Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer’s-like axonal dystrophy

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Abstract

In the hallmark neuritic dystrophy of Alzheimer’s disease (AD), autophagic vacuoles containing incompletely digested proteins selectively accumulate in focal axonal swellings, reflecting defects in both axonal transport and autophagy. Here, we investigated the possibility that impaired lysosomal proteolysis could be a basis for both defects leading to neuritic dystrophy. In living primary mouse cortical neurons expressing fluorescence-tagged markers, LC3-positive autophagosomes forming in axons rapidly acquired the endo-lysosomal markers, Rab7 and LAMP1, and underwent exclusive retrograde movement. Proteolytic clearance of these transported autophagic vacuoles was initiated upon fusion with bi-directionally moving lysosomes that increase in number at more proximal axon levels and in the perikaryon. Disrupting lysosomal proteolysis by either inhibiting cathepsins directly or by suppressing lysosomal acidification slowed the axonal transport of autolysosomes, late endosomes and lysosomes and caused their selective accumulation within dystrophic axonal swellings. Mitochondria and other organelles lacking cathepsins moved normally under these conditions, indicating that the general functioning of the axonal transport system was preserved. Dystrophic swellings induced by lysosomal proteolysis inhibition resembled in composition those in several mouse models of AD and also acquired other AD-like features, including immunopositivity for ubiquitin, APP, and neurofilament protein hyperphosphorylation. Restoration of lysosomal proteolysis reversed the affected movements of proteolytic Rab7 vesicles, which in turn, largely cleared autophagic substrates and reversed the axonal dystrophy. These studies identify the AD-associated defects in neuronal lysosomal proteolysis as a possible basis for the selective transport abnormalities and highly characteristic pattern of neuritic dystrophy associated with AD.

INTRODUCTION

In Alzheimer’s disease (AD), focal swellings develop along the axons and dendrites of neurons throughout affected brain regions. These dystrophic neurites also form a denser spherical plexus intermixed with extracellular deposits of amyloid β, referred to as senile or “neuritic” plaques. Together, neuritic plaques and neurofibrillary tangles within neurons

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constitute the defining neuropathological hallmarks of AD. The accumulation of organelles within dystrophic neurites has long been believed to reflect a disruption of axonal transport (Morfini et al., 2009). Recent ultrastructural observations have shown that the vast majority of structures present in dystrophic neurites in AD brain are organelles specifically related to the autophagic-lysosomal pathway (autophagic vacuoles or AVs) (Nixon et al., 2005), suggesting that transport of this class of organelles is selectively disrupted. In addition, the remarkable abundance of undigested protein within accumulated AVs, many of which contain lysosomal proteases, strongly implies a defect in the proteolytic clearance of autophagy substrates by lysosomes. In this regard, comparable levels of protein “storage” in brain are most often observed in certain primary lysosomal storage diseases associated with severe cognitive disabilities and neurodegenerative phenotypes that share key neuropathological features of AD (Bahr and Bendiske 2002; Ohm et al 2003; Ballabio and Gieselmann 2009). Further implicating primary dysfunction of the lysosomal system in AD is evidence for a continuum of abnormalities within endocytic and autophagic pathways connected to lysosomes, some linked directly to genes causing early onset AD (Nixon et al., 2000; Nixon and Cataldo 2006; Lee et al., 2010).

Autophagy is the cell’s sole mechanism for the degradation of organelles, and a major route for the bulk degradation of cytoplasm, especially of long-lived proteins (Dunn, 1994). Its activation by nutrient deprivation or the presence of protein aggregates promotes survival (Shintani and Klionsky, 2004). Following the highly regulated sequestration of organelles and cytoplasm within a double membrane vacuole, the autophagosome (Nakatogawa et al., 2009; Inoue and Klionsky, 2010), the rate-limiting step of substrate digestion occurs when autophagosomes fuse with lysosomes to form autolysosomes (Tanida et al., 2005). Activation of acid hydrolases, including proteases (cathepsins) within the lysosomes/autolysosomes requires acidification of the intralumenal environment by the vacuolar ATPase (v-ATPase) proton pump (Sun-Wada et al., 2003).

In this study, we investigated the possible relationship between lysosomal dysfunction, neuritic dystrophy, and selective accumulation of AVs and lysosomes. Using live-imaging of fluorescently-tagged compartmental markers in primary cortical neurons, we characterized the fate of autophagosomes in axons and compared the axonal transport of specific organelles after disrupting lysosomal proteolysis. Our findings demonstrate that lysosomal proteolysis inhibition, but not autophagy activation - another potential basis for promoting axonal dystrophy - slowed the axonal transport of autophagy-related organelles, without affecting transport of other organelles. Moreover, the same class of lysosomal system cargoes accumulated within axonal swellings which also acquired additional biomarkers characteristic of dystrophic neurites in AD brains. These results directly link lysosomal proteolysis dysfunction to two additional key aspects of AD pathogenesis, underscoring the importance of this deficit as a target for AD therapies (Yang et al. 2010; Sun et al., 2008).

MATERIALS AND METHODS

Antibodies and plasmids

Mouse monoclonal antibodies to LC3 (NanoTools, Teningen Germany), phosphorylated NF-M/H (SMI-31) and unphosphorylated NF-M/H (SMI-32; Sternberger Monoclonal Inc., Lutherville, MD), dynein intermediate chain (DIC; Sigma, St. Louis, MO), β-tubulin (Sigma, St. Louis, MO), p62 (Abnova, Taiwan), ubiquitin (Millipore, Bedford, MA), microtubule-associated protein 2 (MAP2; Chemicon), rat monoclonal antibody to LAMP-2 (Hybridoma Bank, Iowa City, Iowa), rabbit polyclonal antibodies to LC3 (Novus Biologicals, Littleton, CO), GFP (Abcam, Cambridge, MA), ubiquitin (Dako, Carpinteria, CA), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), guinea pig polyclonal antibody...
to p62 C-terminus (Progen Biotechnik) were purchased from commercial sources as listed above. Rabbit polyclonal antibodies against NF-L and cathepsin D were generated in our laboratory, mouse monoclonal anti-APP for human and murine APP referred to as antibody C1/6.1 has been described previously (Mathews et al., 2002), and mouse monoclonal murine-specific APP antibody M3.2 that recognizes APP holoprotein, sAPPα, β-CTF, and Aβ was provided by Paul Mathews (Nathan Kline Institute, New York University, New York NY).

EGFP-LC3 was a gift from Dr. Noburu Mizushima (Tokyo Medical and Dental University, Tokyo, Japan); GFP-RFP-LC3 (Kimura et al., 2007) was generously provided by Dr. Tamotsu Yoshimori (Osaka University). YFP-LAMP1 was provided by Dr. Joel Swanson (University of Michigan, Ann Arbor, MI). GFP-Rab7 was a gift from Dr. Anne Cataldo (McLean Hospital, Belmont, MA). DsRed-LC3 was made as described previously (Boland et al 2008). pDsRed2-Mito and pAcGFP1-Endo were purchased from Clontech.

