguing trend of reduced protein content in the AV15, AV20, and lysosomal fractions from homozygous Cln3<sup>ex7/8</sup> liver, compared to the wild-type fractions, which worsened with age (data not shown). These observations suggested abnormalities in the AV and lysosomal organelles from homozygous Cln3<sup>ex7/8</sup> liver. Therefore, we sought to examine further the ultrastructural morphology of the purified AV and lysosomal fractions. Comparison of the AV15, AV20, and lysosomal fractions from wild-type (Cln<sup>+/+</sup>) mice revealed an apparent progressive maturation and degradation of contents with increasing sedimentation, where the engulfed components were no longer evident in the vesicles recovered in the lysosomal fraction (Fig. 3), consistent with previous observations (12,13). In contrast, this trend was not observed in the fractions from homozygous Cln3<sup>ex7/8</sup> liver. Instead, vesicles in both AV fractions and in the lysosomal fraction from homozygous Cln3<sup>ex7/8</sup> liver were full of multilamellar membranes, consistent in appearance with the ‘fingerprint’-like storage material observed in JNCL. Moreover, AVs with dense cores were seen in the AV fractions from wild-type (Cln<sup>+/+</sup>) mice, but these structures were not often observed in the homozygous Cln3<sup>ex7/8</sup> AV fractions (Fig. 3).

**Subunit c accumulates in autophagic vacuoles in homozygous Cln3<sup>ex7/8</sup> and aged wild-type mouse liver** - The hallmark storage of mitochondrial ATP synthase subunit c in JNCL is thought to occur at the level of the lysosome (15). However, given that the subunit c protein is predicted to traffic to the lysosome via the autophagic pathway (29), we sought to more precisely pinpoint the organelle in which subunit c predominantly accumulates. Utilizing the purified AV and lysosomal fractions from wild-type and homozygous Cln3<sup>ex7/8</sup> knock-in liver, we performed a semi-quantitative analysis of subunit c distribution by immunoblotting and densitometry. As expected, immunoblots of subunit c revealed enrichment of this protein in mitochondrial fractions (Fig. 4A). Moreover, consistent with autophagic-lysosomal turnover of mitochondria, undegraded subunit c protein was also readily detectable in AV fractions and lysosomes from both wild-type and homozygous Cln3<sup>ex7/8</sup> knock-in liver. Densitometric semi-quantitative analyses of ‘total subunit c’, determined by normalization of densitometric units to total protein content in each fraction (Experimental Procedures), revealed a markedly enhanced recovery of subunit c in the homozygous Cln3<sup>ex7/8</sup> knock-in AV15 fractions, relative to wild-type AV15 fractions (Fig. 4B). The homozygous Cln3<sup>ex7/8</sup> knock-in lysosomal fractions also exhibited more total subunit c relative to the wild-type lysosomal fractions. Intriguingly, the heavier AV20 fractions from Cln3<sup>ex7/8</sup> homozygote liver did not exhibit prominent abnormal subunit c levels (Fig. 4B). No major differences in total subunit c recovery were observed in the tubulovesicular and mitochondrial fractions. Organelle marker immunoblots confirmed the purity of the fractions, and no major differences in distribution of these markers were observed between the wild-type and homozygous Cln3<sup>ex7/8</sup> liver fractions (Fig. 4B). However, LC3-II recovery was higher (~two- to three-fold) in the AV15 fraction from homozygous Cln3<sup>ex7/8</sup> liver, compared with wild-type (Fig. 4A), consistent with our previous observations in whole brain extracts (Fig. 1B). Notably, Lamp 1 and cathepsin D recovery were also increased (~two- to three-fold) in the AV15 fraction from homozygous Cln3<sup>ex7/8</sup> liver, compared with wild-type (Fig. 4B and data not shown).

Given the progressive nature of subunit c accumulations in homozygous Cln3<sup>ex7/8</sup> mice and JNCL patients (18,30), the accumulation of subunit c in the purified AVs was assessed at different ages. As expected, subunit c recovery from purified AV and lysosomal fractions in homozygous Cln3<sup>ex7/8</sup> knock-in liver increased from 4 months to 12 months (Fig. 5). Surprisingly,
however, an age-related increase in subunit c recovery from purified AV and lysosomal fractions was also observed for wild-type liver. Unlike the subunit c accumulation in homozygous Cln3<sup>Δex7/8</sup> knock-in fractions, which occurs largely in the AV15 fraction, subunit c was particularly increased in the AV20 fraction from wild-type aged mouse liver. Moreover, ‘total subunit c’ recovery in both wild-type and homozygous Cln3<sup>Δex7/8</sup> knock-in AV and lysosomal fractions was surprisingly high.

**Downregulation of mTOR in JNCL** - Our observations at the level of LC3 suggest autophagy may be activated in both brain and liver from homozygous Cln3<sup>Δex7/8</sup> knock-in mice, although the maturation of the formed vesicles may be impaired. The mammalian target of rapamycin (mTOR) pathway is known to negatively regulate autophagy (31,32). To examine the involvement of the mTOR pathway in JNCL pathophysiology, we probed the state of mTOR activation in homozygous Cln3<sup>Δex7/8</sup> knock-in mice. As shown in Figure 6A, we found significantly reduced levels of phospho-mTOR in 6-month homozygous Cln3<sup>Δex7/8</sup> brain, compared to Cln3<sup>Δex7/8</sup> heterozygous and wild-type littermates. To determine whether our observations in homozygous Cln3<sup>Δex7/8</sup> mice were also reflected in cell culture, we expanded the analysis of mTOR activation to include JNCL patient lymphoblastoid cells and CbCln3<sup>Δex7/8</sup> cerebellar cells, which we previously established from Cln3<sup>Δex7/8</sup> knock-in mice (22).