Cell culture and transfection
Embryonic mouse cortical neurons from C57B1/6J mice were cultured as described (Boland et al 2008). Primary cortical neurons were harvested from embryonic day 16–17 pups. Pups were decapitated and cerebral cortices were removed and dissociated by incubation in Hibernating Medium (BrainBit) containing Papain (10 mg/ml) and DNase for 15 min at 37°C. Following digestion, cells were centrifuged at 1000 rpm for 3 min and the pellet was re-suspended in Modified Eagle’s Medium with 10% FBS (Hyclone) for counting and plating. Cells were plated (0.75 X 10^5 cells/cm²) on glass-bottom chamber dishes (BD Biocoat) or on circular coverslips (1.5 X 10^5 cells/cm²), both coated with poly-d-lysine (200 μg/ml) and incubated at 37°C for at least 4 hours. After the cells attached, the plating medium was changed to Neurobasal medium containing B27, Penicillin/Streptomycin, and Glutamax. Half the volume of culture media was exchanged every 3 days. Penicillin/Streptomycin was removed from media after the first feeding. Neurons were transfected on DIV 3–4 using Lipofectamine 2000 (Invitrogen), based on the manufacturer’s suggestions with minor modifications. Conditioned media was replaced with fresh Neurobasal media containing 1–3 μg DNA/μL of Lipofectamine 2000 in 500 mL Optimem (Invitrogen). The neurons were transfected for 30 minutes at 37°C, and subsequently washed 3 times with Neurobasal Medium to remove DNA and resuspended in conditioned media. Neurons were grown for 24 to 48 hours after transfection prior to treatments or time-lapse imaging.

For lysosomal inhibition or autophagy activation, cells were treated with leupeptin (20–100μM, International Peptides), E64 (10μM, Sigma), pepstatin (20μM, Sigma) or bafilomycin (10–50nM, Sigma), or rapamycin (10nM, Sigma). For leupeptin-recovery in GFP-Rab7 neurons, transfected neurons were treated with leupeptin (20μM), or normal media (control recovery) for 24 hours starting 6 hours after transfection and recovered in normal Neurobasal media for an additional 24 hours.

Western blot analysis
Neurons (5–7 DIV) grown in 6-well plastic dishes (BD Bioscience, Franklin Lakes, NJ) were washed (3X) in Tris buffered saline (TBS, pH 7.4) at RT, then scraped in 200ml/well of ice-cold lysis buffer (M-PER buffer containing Halt Protease Inhibitor Cocktail (1:100) and 50mM EDTA, Pierce, Rockford, IL). Protein concentration was determined using the Bradford Assay (Pierce) and samples were standardized to 1mg/ml using 70% trichloroacetic acid to precipitate cell lysates that were resuspended in equal volumes of lysis buffer. Sample loading buffer (2X) [62.5mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue, 710mM β-mercaptoethanol] was added to cell lysates at a 1:1 ratio with lysis buffer before heating samples for 5 minutes at 90°C. Tris-glycine gels
(Invitrogen) were loaded with 20 μg/well of protein and separated either on 4–20% gels or 16% gels to separate LC3-I and LC3-II. Separated proteins were transferred onto 0.2 μm nitrocellulose membranes (Protran, Whatman, Florham Park, NJ) for 24 hours at 10V. Membranes were rinsed in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) before being blocked at RT for 1 hour in a 5% non-fat milk/TBS-T solution. Membranes were incubated with primary antibody overnight at 4°C, washed three times in TBS-T and then incubated for 1 hour at RT in secondary antibody solution (3% non-fat milk/TBS-T) containing HRP-conjugated secondary antibodies (Promega, Madison, WI). Membranes were washed three times for 10 minutes in TBS-T before chemiluminescent substrates (ECL, Perkin Elmer) were applied and membranes were exposed to film. Densitometry of bands representing protein expression was performed using Image-J (NIH Image v.1.42i; http://rsb.info.nih.gov/ij) software. For each immunoblot, the band intensity of each lane was normalized relative to the loading control and compared to the control lane. Subsequently, the percent change between treatments was calculated based on the normalized values.

**Immunocytology**

Neurons were fixed for immunocytochemistry analyses by removing culture medium, washing (3x) in cold TBS and adding 4% paraformaldehyde/5% sucrose/TBS (pH 7.4) for 15 minutes at RT, washed 3 times with TBS and permeabilized for 15 minutes in 0.02% Triton-X/TBS. For LC3 and LAMP immunocytochemistry, cells were fixed in −20°C methanol for 5 minutes, washed 3X in TBS and permeabilized with digitonin/TBS (0.1 mg/ml). Non-specific antigens were blocked by incubation in 10% NGS/0.02% Triton-X/TBS blocking solution for 1 hour at RT. Primary antibodies were diluted in 0.5% BSA-TBS and incubated overnight at 4°C. The next day, cells were washed (3x) in TBS for 10 minutes prior to incubation with Alexa-488 or Alexa-546 secondary antibodies (1:1000 in blocking solution, Invitrogen) for 1 hour at RT. Neurons were washed (3x) in TBS for 10 minutes before mounting onto microscope slides with anti-fade Gelmount (Biomed, Foster City, CA) and visualized using a Zeiss confocal microscope.

**Magic Red, LysoTracker-Red, and Bodipy-Pepstatin loading**

Neurons plated on 35mm glass-bottom chamber dishes (BD Biocoat) were incubated with 1μM of either BODIPY-pepstatin-FL (Invitrogen), Magic Red cathepsin B (Marker Gene Technologies) or Lysotracker Red (Invitrogen) in Neurobasal medium for 30–60 minutes at 37°C followed by washing with fresh Neurobasal Medium (2X). Subsequently, Neurobasal medium was replaced with pre-warmed Low Fluorescence Hibernate Medium (Brainbits, Springfield, IL) to reduce fluorescent background. For live imaging, cultures were placed in a humidified chamber maintained at 37°C and 5% CO₂, and mounted on a Zeiss LSM510 confocal microscope. Z-stacks were acquired using LSM 510 software.

**Live Imaging**

35mm-glass bottom dishes (BD) containing normal growth medium (NB supplemented with B27) or Low Fluorescence Hibernate Medium (Brainbits) supplemented with B27, were mounted in a temperature-control stage on the confocal microscope, and maintained at 37°C and 5% CO₂. The imaging was performed using a Zeiss confocal equipped with LSM 510 attachment using a 40X oil immersion lens. Laser lines at 488nm (GFP-tagged constructs, and YFP-construct) and 543 nm (DsRed-constructs) were used. Time-lapse recordings were acquired by scanning single plane images every 3–5 s for at least 5 minutes. Our data was collected in the axon (longest process emerging from the cell body) up to approximately 400 microns away from the cell body in any area where a substantial length of axon was in focus and appropriate to record movements. Kymographs of time-lapse movies were generated using ImageJ (Multiple Kymograph plugin).
Transport Analysis

Transport parameters of vesicle movement were generated using the ImageJ plugin MtrackJ (www.imagescience.org/meijering/software/mtrackj). For each time-lapse movie, manually tracking individual vesicles using this program generates “Points” data (x, y, t-coordinates of each point/frame) as well as “Tracks” data (duration, min/max/mean V, etc.) based on an arbitrary reference point representing the most proximal point in the neurite that is closest in proximity to the cell body. After manually tracking vesicles until they were no longer visible, “Points” and “Tracks” data were transferred to MS Excel to calculate net transport direction, relative frequency of velocity, average velocity, and frequency of direction changes. Minimum velocity threshold was set at 0.1 μm/s, where vesicles with maximum velocity less than 0.1 μm/s for the entire track were categorized as stationary, whereas vesicles with at least one movement with velocity of at least 0.1 μm/s were categorized as motile (moving). The transport direction was calculated based on the algebraic sign of dx/dt from frame to frame (x1−x0)/(t1−t0), where anterograde movement was positive and retrograde movement was negative. Net transport direction was determined by comparing the initial and final (x, y) position of the vesicle, and classified as retrograde, anterograde or stationary (if no movement above 0.1 μm/s occurred despite a net change in position). Motility and net transport direction was expressed as an averaged percent of total vesicles per axon. The relative frequency of instantaneous velocity was obtained by sorting and binning instantaneous velocity values of each track in intervals of 0.2 μm/s, and using the COUNT function of MS Excel for each bin divided by the total number of movements.