**Impaired autophagic trafficking in homozygous CbCln3<sup>Δex7/8</sup> cerebellar cells** – Although autophagy may be upregulated in our genetic models of JNCL, the formed AVs may not mature normally, leading us to consider a possible defect in AV clearance. This prompted a more detailed examination of autophagic trafficking in JNCL utilizing CbCln3<sup>Δex7/8</sup> cerebellar cells.

We have previously demonstrated that homozygous CbCln3<sup>Δex7/8</sup> cells do not exhibit detectable subunit c accumulation under normal growth conditions, although subunit c inclusions were induced by aging cells at confluence, which may upregulate autophagy (22). To directly test whether stimulation of autophagy leads to subunit c accumulation in homozygous CbCln3<sup>Δex7/8</sup> cerebellar cells, we treated cells with the mTOR inhibitor, rapamycin, and followed subunit c inclusion formation. The ability of rapamycin to upregulate autophagy was first confirmed in our CbCln3<sup>Δex7/8</sup> cell lines by following LC3-II levels. Consistent with our *in vivo* observations (Fig. 1B), untreated homozygous CbCln3<sup>Δex7/8</sup> cerebellar cell extracts exhibited more LC3-II, relative to LC3-I, than wild-type cells, and rapamycin upregulated the LC3-II/LC3-I ratio in both wild-type and homozygous CbCln3<sup>Δex7/8</sup> cells (Fig. 7A).

We also examined LC3 by transfection with a plasmid expressing GFP-LC3. In wild-type cerebellar cells, we observed scattered, small AVs and large, perinuclear vesicles that were consistent in appearance with maturing AVs (33) (Fig. 7C). However, homozygous CbCln3<sup>Δex7/8</sup> cerebellar cells infrequently contained the large, perinuclear structures, but contained numerous small AVs (Fig. 7C), consistent with a deficiency in AV maturation.

AV maturation requires fusion with late endosomes and lysosomes, which leads to acidification and proteolytic activation for degradation of the AV cargo (5,6). We have previously shown that both endosomes and lysosomes exhibit abnormalities in homozygous CbCln3<sup>Δex7/8</sup> cerebellar cells (22), leading us to hypothesize that the steps in AV maturation that require endosomal and lysosomal function are disrupted in JNCL. As a first step in examining trafficking and fusion between endocytic and lysosomal compartments in homozygous CbCln3<sup>Δex7/8</sup> cells, we performed a combined endocytic uptake and Lysotracker staining assay,
with or without the induction of autophagy by rapamycin. As we have previously reported (22), the endocytic and Lysotracker (labels lysosomes and autophagolysosomes) dyes, were reduced in untreated homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cerebellar cells, relative to untreated wild-type cerebellar cells (Fig. 7D). Upon rapamycin treatment in both wild-type and homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells, the endocytic and Lysotracker stains were increased, and overlap of the two dyes was more evident (Fig. 7D). Comparison of the rapamycin-treated homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cerebellar cells with the rapamycin-treated wild-type cells revealed a similar reduction in stain to that observed for untreated cells, but we also observed ~4-fold reduction in the co-localization rate of these two markers in homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells, relative to the wild-type cells (wild-type=17±10.5%, vs. 4.6±2.6%, p<.01) (Fig. 7D). These observations support the hypothesis that trafficking between endocytic and autophagolysosomal/lysosomal compartments, which involves vesicle fusion, is abnormal in homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cerebellar cells.

\textit{Inhibition of autophagy reduces cell viability} - Autophagy is both a pro-survival pathway and an important pathway leading to programmed cell death (34). To dissect the relationship of autophagy and cell death in wild-type and homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells, we assessed cell survival following treatment with rapamycin or 3-methyladenine (3-MA), a broad phosphatidylinositol 3-kinase inhibitor widely recognized as an inhibitor of autophagy (13, 35). As shown in Figure 8, doses of rapamycin that induce autophagy (20 and 50 nM) halted cell growth but did not lead to significant cell loss in either wild-type (\textit{Cln3}\textsuperscript{+/+}) or homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells. Higher rapamycin doses (0.25 and 1 \mu M) led to slightly reduced cell survival (~90%) in homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells. Higher rapamycin doses (0.25 and 1 \mu M) led to slightly reduced cell survival (~90%) in homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells over the 48-hour treatment period, but these levels of cell loss never reached significance (p>.05). By contrast, 3-MA treatment, at doses commonly used to inhibit autophagy (5-10 mM) (35), resulted in significant cell loss in both wild-type and homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells, but more markedly in the latter. Survival of homozygous Cb\textit{Cln3}\textsuperscript{ex7/8} cells was significantly reduced, compared to wild-type cerebellar cell survival at all 3-MA doses tested (Fig. 8). Similar studies with control and JNCL patient lymphoblasts supported these observations, where significant cell loss was only observed following 3-MA treatment (data not shown).

\textbf{DISCUSSION}

These studies utilizing JNCL patient cells and genetically accurate mouse and cerebellar cell culture models, which faithfully replicate the common JNCL mutation, demonstrate an important role for autophagy in the turnover of ATPase subunit c and in JNCL disease phenotypes. Furthermore, surprising properties of ATPase subunit c distribution in membrane organelles and in aging were revealed. Most importantly, our studies implicate disruption of autophagy in cells homozygous for the common JNCL mutation, including patient lymphoblastoid cells, as depicted in the hypothetical model in Figure 9.