Morphometric Analysis of GFP-LC3 neurons

The number of GFP-LC3 neurons containing GFP-puncta in the cell body or in neurites, were counted using confocal images of GFP-LC3 transfected neurons, imaged at 40X direct magnification for the various treatment conditions. At least 40 neurons were examined and values are expressed as a percent total of counted cells. Based on visual detection of puncta, we categorized neurons into as either neurons with vesicular structures or neurons with no puncta (diffuse LC3 only). When punctate LC3 was detectable, the number of cells where puncta was visualized in the cell body, axons, or both was counted. All numerical values are expressed as a mean ±/− SEM. For counting number of GFP-LC3 vesicles in axons, the number of LC3 vesicles were manually counted and subsequently divided by the total length of the axon as measured using the NIH ImageJ plugin, NeuronJ.

The number of GFP-LC3 vesicle swellings per length of neurite and the percentage of GFP-LC3 neurons containing swellings were analyzed by measuring the total length of the GFP-LC3 neurite in the 40X field using ImageJ plugin, NeuronJ, and counting the number of visible swellings. At least 30 neurons were examined for each condition.

Electron Microscopy

All animal studies were carried out according to the regulations of the IACUC at the Nathan Kline Institute. Transgenic mice expressing either the Swedish mutation of human APP (APPK670M/N671L; referred to as APP) or APPswe/PS1M140L (referred to as PSAPP) at the indicated plaque-bearing ages were anesthetized and transcardially perfused with 4% paraformaldehyde/0.1M sodium cacodylate buffer, after which brains were dissected and submerged in 2% glutaraldehyde/0.1 M sodium cacodylate buffer overnight at RT. 50 μm coronal sections cut by vibratome were postfixed in 1% osmium tetroxide in Sorensen’s
phosphate buffer for 1 hour at RT and dehydrated in a series of increasing concentration of ethyl alcohols (50–100%). For neurons, DIV 4–5 neurons grown on glass coverslips were fixed by removing culture medium, washing once in 37°C supplement-free Neurobasal medium and adding 4% paraformaldehyde/1% glutaraldehyde/5% sucrose in 0.1M sodium cacodylate buffer (pH 7.2; Electron Microscopy Sciences (EMS), Hatfield, PA) for 24 hours at RT. Following fixation, neurons were washed (x3) in 0.1M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide and progressively dehydrated in a graded series of ethanol (50% – 100%) followed by embedding in Epon (EMS, Fort Washington, PA) for at least 3 days at RT. Tissue was embedded in Epon were cut serially into ultrathin (0.06 μm) sections. Ultrathin sections were stained with uranyl acetate and lead citrate. Ultrathin sections were cut from Epon-embedded blocks and placed on copper grids for structural analysis.

Quantitative analysis of AVs from EM images

For quantification of organelles in neuritic swellings after leupeptin treatment, 30 randomly selected EM images with accumulated organelles were captured at a final magnification of 10,500×, and the number of each type of AV, mitochondria and single membrane vesicles was counted using the criteria for AV identification previously established (Nixon et al., 2005). For APP and PSAPP mice, the same procedure was applied, but 50 dystrophic neurites in each of two mice per genotype were used at the indicated ages.

RESULTS

Autophagosome identification in primary cortical neurons

To study the dynamic behavior of autophagosomes in primary cortical neurons (4–5 DIV), we monitored the dynamics of LC3-positive vesicles in the cell bodies and processes after EGFP-LC3 transfection, an established method for autophagosome detection (Kabeya et al., 2000; Bampton et al., 2005; Klionsky et al., 2008). By 24–48 hours after transfection, GFP-LC3 distributed uniformly and predominantly as cytosolic LC3-I and the majority (>90%) of GFP-LC3 neurons exhibited smooth processes and a healthy appearance without evident toxicity (Figure 1A). The axon of each neuron, the longest projection emerging from the cell body, was clearly identified by its strong immunolabeling for the axonal marker neurofilament (Figure 1B), but not for MAP2, a marker of dendrites (Figure 1C). LC3 was also present in MAP2-positive dendrites; however, these dendrites were comparatively much shorter in length than axons (Figure 1D).

Autophagosomes were identified as strongly GFP-LC3-positive, or LC3-immuno-positive, vesicular compartments, and nearly all LC3-vesicles fell into a size range of 0.5–1μm, consistent with the reported size of approximately 90% of RFP-LC3 vesicles in healthy Purkinje neurons, which were smaller than 0.75μm in diameter (Bains and Heidenreich, 2009). Under normal conditions, GFP-LC3 vesicles distributed throughout the somatodendritic region and axons (Figure 1A; insets). In live imaging analyses, de novo formation of LC3-vesicles in cell bodies could be detected occasionally (Figure 1 G), whereas LC3 vesicles in axons often originated by budding from existing vesicles (Figure 1 H). The dynamic behaviors of these profiles as well as the co-localization of intrinsic membrane proteins in those structures excluded the possibility that they represented artifactual aggregates of over-expressed LC3 (Ciechomska and Tolkovsky, 2007). Of the approximately 70% of GFP-LC3 neurons (n=42) displaying LC3-positive vesicles, LC3 puncta were more frequently found in axons (83 ± 5.29 % of neurons contain an average of 5 puncta per 100μm of axon length) than in perikarya (33 ± 6.6% of neurons displayed LC3 puncta in their perikarya). Neurons transfected with Red LC3 constructs (DsRed-, RFP-) exhibited a similar pattern but displayed more vesicles in cell bodies (Figure 1E and F),

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consistent with the relatively greater stability of red fluorescence compared to green in fully acidified autolysosomes following autophagosome-lysosome fusion (Kimura et al., 2007), which is an event that is most active in the cell body (Jahreiss et al., 2008).

**Autophagosomes undergo predominantly retrograde transport**

To analyze the movement of axonal LC3 vesicles in relation to other organelles, we co-expressed the mitochondrial marker DsRed-Mito with GFP-LC3. The two markers rarely co-localized in axons (Figure 2 A and B) and exhibited very different patterns of transport. LC3 vesicles moved in a processive pattern almost exclusively in the retrograde direction (Figure 2A). Among over 3500 total individual measurements, there were less than 50 anterograde movement events. Moreover, the net direction of movement occurred mostly in the retrograde direction (Figure 2). In contrast to the active retrograde movement of LC3 vesicles, mitochondria were most frequently paused for long periods (≥5 min), and when moving, traveled in either an anterograde or retrograde direction (Hollenbeck and Saxton, 2005) (Figure 2B). The relatively uniform distribution of autophagosomes along axons and their strong net movement in the retrograde direction implies that autophagosome formation is an active process in axons.

**Late endosomes and lysosomes undergo bidirectional movement**

To investigate the trafficking of lysosomes in axons, we identified compartments that were strongly acidified and contained active cathepsins or LAMP1 (lysosome-associated membrane protein 1), a marker of late endosomes and lysosomes. After expression of YFP-tagged LAMP-1, LAMP1-positive vesicles were concentrated near the somatodendritic region, although LAMP1 vesicles were also scattered throughout the axon (Figure 3A and B). YFP-tagged-LAMP1 (Figure 3A and B) or acidified vesicles identified using LysoTracker-Red exhibited a similar distribution pattern (Figure 3C), as did endogenous lysosomes detected by LAMP1/cathepsin D double immunofluorescence in non-transfected neurons (Figure 3D).

To confirm that YFP-LAMP1 vesicles contained active cathepsins, we double-labeled YFP-LAMP1-vesicles with a cathepsin B substrate linked to Magic-Red, an indicator dye that fluoresces only when the cathepsin B substrate is cleaved. Active cathepsin B was detected in virtually all LAMP1-vesicles in the cell body and within proximal regions of the axon. By contrast, at more distal levels of the axon, the intensity of Magic-Red in YFP-LAMP1 vesicles progressively declined (Figure 3B). This co-localization pattern resembled the distribution pattern of cathepsin D immuno-reactive LAMP vesicles, where less than 20% or LAMP vesicles in axons were cathepsin D-positive (Figure 3D; inset). LC3 vesicles had matured to mainly autolysosomes, which was confirmed by the detection of Bodipy-pepstatin-FL, a probe for active cathepsin D and a marker for autolysosomes/lysosomes in DsRed-LC3 vesicles (Figure 3K). In comparison to axons, dendrites contained abundant lysosomes suggesting that lysosomal degradation efficiency in dendrites is more comparable to that in the cell body than in the axon (not shown).

Compared to the transport of LC3 vesicles, LAMP1-vesicles moved more frequently in the anterograde direction (anterograde LAMP-37.9 ± 7.4% versus anterograde LC3: < 2% total), frequently changed direction (5 events/min on average) and also exhibited more rapid velocities in either direction (0.5 μm/s for LAMP vs. 0.3 μm/s for LC3, Figure 4D). LAMP1-vesicle and LysoTracker Red (LT)-positive vesicles in non-transfected neurons had nearly identical transport behaviors, which were consistent with parameters of LysoTracker-positive vesicles reported in other neuronal culture systems (Lalli and Schiavo, 2002). Thus, the active bidirectional movements of lysosomes in axons are likely to facilitate efficient fusion with LC3 vesicles. As LC3 is delivered by retrograde transport to proximal axons and

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the perikarya, a higher incidence of lysosomes in the cell body make AV-lysosomes fusion (i.e. complete autophagosome clearance) more likely (Kimura et al., 2007, 2008).

**Autophagosomes mature to late endosomes and lysosomes during retrograde transport**

We confirmed that autophagosomes fuse with lysosomes in axons by finding that over 97% of LC3 vesicles co-localized with GFP-Rab7 (Figure 3J) or YFP-LAMP (Figure 3I). We determined whether LC3 vesicles in axons fuse with those containing Rab7 or LAMP after co-expressing DsRed-LC3 with GFP-Rab7 or YFP-LAMP1. Even though smaller proportions of LAMP1 vesicles (31 ± 3%, n=179 vesicles) or Rab7 vesicles (43±5%, n=159 vesicles) were also LC3-positive, LC3-Rab7/LAMP vesicles retained the transport properties of predominant retrograde transport that characterize GFP-LC3 vesicles (Figure 3E–H) rather than assuming the predominantly anterograde movement of LAMP-positive vesicles lacking LC3 (Figure 3F, arrowheads). To eliminate the possibility that combined over-expression of an endosomal protein and LC3 would promote their co-localization, we co-expressed DsRed-LC3 with AcGFP-RhoB, a protein present on endocytic membranes and widely used as an early endosome marker (Rondanino et al., 2007) and found minimal co-localization (Figure 3L). In further support of this observation, efficient fusion between axonal LC3 and LAMP vesicles required microtubule-dependent transport, as the microtubule destabilizing agent vinblastine (1 μM, 1 hour) abolished co-localization (Figure 3M). These data combined with the distribution of LAMP/cathepsin-positive vesicles (Figure 3A–D) indicate that regardless of the proximity to the cell body, autophagosomes in axons acquire Rab7/LAMP rapidly such that LC3 vesicles observed along the axon are almost always observed to be fused with endosomes (to form amphisomes) or with a lysosome. However, given that autophagosomes undergo multiple fusion events with lysosomes for complete degradation (Kimura et al., 2007), full maturation to autolysosomes containing activated lysosomal hydrolases is most likely to occur when AVs are transported to proximal regions of axons or to the cell body where lysosomes are concentrated.

**Lysosomal acidification failure or cathepsin inhibition selectively impairs axonal transport of cathepsin-containing vesicles**

In light of evidence pointing to significant impairment of both autophagic proteolysis and axonal transport in AD, we investigated the effects on transport dynamics of blocking lysosomal proteolysis by either of two independent mechanisms. The lysosomal inhibitors, leupeptin, a cysteine protease inhibitor (20μM for 24 hours), or bafilomycin A, an inhibitor of lysosomal v-ATPase (50nM for 4 hours), effectively blocks lysosomal cysteine protease activity or intralumenal acidification respectively, resulting in impaired degradation of the autophagic vesicles reflected by the increase in the ratio of LC3-II (autophagosome membrane-associated) to LC3-I (cytosolic)- an index of autophagy turnover (Klionsky et al, 2008)- and higher levels of autophagy substrate p62 (Figure 9C). Both treatments significantly inhibited movements of LC3-vesicles based on time-lapse movies of GFP-LC3 taken from axonal regions that did not show obvious changes in axonal diameter or organelle accumulation. Quantification of kymographs from GFP-LC3 time-lapse movies confirmed that the percentage of stationary LC3 vesicles significantly increased from less than 5% under normal conditions to over 50% after leupeptin and over 75% in bafilomycin (Figure 4 A, B). Furthermore, the mean velocity during retrograde transport was slowed (Figure 4B) especially after bafilomycin treatment. However, within LC3 vesicle that retained movement after leupeptin, budding events were occasionally observed, suggesting that budding may not be affected by proteolysis inhibition.

To investigate whether or not the higher incidence of stationary LC3 vesicles after proteolysis inhibition may involve detachment from its retrograde transport motor, we probed for the minus-end motor dynein immunoreactivity on LC3 vesicles using a dynein
intermediate chain (DIC) antibody. Consistent with evidence that LC3 retrograde transport is predominantly mediated by dynein (Kimura et al., 2008; Katsumata et al., 2010), we found that 93 ± 3.9 % of axonal LC3 vesicles were normally associated with dynein (n= 60 LC3 vesicles for control; Figure 4 C and D). By contrast, 27 ± 6.3 % of LC3 vesicles along axons were not co-localized with DIC after leupeptin treatment (p < 0.05; n= 163 vesicles for leupeptin treatment; Figure 4C and D), and this fraction of LC3 vesicles was slightly less than the percentage increase in stationary behavior of LC3 vesicles after leupeptin (approximately 40%), suggesting that detachment from the retrograde motor may partially account for the increased proportions of stationary LC3 vesicles resulting from cathepsin inhibition.