The observation that subunit c accumulation in homozygous \textit{Cln3}\textsuperscript{ex7/8} liver is not restricted to the lysosome, but is most evident in the light AV fraction, suggests that there is a delay in the maturation of AVs to fully degradative autolysosomes in JNCL. The ultrastructural studies showing marked changes in the degradation of the AV contents supports this hypothesis. Although the different properties of the two AV populations are not yet entirely understood, the light AV15 fraction exhibits properties consistent with autophagosomes, while the denser AV20 fraction is more autophagolysosomal, with acidifying pH and proteolytic enzymatic activity (36). Indeed the ultrastructure of the purified organelles in this study supports this distinction between the two AV populations. Thus, our observations of predominant accumulation of subunit c in the light AV15 fraction are consistent with a delay in maturation of AVs in JNCL. A similar expansion of the autophagic intermediates has been described previously in other conditions, such as Alzheimer’s disease, where autophagic clearance through to the lysosome is thought to be impaired (12,13). The specific defect that leads to the accumulation in JNCL may involve fission and/or fusion defects between the formed AVs and late
endosomes and lysosomes, leading to a reduction in AVs containing mitochondria and subunit c, which normally sediment in the AV20 fraction. This notion is supported by our cell biological studies in homozygous CbCln3<sup>ex7/8</sup> cerebellar cells in which trafficking between endosomal and lysosomal compartments is altered. Moreover, the observation that battenin is highly enriched in the membranes of AVs is suggestive for the first time that battenin plays a direct role in AV organelle maturation towards the lysosome. The specific battenin function that contributes to AV maturation could involve trafficking of the AVs, endosomes, and/or lysosomes, or it may be that battenin helps guide fission/fusion via membrane protein interactions or by directly regulating organelle properties, such as membrane dynamics, enzyme activation, pH and/or ion homeostasis. Consistent with these hypotheses, endosomal and lysosomal trafficking defects (21,22) and lysosomal pH alterations (19,37) are common findings in JNCL.

That subunit c was also highly enriched in AV and lysosomal fractions from wild-type mice was a remarkable finding. This suggests that subunit c accumulation may not be specific to NCL, although the precise mechanisms underlying this phenomena may be different in aging, compared to JNCL, given the distinct profiles of subunit c accumulation in the AV and lysosomal compartments from wild-type aged liver and homozygous Cln3<sup>ex7/8</sup> knock-in mice. Consistent with this hypothesis, declining autophagic-lysosomal function is an established occurrence in normal aging (38), and subunit c accumulation is observed in other lysosomal disorders, such as mucolipidosis type 1 and 2 (30). Moreover, knock-out mice deficient for cathepsins (including cathepsins D, B, and L) exhibit significant autophagy-mediated subunit c accumulation (39). However, we previously reported that cathepsin D enzymatic activity is not significantly altered in homozygous Cln3<sup>ex7/8</sup> mice and CbCln3<sup>ex7/8</sup> cerebellar cells, although cathepsin D trafficking may be altered (22). These observations argue that subunit c accumulation in JNCL does not arise from cathepsin D deficiency, but more likely occurs via distinct mechanisms from those giving rise to subunit c accumulation in cathepsin D knock-out mice. Therefore, further study of the autophagic pathway in other lysosomal disorders and in aging, to elucidate the distinct mechanisms and consequences of subunit c accumulation in AVs, is warranted.

The events that lead to downregulation of mTOR signaling and upregulation of autophagy in JNCL are not yet clear, but our results suggest this may be a pro-survival response during the JNCL disease process (see Fig. 9), since further upregulation of autophagy fails to significantly impact cell survival, but inhibition of autophagy leads to cell death, most likely by apoptotic mechanisms (35,40). mTOR-mediated autophagy is known to be stimulated by cellular stress, energy depletion, and nutrient deprivation (2). These are all established phenotypes in JNCL cells. For example, we and others have reported reduced ATP and increased sensitivity to cellular stressors in the presence of the common JNCL mutation (22,41), and amino acid transport defects lead to reduced cytoplasmic levels of the amino acid arginine in JNCL (20). Moreover, utilization of extracellular nutrients requires endocytosis, and intracellular nutrients are generated by autophagic turnover of organelles and long-lived proteins, both pathways that are compromised in JNCL (21,22). However, the upregulation of autophagy in JNCL is unlikely to produce long-term benefits for the cell, given that our data strongly suggest that autophagy may be deficient at the level of AV maturation due to the lack of normal battenin function.

Aging is likely to further aggravate the disruption of autophagy in JNCL, and indeed the disease is progressive over several decades, culminating in massive neuronal loss and death. Autophagy exhibits a functional decline with age, both at the level of activation of the pathway and in fusion of AVs with lysosomes (38). Intriguingly, undigested material in AVs, in the form of lipofuscin, may further disrupt the fusion process (38). Therefore, although the primary defect in AV maturation may be due to loss of normal battenin function at the AV/lysosomal membrane, given that we observe maturation defects in the absence of subunit c accumulation in homozygous CbCln3<sup>ex7/8</sup> cells [this study and (22)], the accumulation of subunit c could worsen this critical step in the pathway. Moreover, it has been proposed that neurons and
cardiac myocytes are most vulnerable to the age-related changes in autophagy and that this may lead to the accumulation of damaged mitochondria (42,43). It is therefore tempting to speculate that the defect in autophagy, resulting from the loss of normal battenin function, may explain the clinical specificity of the symptoms of JNCL, which are predominantly to the nervous system (44) but also have been shown to include the myocardium (45).

Finally, the results of our studies suggest that the accurate genetic mouse and cellular models of JNCL, the most common neurodegenerative disease afflicting children, will be useful systems for study of other disorders with altered autophagic-lysosomal trafficking or turnover of mitochondria through this pathway. Further investigation of the biology of battenin and the subunit c protein in this pathway will not only advance NCL research but will also contribute to a greater understanding of neurodegenerative disease and aging.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Vamsi Moortha for insightful discussion regarding the mitochondrial subunit c protein and Dr. Christopher Korey for thoughtful discussion and review of the manuscript. We also thank Drs. Junji Ezaki and Eiki Kominami for their gifts of anti-battenin and anti-mCln3p antibodies, Dr. Noboru Mizushima for his gifts of anti-LC3 antibody and GFP-LC3 expression plasmid, and the Harvard Electron Microscopy Core for assistance with TEM studies. YC is funded by a postdoctoral fellowship from the Batten Disease Support and Research Association. This work was supported by NIH/NINDS grant NS33648 (MEM) and NIH/NIA grant AG02194 (AMC).

REFERENCES

1. Cuervo, A. M. (2004) Trends Cell Biol 14, 70-77
2. Yorimitsu, T., and Klionsky, D. J. (2005) Cell Death Differ 12 Suppl 2, 1542-1552
3. Tsukada, M., and Ohsumi, Y. (1993) FEBS Lett 333, 169-174
4. Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M., and Wolf, D. H. (1994) FEBS Lett 349, 275-280
5. Mizushima, N., Ohsumi, Y., and Yoshimori, T. (2002) Cell Struct Funct 27, 421-429
6. Levine, B., and Klionsky, D. J. (2004) Dev Cell 6, 463-477
7. Anglade, P., Vyas, S., Javoy-Agid, F., Herrero, M. T., Michel, P. P., Marquez, J., Mouatt-Prigent, A., Ruberg, M., Hirsch, E. C., and Agid, Y. (1997) Histol Histopathol 12, 25-31
8. Stefanis, L., Larsen, K. E., Rideout, H. J., Sulzer, D., and Greene, L. A. (2001) J Neurosci 21, 9549-9560
9. Kegel, K. B., Kim, M., Sapp, E., McIntyre, C., Castano, J. G., Aronin, N., and DiFiglia, M. (2000) J Neurosci 20, 7268-7278
10. Petersen, A., Larsen, K. E., Behr, G. G., Romero, N., Przedborski, S., Brundin, P., and Sulzer, D. (2001) Hum Mol Genet 10, 1243-1254
11. Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Scaravilli, F., Easton, D. F., Duden, R., O'Kane, C. J., and Rubinsztein, D. C. (2004) Nat Genet 36, 585-595
12. Yu, W. H., Kumar, A., Peterhoff, C., Shapiro Kulnane, L., Uchiyama, Y., Lamb, B. T., Cuervo, A. M., and Nixon, R. A. (2004) Int J Biochem Cell Biol 36, 2531-2540
13. Yu, W. H., Cuervo, A. M., Kumar, A., Peterhoff, C. M., Schmidt, S. D., Lee, J. H., Mohan, P. S., Mercken, M., Farmery, M. R., Tjemery, L. O., Jiang, Y., Duff, K.,
Uchiyama, Y., Naslund, J., Mathews, P. M., Cataldo, A. M., and Nixon, R. A. (2005) J Cell Biol 171, 87-98