Because both leupeptin and bafilomycin inhibit lysosomal proteolytic activity and LC3 compartments rapidly acquire lysosomal membrane protein LAMP-1, we quantified the movements of LAMP-1 and LysoTracker Red vesicles after these treatments. Proteolysis inhibition significantly increased the number of LAMP or LysoTracker Red vesicles that were stationary (Figure 5A) and reduced the frequency of direction changes and mean anterograde or retrograde velocities of still-moving vesicles (Figure 5D). Leupeptin or bafilomycin had similar effects on GFP-Rab7-positive late endosomes, another organelle containing cathepsins (Bucci et al., 2000)(Figures 5B, E). By contrast, lysosomal proteolysis inhibition did not affect the transport behaviors of vesicular organelles lacking cathepsins. AcGFP-RhoB-positive early endosomes displayed a vesicular distribution pattern, and immunofluorescence co-labeling with cathepsin D confirmed that these compartments do not contain cathepsin (not shown). Axonal transport of AcGFP-RhoB vesicles was very active and bidirectional (Figure 5C) with a bias toward retrograde movement. Unlike protease containing vesicles, however, GFP-RhoB-vesicles in axons were transported normally either before or after treatment with leupeptin or bafilomycin (Figure 5 C, E). Similarly, mitochondrial transport was unaltered by leupeptin treatment (Figure 4E, F). In contrast to the effects of lysosomal proteolysis inhibition on the transport of LC3, LAMP-1, Rab7 and LysoTracker-positive vesicles, strong induction of autophagy with rapamycin (RM) had no detectable effect on transport (Table 1).

**Disrupted lysosomal proteolysis causes AD-like axonal dystrophy**

The selective organelle trafficking disruption induced by lysosomal proteolysis inhibition caused LC3 vesicles to accumulate in axons, especially within focal swellings (Figures, 6A, D). The percentage of GFP-LC3 neurons that displayed axonal swelling (Figure 6F) as well as the frequency of swellings (Figure 6G) increased approximately 3-fold after leupeptin, E64 (a more selective cysteine protease inhibitor), pepstatin (an aspartic protease inhibitor) or bafilomycin treatment (Figure 6F). The frequency of GFP-LC3 vesicles between axonal swellings was also increased over two fold by leupeptin and bafilomycin (Figure 6 H). Other cathepsin-containing vesicular organelles such as LysoTracker-positive lysosomes and Rab7-positive late endosomes were also sequestered in swellings following leupeptin treatment (Figure 7 A–B), whereas DsRed-positive mitochondria (Figure 7C) or early endosomes detected by immunocytochemistry for early endosome membrane protein Rab5 (Figure 7D), were only occasionally observed in the swellings. In live-imaging analyses of GFP-LC3 and DsRed-Mito in relation to axonal swellings, we observed that mitochondria enter and exit the swollen region whereas LC3 vesicles accumulate (Figure 7J), establishing that cathepsin inhibition or accumulated AVs in axonal swellings did not block the trafficking of mitochondria. In further support of our observation that global axonal transport is not affected by proteolysis inhibition, additional immunocytochemical analyses showed that non-vesicular axonal constituents such as β-tubulin and NF-L were also not enriched in swellings (Figure 7E, F). These organelles have been previously observed to
accumulate above and below a global block in axonal transport (Griffin et al., 1977; Nagatsu et al., 1978).

We also investigated the presence of other antigens related to neuritic dystrophy that are found to be associated with autophagic vacuoles. Vesicles immuno-labeled with antibodies to APP and ubiquitin, which are commonly seen in dystrophic neurites in senile plaques in the AD brain (Dickson et al., 1990), were enriched within LC3-positive swellings (Figure 7G, H). Notably, endogenous APP C-terminal fragments were accumulated in the lysates in leupeptin-treated neurons (Figure 7K). Phosphorylation of neurofilaments, another marker of dystrophic neurites, was also increased in swellings with LC3-accumulation (Figure 7I), although the total levels of these proteins (the sum of phosphorylated and non phosphorylated NF) were not elevated (Figure K), suggesting that neurofilament kinases are locally activated within axonal swellings.

Consistent with the transport analyses, neuritic swellings in leupeptin-treated neurons viewed ultrastructurally were filled predominantly with autophagy-related structures (Boland et al, 2008; Figure 7 M), including double-membrane AVs, amphisomes, and autolysosomes, and were similar in composition of profiles to the dystrophic neurites seen in PSAPP and APP at ages after plaques have developed (6–10 month-old PSAPP; Figure 7P, 23 month-old APP; Figure 7O). Morphometric analysis of over 50 dystrophic neurites in these mouse models showed that AVs constitute nearly 90% of the organelles in dystrophic neurites, whereas mitochondria and clear lumen vesicles each accounted for fewer than 5% of accumulated organelles (Figure 7N). Dystrophic neurites from leupeptin-treated neurons exhibited a similar organelle composition to dystrophic neurites in vivo (Figure 7N) whereas autophagosomes are absent in normal neurites (Figure 7L).

Reversibility of proteolysis inhibition induced axonal transport deficits and axonal dystrophy

We replaced leupeptin-containing media with normal media after 24 hours to investigate the possibility of recovery. During 3 days in normal media, cathepsin B activity was restored to normal levels (Figure 8A, B). To determine whether axonal transport of protease-containing vesicles could also be recovered, we measured the movements of GFP-Rab7 vesicles after replacing from leupeptin-containing media with normal media. At 24 hours of recovery, the motility and percentage of net retrograde movements of GFP-Rab7 vesicles were nearly identical to the levels of control-recovery neurons (Figure 8C, D). The significant increase in stationary behavior and reduction in retrograde movement of GFP-Rab7 vesicles caused by leupeptin (Figures 5A; 8C, D— independent experiments) was completely reversed, although the mean velocity of retrograde vesicles remained slower after recovery (Table 2).

Regarding biochemical markers of axonal dystrophies and autophagic contents, recovery of lysosomal proteases by replacing leupeptin-containing media with normal media resulted in a decline in the phosphorylation of neurofilaments (SMI-31; Figure 9C) and in levels of p62 to the level seen in untreated neurons (Figure 9C, D). Using SMI-31, an antibody used to label axonal swellings (defined as focal regions of 7x-fold increased diameter), the 3-fold increase in the number of SMI-31 positive axon swellings after leupeptin (Figure 9B) was nearly completely reversed during the recovery period, although LC3-II/I ratios remained elevated (Figure 9C). Recovery was also evident ultrastructurally. By 4 days of recovery, AVs in both the cell bodies and neurites compared to those in leupeptin-treated neurons (large and compact with electron-dense material; Figure 9E, F) had now become clear single membrane vesicles (i.e., autolysosomes; see Boland et al 2008; Figure 9G, H), indicative of maturation due to restoration of intra-lumenal proteolysis. The reversibility of this pathology
indicates that dystrophies that are produced following lysosomal failure are not committed to irreversible neurodegeneration.