14. Wisniewski, K. E. (2001) Neurology 57, 576-581
15. Palmer, D. N., Fearnley, I. M., Walker, J. E., Hall, N. A., Lake, B. D., Wolfe, L. S., Haltia, M., Martinus, R. D., and Jolly, R. D. (1992) Am J Med Genet 42, 561-567
16. Mole, S. E. (2004) Brain Pathol 14, 70-76
17. International Batten Disease Consortium. (1995) Cell 82, 949-957
18. Cotman, S. L., Vrbanac, V., Lebel, L. A., Lee, R. L., Johnson, K. A., Donahue, L. R., Teed, A. M., Antonellis, K., Bronson, R. T., Lerner, T. J., and MacDonald, M. E. (2002) Hum Mol Genet 11, 2709-2721
19. Pearce, D. A., Ferea, T., Nosel, S. A., Das, B., and Sherman, F. (1999) Nat Genet 22, 55-58
20. Ramirez-Montealegre, D., and Pearce, D. A. (2005) Hum Mol Genet 14, 3759-3773
21. Luio, K., Yliannala, K., Ahtiainen, L., Maunu, H., Jarvela, I., Kyttala, A., and Jalanko, A. (2004) Hum Mol Genet 13, 3017-3027
22. Fossale, E., Wolf, P., Espinola, J. A., Lubicz-Nawrocka, T., Teed, A. M., Gao, H., Rigamonti, D., Cattaneo, E., MacDonald, M. E., and Cotman, S. L. (2004) BMC Neurosci 5
23. Ezaki, J., Takeda-Ezaki, M., Koike, M., Ohsawa, Y., Taka, H., Mineki, R., Murayama, K., Uchiyama, Y., Ueno, T., and Kominami, E. (2003) J Neurochem 87, 1296-1308
24. Kominami, E., Ezaki, J., Muno, D., Ishido, K., Ueno, T., and Wolfe, L. S. (1992) J Biochem 111, 278-282
25. Marzella, L., Ahlberg, J., and Glaumann, H. (1982) J Cell Biol 93, 144-154
26. Chen, R., Fearnley, I. M., Palmer, D. N., and Walker, J. E. (2004) J Biol Chem 279, 21883-21887
27. Anderson, M. A., and Gusella, J. F. (1984) In Vitro 20, 856-858
28. Mizushima, N. (2004) Int J Biochem Cell Biol 36, 2491-2502
29. Kominami, A. E. (2002) IUBMB Life 54, 89-90
30. Elleder, M., Sokolova, J., and Hrebicek, M. (1997) Acta Neuropathol 93, 379-390
31. Noda, T., and Ohsumi, Y. (1998) J Biol Chem 273, 3963-3966
32. Cutler, N. S., Heitman, J., and Cardenas, M. E. (1999) Mol Cell Endocrinol 155, 135-142
33. Jager, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., and Eskelinen, E. L. (2004) J Cell Sci 117, 4837-4848
34. Mizushima, N. (2005) Cell Death Differ 12 Suppl 2, 1535-1541
35. Boya, P., Gonzalez-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., and Kroemer, G. (2005) Mol Cell Biol 25, 1025-1040
36. Kovacs, J., and Rez, G. (1989) Revis Biol Celular 20, 63-78
37. Holopainen, J. M., Saarikoski, J., Kinnunen, P. K., and Jarvela, I. (2001) Eur J Biochem 268, 5851-5856
38. Cuervo, A. M., Bergamini, E., Brunk, U. T., Droge, W., and Terman, A. (2005) Autophagy 1, 131-140
39. Koike, M., Shibata, M., Waguri, S., Yoshimura, K., Tanida, I., Kominami, E., Gotow, T., Peters, C., von Figura, K., Mizushima, N., Saftig, P., and Uchiyama, Y. (2005) Am J Pathol 167, 1713-1728
40. Persaud-Sawin, D. A., and Boustany, R. M. (2005) Apoptosis 10, 973-985
41. Jolly, R. D., Brown, S., Das, A. M., and Walkley, S. U. (2002) *Neurochem Int* **40**, 565-571
42. Terman, A., Dalen, H., Eaton, J. W., Neuzil, J., and Brunk, U. T. (2003) *Exp Gerontol* **38**, 863-876
43. Terman, A., Dalen, H., Eaton, J. W., Neuzil, J., and Brunk, U. T. (2004) *Ann N Y Acad Sci* **1019**, 70-77
44. Goebel, H. H., and Wisniewski, K. E. (2004) *Brain Pathol* **14**, 61-69
45. Hofman, I. L., van der Wal, A. C., Dingemans, K. P., and Becker, A. E. (2001) *Eur J Paediatr Neurol* **5 Suppl A**, 213-217

FOOTNOTES

1 Abbreviations used are: 3-MA=3-methyladenine, µg=microgram, µM=micromolar, A.U.=arbitrary units, AV=autophagic vacuole, AV15=light autophagic vacuolar fraction, AV20=heavy autophagic vacuolar fraction, Cb=cerebellum, DM=double membrane, DMSO=dimethyl sulfoxide, E=tubulovesicular fraction, ER=endoplasmic reticulum, GCL=granule cell layer, Hc=hippocampus, JNCL=juvenile neuronal ceroid lipofuscinosis, kDa=kilodaltons, L=lysosome, LE=late endosome, Lys=lysosomal fraction, MD=multidense bodies, Mito=mitochondria, ML=multilamellar, mM=millimolar, MOL=molecular layer, mTOR=mammalian target of rapamycin, MV=multivesicular bodies, MW=molecular weight, n=nucleus, NCL=neuronal ceroid lipofuscinosis, nM=nanomolar, P=Purkinje cell layer, P4=postnatal day 4, P8=postnatal day 8, p70 S6K=p70 S6 kinase, SD=standard deviation, SDS-PAGE=sodium dodecyl sulfate polyacrylamide gel electrophoresis, TEM=transmission electron microscopy.

FIGURE LEGENDS

**Figure 1. Enhanced LC3 levels in homozygous Cln3ex7/8 brain.** A. Wild-type (*Cln3<sup>+/+</sup>*) or homozygous *Cln3<sup>ex7/8</sup> (Cln3<sup>ex7/8/ex7/8</sup>) paraffin-embedded brain sections are immunostained for the LC3 protein. Shown are the CA2/CA3 region of hippocampus (Hc) and cerebellum (Cb) from 10-month mice. Note that few immunopositive puncta are present in wild-type sections, while homozygous *Cln3<sup>ex7/8</sup> sections contain clusters of LC3-positive puncta around pyramidal neurons and Purkinje cells (P). Sections are from 10-month old mice. MOL=molecular layer, GCL=granule cell layer, 40x magnification. B. Representative immunoblots of total brain extracts are shown, probed for LC3. The higher apparent molecular weight (MW) band represents the soluble form of LC3-I, and the lower apparent MW band represents the autophagosome-associated LC3-II (P4 and P8 blots are shown). Typically, densitometry detects 2-fold higher LC3-II/LC3-I ratios in homozygous *Cln3<sup>ex7/8</sup>* extracts (red bars), compared to wild-type extracts (blue bars). A.U.=arbitrary units, P4=postnatal day 4, P8=postnatal day 8, 10 wk=ten weeks old.

**Figure 2. Battenin is highly enriched in autophagic vacuole and lysosomal fractions.** Immunoblots of wild-type (*Cln3<sup>+/+</sup>*) or homozygous *Cln3<sup>ex7/8</sup> (Cln3<sup>ex7/8/ex7/8</sup>) liver fractions are shown, probed with anti-battenin antibody. A broad ~60-65 kDa wild-type battenin band (*) is detected faintly in the homogenate (Hom), the postnuclear pellet (PNP), and the tubulovesicular fraction (E, contains ER, Golgi and endosomes), and this band is highly enriched in the autophagic vacuole fractions (AV15, AV20) and in the lysosomal (Lys) fraction. This band is not seen in homozygous *Cln3<sup>ex7/8</sup>* fractions, although a faint smear in that region is present in the AV20 fraction. Faint, novel, low MW bands (~30 kDa, arrows) are detected on homozygous *Cln3<sup>ex7/8</sup>* immunoblots in the E, AV15, AV20 and lysosomal fractions. A longer exposure (AV15*, AV20*, Lys*) is shown, for better visualization of these faintly detected bands. This antibody also detects prominent bands at ~28 kDa and ~42 kDa that fractionate predominantly with the
cytosol, and a cross-reactive band at ~50 kDa that fractionates with mitochondria (Mito). The identity of these bands is unknown, but they do not differ between wild-type (Cln3<sup>+/+</sup>) and homozygous Cln3<sup>ex7/8</sup> immunoblots.

**Figure 3. Morphometric analysis of purified autophagic vacuole and lysosomal fractions.** Typical TEM micrographs of AV15, AV20 and lysosomal (LYS) fractions from wild-type (Cln3<sup>+/+</sup>, top panels) or homozygous Cln3<sup>ex7/8</sup> (Cln3<sup>ex7/8/ex7/8</sup>, bottom panels) liver are shown. Bar graphs represent results from morphometric analyses of micrograph images (mean per frame ± SD). Double membrane (DM) vesicles, typical of AVs, are most abundant in the AV fractions, and the recovered lysosomal fraction is enriched in lysosomes (L), as expected. Double membrane structures are distributed similarly for wild-type (blue bars) and homozygous Cln3<sup>ex7/8</sup> knock-in (red bars) fractions, although a slight elevation in these structures is seen in the homozygous Cln3<sup>ex7/8</sup> AV20 fraction (*p<.05). The abundance of double-membrane multivesicular bodies (MV) is the same across all of the fractions, although a slight reduction of MVs is observed in the lysosomal fraction from homozygous Cln3<sup>ex7/8</sup> liver (**p<.01). Multilamellar (ML) structures are abundant throughout the AV and lysosomal fractions from homozygous Cln3<sup>ex7/8</sup> liver, but these are only occasionally observed in the wild-type fractions (***p<.001). Multidense bodies (MD, white asterisk) are often seen in wild-type AV fractions, but these are reduced in the AV fractions from homozygous Cln3<sup>ex7/8</sup> liver (AV15 did not reach significance, p=.08, AV20, **p<.01). Note that MDs are not typical of the lysosomal fraction. In the micrograph images, arrows indicate ML structures and the arrowhead points to a large AV filled with membranes. Scale bar for all images is shown (500 nm).

**Figure 4. Subunit c accumulation in autophagic vacuole and lysosomal fractions from homozygous Cln3<sup>ex7/8</sup> liver.** A. Representative immunoblots of wild-type (Cln3<sup>+/+</sup>, left panels) and homozygous Cln3<sup>ex7/8</sup> (Cln3<sup>ex7/8/ex7/8</sup>, right panels) liver fractions are shown, probed with anti-subunit c or organelle marker antibodies. Note that subunit c migrates as multimers and monomers on these gels; the signal from both forms was used for determination of total subunit c (Exp. Procedures). Marker antibodies for purified fractions are: anti-LC3=AV and lysosomal, anti-Lamp 1=AV and lysosomal, anti-Grp75=mitochondria, anti-GRP94=E (an ER resident protein). B. The bar graph displays the ratio of total subunit c in homozygous Cln3<sup>ex7/8</sup> versus wild-type fractions, as determined by densitometry of representative immunoblots. The horizontal line on the y ordinate highlights equivalent amounts of total subunit c in wild-type and homozygous Cln3<sup>ex7/8</sup> samples. Hom=homogenate, PNP=postnuclear pellet, Cyto=cytosol, E=tubulovesicular fraction (ER, some Golgi and endosomes), Mito=mitochondria, AV15=light AV fraction, AV20=heavy AV fraction, Lys=lysosomes.

**Figure 5. Subunit c accumulation in aging wild-type and homozygous Cln3<sup>ex7/8</sup> liver.** The bar graphs display the fold enrichment of total subunit c in fractions, over that observed in the homogenate, from 4 month (left graph) or 12 month (right graph) wild-type (blue bars) and homozygous Cln3<sup>ex7/8</sup> (red bars) liver, as determined by densitometry of representative immunoblots (Experimental Methods). Note the expected enrichment in mitochondrial fractions, which did not change with age. Surprising enrichment of subunit c protein is observed in all AV and lysosomal fractions, which becomes more pronounced with age in both wild-type and homozygous Cln3<sup>ex7/8</sup> liver. Also note that the subunit c distribution pattern for homozygous Cln3<sup>ex7/8</sup> mice consistently displays more enrichment in the AV15 and lysosomal fractions, whereas the wild-type subunit c is more equally distributed, or enriched in the AV20 fraction. Hom=homogenate, PNP=postnuclear pellet, Cyto=cytosol, E=tubulovesicular fraction (ER, some Golgi and endosomes), Mito=mitochondria, AV15=light AV fraction, AV20=heavy AV fraction, Lys=lysosomes.