**DISCUSSION**

Growing genetic and biochemical evidence indicates that lysosomal proteolysis is markedly impaired in neurons in AD (Boland et al., 2008, Lee J-H et al., 2010, Yang et al., 2010). Here we show that lysosomal proteolysis inhibition, but not strong autophagy induction, selectively disrupts the transport of cathepsin-containing AVs, late endosomes and lysosomes. The impaired transport of these cargoes resulted in the formation of axonal swellings selectively enriched in this same class of autophagic and endosomal-lysosomal vesicles and are also immuno-positive for APP, ubiquitin and hyper-phosphorylated neurofilaments, three well-known markers of the hallmark dystrophic neurites seen in AD brain. The strong resemblance of these axonal swellings to the dystrophic neurites in AD brain suggests that lysosomal proteolysis impairment is a driving force behind the highly characteristic axonopathy that develops in AD.

**Origin and fate of autophagosomes in axons**

In agreement with previous evidence that neuronal autophagy is constitutive (Komatsu et al., 2005; Boland et al., 2008), autophagosome formation of GFP-LC3 positive vesicles was active in both the somato-dendritic and axonal regions. The higher frequency of GFP-LC3 vesicles in axons than cell bodies was expected given the longer residence time of AVs in an axon than in the cell body where degradation is facilitated by the higher density of lysosomes. It is also possible that autophagosomes form at higher rates at axon terminals, as previously proposed (Kaasinen et al., 2008).

The extreme polar shapes of neurons, which prolong the maturation of LC3 vesicles, facilitated our investigations on the fates of AVs. Based on quantitative data on nearly 300 GFP-LC3 vesicles, we observed that the overwhelming majority of LC3-positive vesicles in axons move in the retrograde direction (Figure 2). After they appear, LC3 vesicles rapidly fuse with late-endosomes (Rab7/LAMP2 vesicles) abundant all along the axons to form amphisomes or with a lysosome to form an autolysosome: very few LC3 vesicles were seen that were not fused with Rab7 or LAMP. Interestingly, the extensive co-localization of LC3 with Rab7 supports previous findings that fusion between AVs and endosomes is active (Morvan et al., 2009) and may be necessary for autophagy completion (Filimonenko et al., 2007; Fader et al., 2008; Razi et al., 2009).

Although lysosomes are often assumed to be restricted to the perikarya, lysosomes have been identified in axons of cells in pituitary axons in human brain (Cataldo and Broadwell, 1984), hippocampal pyramidal neurons (McGuinness et al., 2007) and dorsal root ganglia (Perrot and Julien, 2009). We observed vesicles containing LAMP1, active cathepsins (B and D), and LysoTracker in axons, and established that AVs fuse with proteolytically active lysosomes in axons, although fusion is more active near the cell body where the lysosomes are most concentrated. This mechanism of progressive maturation of AVs as they approach the cell body was proposed in earlier studies that provided strong support for this proposal (Hollenbeck, 1993; Overly and Hollenbeck, 1996). Although LC3 has been shown to bind to the anterograde motor kinesin via FYCO (Pankiv et al., 2010), the extensive acquisition of Rab7/LAMP on LC3-positive vesicles likely converts them into mainly retrogradely moving AVs, thus favoring transport to the cell body for complete degradation.
Cathepsin inhibition selectively disrupts the axonal transport of cathepsin-containing organelles leading to AD-like axonal dystrophy

Proteolysis inhibition, by either of two independent mechanisms, selectively slowed the anterograde and retrograde transport of LAMP or LysoTracker-positive vesicles and rendered a greater number of them stationary for relatively long periods. Lysosomal protease inhibition also slowed or halted the axonal transport of LC3-positive AVs, nearly all of which had fused with cathepsin-containing LAMP1/Rab7-positive late endosomes. Significantly, the negative effects of protease inhibition on Rab7 vesicles (which overlap extensively with LC3, and LAMP vesicles, Figure 3) were completely reversible upon removal of the proteolysis block. By contrast, the movement of RhoB vesicles or mitochondria that lack cathepsins was unaffected. The similar effects on transport of cathepsin inhibitors or bafilomycin A which inhibits lysosome/autolysosome-acidification, suggest that altered motility of AVs/endo-lysosomes involves a change in the intralumenal environment of lysosomes that, in turn, alters the transport properties of these vesicles.

The selective transport failure of AV/endo-lysosomal compartments and axonal accumulation within dystrophic axonal swellings in response to proteolysis inhibition, provides a possible molecular basis for axonal dystrophy in AD. This selective organelle accumulation is highly characteristic of AD, and distinct from dystrophies that result from a global disruption of microtubule-based axonal transport, where all varieties of vesicular and non-vesicular cargoes are impeded and accumulate above or below the site of blockade (Griffin et al., 1977). Moreover, global transport failure is often seen in the context of neurodegeneration, and distinguishing primary effects on transport from those secondary to neurodegenerative events is usually difficult. By contrast, we showed that transport impairments and axonal dystrophy are reversible upon withdrawal of the proteolytic block, consistent with evidence that a given dystrophic neurite may persist in AD brain for relatively long periods of time (Meyer-Luehmann et al., 2008; Adalbert et al., 2009) and is potentially reversible (Garcia-Alloza et al., 2007). Dystrophic axons with a more selective content of fibrillar and vesicular components have been seen in diseases associated with early axonal transport deficits and axonal dystrophy (Williamson and Cleveland, 1999; Morfini et al., 2009; Hadano et al., 2010); however, the accumulated organelles in these conditions are distinguishable from those in the dystrophic neurites in AD, which are predominantly composed of autophagy-related structures (Nixon et al., 2005) (Figure 7).

Several of our observations may be relevant to the molecular mechanism by which lysosomal proteolysis inhibition impedes transport of specific organelles. Cathepsin inhibition significantly lowered the proportion of LC3-vesicles containing dynein, which roughly matched the proportion of LC3-positive vesicles that became stationary after protease inhibition, suggesting that detachment from dynein motors from these compartments may contribute to their defective transport, although we cannot exclude the possibility that protease inhibition stabilizes a population of AVs that are normally detached from dynein. Dynein malfunction disrupts axonal transport ultimately causing neurodegeneration (LaMonte et al., 2002; Hafezparast et al., 2003) although structures accumulating in axonal swellings are different from those observed in AD models. However, organelle-specific deficits in axonal transport, as we propose in AD, have been seen in several other diseases where, interestingly, the pathogenic protein is a constituent of the affected organelle (Lim and Kraut, 2009)(Kasher et al., 2009). Notably the ablation of snapin, a Rab7 binding protein that interacts with dynein, was shown to selectively impede Rab7-positive vesicle transport and disrupt autophagic and lysosomal turnover (Cai Q et al., 2010). The dynein mechanism suggested by our preliminary observations represents only one of various possible mechanisms, which may even include alterations of a different motor than dynein or involve more than one motor since, for example, protease inhibition impaired the anterograde movement of LAMP-vesicles. An excess of APP or specific APP
metabolites may also alter vesicular transport (Stokin et al., 2008; Muresan et al., 2009). APP metabolites including, notably, the trans-membrane C-terminal fragments, which accumulation after leupeptin treatment (Siman et al., 1993; Boland et al., 2010), are known to disturb endosomal trafficking and size (Jiang et al., 2010). Consistent with these findings, the increase in APP-CTFs levels after leupeptin in our experiments was robust and may represent an additional pathogenic consequence of lysosomal dysfunction relevant to AD (Jiang et al., 2010).