**Figure 6. mTOR downregulation in homozygous Cln3<sup>ex7/8</sup> brain and ChCln3<sup>ex7/8</sup> cerebellar cells, and JNCL patient samples.** A. Representative immunoblots of wild-type (Cln3<sup>+/+</sup>), heterozygous (Cln3<sup>ex7/8</sup>), and homozygous Cln3<sup>ex7/8</sup> (Cln3<sup>ex7/8/ex7/8</sup>) brain extracts are shown, probed to determine...
the activation state of mTOR. A triplet of bands at ~290 kDa is more faintly labeled with an antibody directed against phosphorylated Ser2448 (phospho-mTOR), relative to total mTOR, in homozygous Cln3Δex7/8 brain extracts, compared to wild-type or heterozygous Cln3Δex7/8 brain extracts. Densitometry showed a ~1.4-fold reduction in phospho-mTOR, in homozygous Cln3Δex7/8 extracts (p<.05). B. Representative immunoblots of control and JNCL patient lymphoblast extracts and wild-type (CbCln3+/+) and homozygous CbCln3Δex7/8 (CbCln3Δex7/8Δex7/8) cerebellar cell extracts are shown, probed to determine the activation state of the immediate downstream target of mTOR, p70 S6 kinase (p70 S6K). A ~70 kDa band is more faintly labeled with an antibody directed against phosphorylated Thr389 (phospho-p70 S6K) in the activation state of the immediate downstream target of mTOR, p70 S6 kinase (p70 S6K). A ~70 kDa band is more faintly labeled with an antibody directed against phosphorylated Thr389 (phospho-p70 S6K) in the activation state of the immediate downstream target of mTOR, p70 S6 kinase (p70 S6K).

**Figure 7. Trafficking defects involving autophagy and the endosomal-lysosomal system in homozygous CbCln3Δex7/8 cells**

A. Typical LC3 immunoblots are shown for extracts from untreated (DMSO only) or rapamycin-treated (250 nM, 4 hours) cultures of wild-type (CbCln3+/+) or homozygous CbCln3Δex7/8 cerebellar cells (CbCln3Δex7/8Δex7/8). Rapamycin increases the LC3-II/LC3-I ratio in wild-type and homozygous CbCln3Δex7/8Δex7/8 extracts (2.6X and 2.2X, respectively), consistent with activation of autophagy. The LC3-II/LC3-I ratio for homozygous CbCln3Δex7/8 cell extracts is consistently higher than the ratio observed in wild-type extracts (3.4X and 2.9X, respectively, p<.05). B. The bar graph depicts results of a typical subunit c inclusion assay, following 48-hour rapamycin treatment (25nM). Untreated (DMSO only) wild-type (blue bars) and homozygous CbCln3Δex7/8 cells (red bars) display ~2 subunit c immunopositive inclusions per frame (60x magnification). Rapamycin treatment does not increase subunit c inclusions in wild-type cells, but approximately doubles subunit c immunopositive inclusions to ~5 per frame in homozygous CbCln3Δex7/8 cerebellar cells (**p<.001). C. Representative micrographs are shown for wild-type and homozygous CbCln3Δex7/8 cerebellar cells, transiently transfected with plasmid expressing GFP-LC3 to mark autophagic vacuoles. The typical large, mature GFP-LC3-positive autophagic vacuoles in the perinuclear region of the wild-type micrograph are not frequently observed in homozygous CbCln3Δex7/8 cells. Numerous smaller GFP-LC3-positive autophagic vacuoles are commonly observed in the perinuclear region and in the cytoplasm of homozygous CbCln3Δex7/8 cerebellar cells. D. Representative micrographs are shown for a combined endocytic uptake (green) and Lysotracker (red) assay. A reduction in Lysotracker stain and endocytic uptake is observed in untreated homozygous CbCln3Δex7/8 cerebellar cells (CbCln3Δex7/8Δex7/8), compared to wild-type cells (CbCln3+/+). Rapamycin treatment (100 nM, 4 hours) upregulates both endocytic uptake and Lysotracker stain in wild-type and homozygous CbCln3Δex7/8 cells. Note that rapamycin treatment also enhances the overlap of the endocytic stain and Lysotracker in the wild-type cells, but less overlap is observed in the homozygous CbCln3Δex7/8 cells (~4-fold reduced, p<.01, see Experimental Methods). 60x magnification, n=nucleus.

**Figure 8. Inhibition of autophagy, but not induction, leads to decreased cerebellar cell survival.** Representative graphs of cerebellar cell survival are shown, following rapamycin treatment or 3-MA treatment to induce or inhibit autophagy, respectively. 48-hour rapamycin treatment (20 nM-1 μM) halts cell division but does not significantly (p>.05 for all) impact wild-type (CbCln3+/+) or homozygous CbCln3Δex7/8 (red squares) cell survival. Conversely, 3-MA treatment (0.5-10 mM) reduced cell survival (did not reach significance for wild-type, p=.08; homozygous CbCln3Δex7/8 cell loss was significant at 2.5, 5, and 10 mM, p<.05). Homozygous CbCln3Δex7/8 cells were more severely affected by 3-MA than wild-type cells, at all doses tested (p<.05). nM=nanomolar, mM=millimolar.