We did not observe significant changes in AVs/lysosome transport or axonal morphology after strong autophagy induction by rapamycin. Nerve growth factor (NGF) deprivation in PC12 cells (Yang et al., 2008) and excitotoxicity induce axonal swellings and activates autophagy but these changes are associated with neurodegeneration (Yue et al., 2002; Wang et al., 2006)(Batistatou and Greene, 1991). In our study, however, stimulating autophagy by TOR inhibition (rapamycin) did not induce axonal swellings or alter the dynamics of AV or lysosome transport consistent with similar observations in non-neuronal cell types using similar treatments (Jahreiss et al., 2008; Katsumata et al., 2010). We cannot, however, exclude the possibility that autophagy over-activation via non-TOR mediated pathways as occurs in some neuronal injuries, differentially affects axonal transport.

Although the detailed mechanism by which changes in the proteolytic activity within a vesicle regulates its transport requires further investigation, our data strongly support a novel hypothesis linking deficient endo-lysosomal proteolysis to the development of a neuritic dystrophy resembling the highly characteristic pattern seen in AD. Our further studies indicate that the mechanism involves a selective disruption of axonal transport of the same types of vesicular organelles that accumulate in dystrophic neurites in AD. The results provide insight into the mechanism by which mutations of Presenilin 1, which cause lysosomal proteolysis inhibition, dramatically accelerates neuritic dystrophy and associated pathologies in familial AD (Cataldo et al., 2004; Lee et al., 2010). Finally our observation that the dystrophy may be reversed by relieving the inhibition of lysosomal proteolysis is consistent with the significant therapeutic effects of restoring a more normal level of lysosomal proteolysis in mouse models of AD (Yang et al., 2010), and underscores the promise of this approach for developing effective AD therapies.

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Figure 1. Identification of axonal AVs using fluorescent LC3

(A) GFP-LC3 vesicles (arrows) are detected in the cell body and in neurites. Cell body and boxed area of the neurite are enlarged. (B) Long projections in GFP-LC3 neurons are immuno-reactive for phosphorylated neurofilaments but not MAP2 (C). (D) Short dendrites immuno-reactive for MAP2 (arrows) also express GFP-LC3. Live image of DsRed-LC3 (E) or GFP-RFP-LC3 (F) neuron, where LC3 vesicles are present in axons (left) and the cell body (right). (G) Time lapse frames of GFP-LC3 in the cell body demonstrating de novo synthesis (arrows). (H) Kymograph of GFP-LC3 in axons and corresponding time-lapse frames illustrating a new LC3 vesicle (arrow) budding off an existing vesicle (arrowhead). All scale bars are as indicated.
Figure 2. LC3 vesicles primarily undergo retrograde movement
Representative live image of axon co-expressing GFP-LC3 and DsRed-Mito. LC3-positive vesicles (A; arrows) and mitochondria (B; arrowheads) do not colocalize. 15-min kymograph of LC3 movement (A) demonstrates that LC3 undergoes predominantly retrograde movement with intermittent pausing indicated by vertical segments whereas mitochondria (B) are frequently stationary (vertical lines), although movement occasionally occurs. All scale bars are as indicated.
Figure 3.
LC3 vesicles undergo maturation during retrograde transport
(A) Representative axon image from YFP-LAMP neuron. Low magnification image of neuron is shown in inset. (B) YFP-LAMP double-labeled with active cathepsin B marker Magic-Red. Cell body LAMP vesicles are enriched with active lysosomal cathepsin B, whereas more distal LAMP vesicles have relatively lower levels of active protease (arrows). (C) Lysotracker Red-vesicles are concentrated near the somatodendritic area. (D) Double labeling of endogenous LAMP and cathepsin D under normal conditions. LAMP vesicles in the perinuclear area contain cathepsins (arrows). Proximal area is enlarged. (E–F) Representative kymographs of DsRed-LC3 (E) and YFP-LAMP (F) movement. LC3/LAMP vesicles undergo retrograde movement (arrows) whereas LAMP vesicles not colocalized with LC3 are anterograde (F; arrowheads). (G–H) Representative kymographs of DsRed-LC3 (G) and GFP-Rab7 (H) movement. LC3/Rab7 vesicles undergo retrograde movement (arrows). (I) Representative axon co-expressing YFP-LAMP and DsRed-LC3. LC3 vesicles are LAMP-positive (arrows). (J) Representative axon co-expressing DsRed-LC3/GFP-Rab7. Most LC3 vesicles are fused with Rab7 vesicles (arrows) whereas a subset of Rab7 vesicles are not co-localized with LC3 (arrowheads). (K) Representative DsRed-LC3 vesicles double-labeled with active cathepsin D marker Bodipy-pepstatin-FL. LC3 vesicles contain active cathepsin D. (L) Representative axon co-expressing GFP-RhoB and DsRed-LC3.
DsRed-LC3 vesicles (arrowheads) do not co-localize with GFP-RhoB (L; arrows) (M) Vinblastine treatment (1μM, 1hour) prevents colocalization between LC3- and LAMP- vesicles. Scale bars in I-N represent 5 μm, or as indicated.
Figure 4. Lysosomal proteolysis inhibition slows LC3 vesicle transport without causing generalized axonal transport defects

(A) Representative 5-min kymographs of GFP-LC3 movies after leupeptin (20µM, 24hours), or bafilomycin A (10nM, 2 hours; compared to controls - Figure 2 A). (B) Quantification of GFP-LC3 movements after leupeptin (n=81 vesicles), bafilomycin A (n=44 vesicles) or controls (n= 96 vesicles). The percentage of moving LC3 vesicles (motility) and LC3 vesicles undergoing net retrograde movement are significantly reduced by leupeptin or bafilomycin treatment. LC3 vesicles also have slower retrograde velocities after leupeptin or bafilomycin, and the frequency of instantaneous retrograde velocities show a depression after leupeptin or bafilomycin. (C) Dynein-intermediate chain (DIC) immunolabeling on GFP-LC3 vesicles under normal conditions and after treatment with leupeptin. A portion of LC3-vesicles did not co-localize with (DIC) after leupeptin (arrowheads). (D) Quantification of the percent of LC3-vesicles that colocalized with DIC (per axon) shows reduction after leupeptin (n= 60). (E) Motility and (F) net direction of DsRed-Mito-positive mitochondria (control, n=69; leupeptin, n=78) show that mitochondria movements were not affected by leupeptin (20µM, 24hours). Values represents means ± sem * p < 0.05; ** p<0.01
Figure 5. Lysosomal proteolysis inhibition slows endo-lysosome transport
(A–C) Representative 5-min kymographs of YFP-LAMP (A), GFP-Rab7 (B) and GFP-RhoB (C) after leupeptin (20μM, 24hours), or bafilomycin A (10nM, 2hours) compared to controls. (D) Quantification of YPF-LAMP and LysoTracker (LT) Red-vesicle motility, net transport direction and frequency of direction changes (YFP-LAMP: control, n= 82 vesicles; leupeptin, n=68 vesicles; bafilomycin, n= 44. LT-Red: control, n= 62 vesicles; leupeptin, n= 71 vesicles; see methods for details). Both leupeptin and bafilomycin significantly reduce the percentage of moving LAMP/LT-vesicles, anterograde and retrograde net movements, and decrease the frequency of direction changes. (E) Quantification of GFP-Rab7 and GFP-RhoB movements after leupeptin (Rab7, n= 66 vesicles; RhoB, n= 54 vesicles), bafilomycin
A (Rab7, n=94 vesicles; RhoB, n= 43 vesicles) or controls (Rab7, n= 109 vesicles; RhoB, n=54). Leupeptin and bafilomycin A reduce the percentage of moving vesicles and the retrograde transport of Rab7 without affecting the retrograde transport of RhoB. Values represents means ± sem * p < 0.05; ** p<0.01
Figure 6. Lysosomal proteolysis inhibition accumulates LC3 vesicles

(A) Endogenous LC3-immunoreactive vesicles (arrows) are abundant and accumulated (arrowhead) after treatment with bafilomycin (10nM, 4 hours). The cell body contains relatively few LC3 puncta compared to the axon. (B and C) Representative live image of GFP-LC3 neuron before (B) or after treatment with leupeptin (20μM, 24 hours; C). LC3 vesicles accumulate in swellings (arrows). Enlarged area of axonal swellings shown below. (D and E) Live image of axonal GFP-LC3 vesicles after treatment with leupeptin (20–40 μM, 24 hours) (D) or Bafilomycin A (50nM, 4 hours) (E) where vesicles are both dispersed (arrows) and accumulated in a focal swelling (arrowhead). (F) Quantification of the percent of GFP-LC3 neurons with neuritic swellings containing GFP-LC3. (G) The number of GFP-LC3 vesicle enriched swellings per millimeter length of neurite after treatment with various protease inhibitors (see methods). (H) Quantification of the number of GFP-LC3 vesicles per 100 microns in control or after leupeptin treatment (n=50). The frequency of GFP-LC3 vesicles increases approximately 2.5 fold after leupeptin. Values represents means ± sem. All scale bars are as indicated. ** p<0.01; *** p <0.0001
Figure 7. Biochemical and ultrastructural profiles of neurites after lysosomal clearance inhibition resemble AD dystrophic neurites

(A–F) Swellings preferentially accumulate with lysosomal (proteolytic) vesicles after treatment with leupeptin (20 μM, 24hours). Lysotracker Red-vesicles (A) Rab7-vesicles (B) and, LC3 vesicles (C; arrows) are preferentially accumulated whereas mitochondria (C; arrowheads) Rab5-positive early endosomes (D), neurofilament-light chain (E) or β-tubulin (F) are relatively evenly distributed along the axon. Swellings accumulate proteins that identify AD-dystrophic neurites including APP-containing autophagic vesicles (G; C1/6.1 antibody for C-terminus of APP double labeled with LC3), ubiquitinated proteins (H) and phospho-neurofilaments (I; NF-M/H; double labeled with LC3). Phosphorylated neurofilament accumulation occurs in swollen regions containing accumulated LC3 vesicles. All scale bars represent 5 microns. (J) Time lapse of swelling with GFP-LC3 and DsRed-Mito co-transfection and treatment with leupeptin (20μM, 5hours). Cell body is at the top. Although both GFP-LC3 vesicles and DsRed-mitochondria are accumulated, mitochondria occasionally resume transport (red arrowheads), whereas LC3 movement out from the swelling is not observed. (K) Western blots with indicated antibodies in leupeptin -treated neurons compared to controls. Leupeptin increases the ratio of LC3-II/L, phospho-neurofilaments (SMI-31) and APP-CTFs, without increasing overall levels of neurofilaments or APP holoprotein (full length). Molecular mass shown in kDa. (L, M) Representative electron micrographs of neurites after leupeptin (20 μM, 24hours; M) compared to controls (L). Leupeptin-induced accumulation of double membrane, amorphous electron dense AVs in neurites. (N) Quantification of morphometric analysis of organelles in dystrophic neurites from leupeptin treated neurons (n= 20), or AD mouse models (APP;
n=25 and PSAPP; n= 25). (O, P) Representative electron micrographs of dystrophic neurites from APP (O) and PSAPP (P) mouse brain. Organelles within dystrophic neurites are mostly double membrane AVs. Values represents means ± sem Scale bars in (A–I, and J) represent 5 microns.
Figure 8. Recovery of lysosomal proteolysis restores transport of GFP-Rab7 positive late endosomes and autolysosomes

(A) Active cathepsin B (Magic Red) is severely reduced by leupeptin treatment (20mm, 24h), but restored upon recovery for 3 days in normal media. (B) Quantification of Magic-Red Cathepsin B loading in (A). Values represent mean intensity. (C) Quantification of GFP-Rab7 movements after leupeptin (n= 55 vesicles), or 24 hour recovery-after leupeptin (leupeptin recovery; n=119) compared to untreated controls (n = 101 vesicles), and recovery-controls (normal media replacement without leupeptin treatment n= 119 vesicles). Leupeptin-recovery restores the percentage of moving vesicles and the retrograde transport of GFP-Rab7 vesicles (C and D). Values represents means ± sem * p < 0.05; ** p<0.01, *** p<0.001.
Figure 9. Recovery of lysosomal proteolysis reverses axonal dystrophies and enhances maturation of accumulated AVs

(A) Phosphorylated neurofilament immunofluorescence using SMI-31 antibody in neurons after leupeptin (20μM, 24hours) followed by 24 hours in normal media. (B) Quantification of the number of SMI-31–enriched swellings per 10^3 μm axon length after leupeptin recovery is similar to controls. (number of 40x fields quantified: n = 29 control; n=26 leupeptin; n=33 recovery) (C) Western blots of LC3, phosphorylated neurofilaments (SMI-31), β-tubulin and P62 in leupeptin and leupeptin-recovery neurons compared to untreated neurons. (D) Quantification of LC3 and P62 western blot densitometry for leupeptin-treated and leupeptin-recovered neurons for 1–4 days. (n = 4) for each treatment. (E and F) Representative ultrastructural images of AVs accumulated in the cell body (E) or neurites (F) after leupeptin treatment (20μM, 24hours). AVs are filled with undegraded electron-dense material (black arrowheads). (G and H) Representative ultrastructural images of AVs in the cell body (G) or neurites (H) after 4 days of recovery in normal media following 24 hour leupeptin treatment (20μM). Most AVs in the cell body and neurites have a clear lumen (white arrows). Values represents means ± sem * p < 0.05; ** p<0.01; *** p <0.001
Time lapse summary of GFP-LC3, YFP-LAMP and Lysotracker Red vesicles after rapamycin treatment

Axonal transport of GFP-LC3, YFP-LAMP and Lysotracker Red vesicles under normal conditions (control) or following rapamycin (RM) treatment (10nM, 5 hours). Rapamycin does not significantly alter motility, movement direction or velocity for GFP-LC3, YFP-LAMP or LysoTracker–Red vesicles.

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Table 2
Time lapse summary of GFP-Rab7 vesicles after recovery from leupeptin

Axonal transport of GFP-Rab7 vesicles under normal conditions (control) or following Leupeptin treatment (20μM, 24hours), leupeptin and 24-hour recovery (leupeptin-recovery) or control-recovery (no leupeptin). Leupeptin recovery significantly improves motility and net retrograde movement direction, although the mean retrograde velocity is not yet significantly recovered.

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