**Figure 9. Hypothetical model of the disrupted autophagic pathway in JNCL.** A hypothetical feedback cycle that regulates autophagy is depicted for a normal cell and a JNCL cell. In both cases, the initiation of autophagy is negatively regulated by mTOR (grey); phosphorylated (P) mTOR inhibits initiation of the pathway and dephosphorylation of mTOR leads to upregulation of autophagy. LC3-II (yellow circles) coats the membrane of the forming autophagic vacuoles (AV, light blue) and remains...
associated with the AVs as they mature to become autolysosomes (orange). The forming AVs engulf mitochondria and the subunit c protein, which are then matured through fusion with late endosomes (LE, green) and lysosomes (L, red). In the normal cell, full-length CLN3/battenin (dark blue triangles) likely sits on the membrane of late endosomes, lysosomes, intermediate AVs, and autolysosomes, where it may function to regulate AV maturation via modulating trafficking, vesicle fission/fusion, and/or endosomal/lysosomal/AV function. In JNCL, full-length, functional CLN3/battenin is absent from these membranes, which likely leads to deficient AV maturation and a build-up of AV intermediates, which contain subunit c and the membranous material observed by TEM. A failure to efficiently turn over this AV cargo in the JNCL cell may lead to nutrient deprivation and a cellular response to downregulate mTOR and upregulate autophagy. This upregulation of autophagy in the JNCL cell, which may also arise via ATP depletion, may further contribute to the build-up of AV intermediates containing undegraded cargo, such as subunit c. Solid lines depict membrane trafficking pathways and dotted lines depict signaling pathways. Arrows depict positive regulation and perpendicular lines depict negative regulation. The line thickness depicts the relative state of pathway activity.
Figure 1

A

\[\text{Cln3}^{+/+} \quad \text{Cln3}\Delta \text{Δex7/8}/\Delta \text{Δex7/8}\]

Hc

Cb

\text{MOL} \quad \text{P} \quad \text{GCL}

B

\[\Delta \text{Δex7/8} \quad \Delta \text{Δex7/8} \quad ++ \quad \Delta \text{Δex7/8} \quad +/+ \quad \Delta \text{Δex7/8}\]

LC3-I

LC3-II

| Time  | P4 | P8 | 10wk |
|-------|----|----|------|
| Ratio | 10 | 20 | 50   |
Figure 2

Cln3<sup>+/+</sup> vs. Cln3<sup>Δex7/8</sup>

| Hom | PNP | Cyo | E | Mito | AV15 | AV20 | Lys | Hom | PNP | Cyo | E | Mito | AV15 | AV20 | Lys | AV15<sup>*</sup> | AV20<sup>*</sup> | Lys<sup>*</sup> |
|-----|-----|-----|---|------|------|------|-----|-----|-----|-----|---|------|------|------|-----|---------|---------|-----|
|     |     |     |   |      |      |      |     |     |     |     |   |      |      |      |     |         |         |     |

- 64 kDa
- 51 kDa
- 39 kDa
- 28 kDa

*
Figure 4

(A) Western blot analysis of subunit c, monomer, LC3-II, Lamp 1, Grp75, and GRP94 in Cln3+/+ and Cln3Δex7/Δex7 cells. Homogenate (Hom), PNP, Cyto, E, Mito, AV15, AV20, and Lysates (Lys).

(B) Bar graph showing the ratio of total subunit c (Chn3Δex7/Δex7/Chn3+/+) in different subcellular compartments. HOM, PNP, CYT, E, MITO, AV15, AV20, and LYS.
Figure 5

4 months

Fold enrichment over homogenate
(AU)

12 months

Hom  PNP  Cyto  E  Mito  AV15  AV20  Lys

Fold enrichment over homogenate
(AU)
Figure 6

A

B

control  JNCL  JNCL

phospho-p70 S6K  total p70 S6K

phospho-mTOR  total mTOR

CbCln3<sup>+/+</sup>  CbCln3<sup>+/−</sup>  CbCln3<sup>−/−</sup>
Figure 7

A

|                | untreated | +rapamycin |
|----------------|-----------|------------|
| CbCln3<sup>+/+</sup> | ![Image](https://example.com) | ![Image](https://example.com) |
| CbCln3<sup>+/+<sub>mut</sub></sup> | ![Image](https://example.com) | ![Image](https://example.com) |

B

|                | untreated | +rapamycin |
|----------------|-----------|------------|
| Submit inclusion/minute | ![Image](https://example.com) | ![Image](https://example.com) |

C

|                | CbCln3<sup>+/+</sup> | CbCln3<sup>Δex2/8;Δex7/8</sup> |
|----------------|-----------------------|----------------------------------|
| GFP-LC3        | ![Image](https://example.com) | ![Image](https://example.com) |

D

|                | CbCln3<sup>+/+</sup> | CbCln3<sup>Δex2/8;Δex7/8</sup> |
|----------------|-----------------------|----------------------------------|
| untreated      | ![Image](https://example.com) | ![Image](https://example.com) |
| +rapamycin     | ![Image](https://example.com) | ![Image](https://example.com) |
Figure 9
Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis
Yi Cao, Janice A. Espinola, Elisa Fossale, Ashish C. Massey, Ana Maria Cuervo, Marcy E. MacDonald and Susan L. Cotman

J. Biol. Chem. published online May 19, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602180200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts