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Supplementary Materials for

Axonal transport of late endosomes and amphisomes is selectively modulated by local Ca^{2+} efflux and disrupted by PSEN1 loss of function

Pearl P. Y. Lie, Lang Yoo, Chris N. Goulbourne, Martin J. Berg, Philip Stavrides, Chunfeng Huo, Ju-Hyun Lee, Ralph A. Nixon*

*Corresponding author. Email: nixon@nki.rfmh.org

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Figure S1

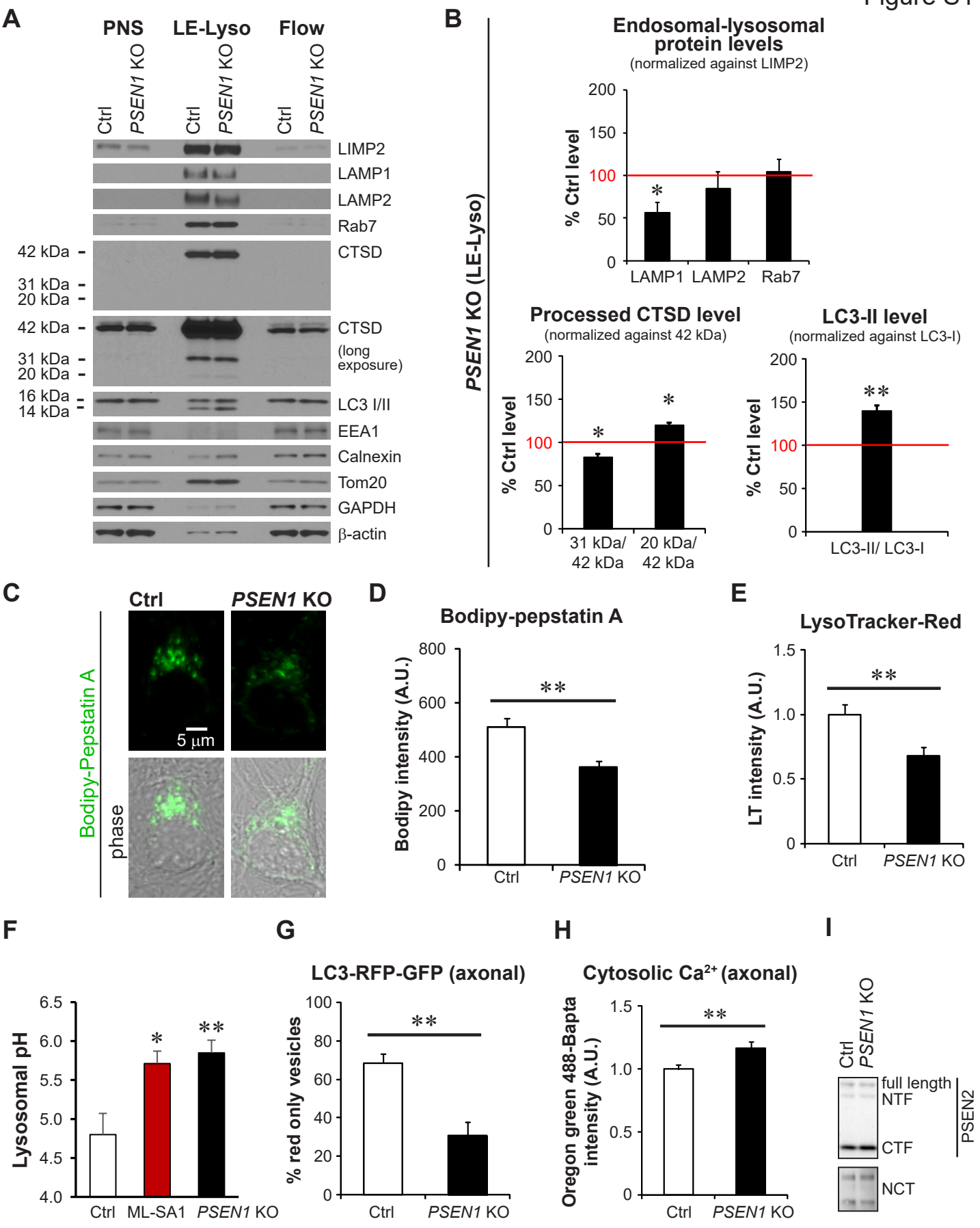

A
PNS
LE-Lyso
Flow
Ctrl
PSEN1 KO
LIMP2
LAMP1
LAMP2
Rab7
CTSD
42 kDa
31 kDa
20 kDa
CTSD (long exposure)
16 kDa
14 kDa
LC3 I/II
EEA1
Calnexin
Tom20
GAPDH
β-actin
B
PSEN1 KO (LE-Lyso)
Endosomal-lysosomal protein levels
(normalized against LIMP2)
% Ctrl level
LAMP1
LAMP2
Rab7
Processed CTSD level
(normalized against 42 kDa)
31 kDa/ 42 kDa
20 kDa/ 42 kDa
LC3-II level
(normalized against LC3-I)
LC3-II/ LC3-I
C
Ctrl
PSEN1 KO
Bodipy-Pepstatin A
phase
5 μm
D
Bodipy-pepstatin A
Bodipy intensity (A.U.)
E
LysoTracker-Red
LT intensity (A.U.)
F
Lysosomal pH
ML-SA1
G
LC3-RFP-GFP (axonal)
% red only vesicles
H
Cytosolic Ca2+ (axonal)
Oregon green 488-Bapta intensity (A.U.)
I
full length
NTF
CTF
NCT
PSEN2

Fig. S1. Lysosomal deficits and calcium dysregulation in *PSEN1* KO neurons (related to Fig. 1).

(A-B) Fractionation of the post-nuclear supernatant (PNS) resulted in the "LE-Lyso" fraction (mixture of LEs, amphisomes and lysosomes) and the "Flow" fraction (flow through; other organelles and cytosol). **(A)** Representative immunoblots of various organelle markers. 8 µg protein per lane. LIMP2 blot has been shown in Figure 4. **(B)** Relative protein levels in the LE-Lyso fraction of *PSEN1* KO. Ctrl level in each experiment is set as 100% (red line). n = 3-4 experiments. **(C-D)** Active CTSD was labeled with Bodipy-pepstatin A in live neurons. (C), representative images of perinuclear areas. (D), quantification of average signal intensities. n = 43 Ctrl, 52 *PSEN1* KO neurons. **(E)** Acidic organelles were labeled with LysoTracker (LT)-Red. Average signal intensities in the perinuclear area were quantified. n = 22 Ctrl, 20 *PSEN1* KO neurons. **(F)** Lysosomal pH measurement in Ctrl, ML-SA1-treated Ctrl and *PSEN1* KO neurons. Bars = mean + SEM (n = 27, 31 and 30 wells, respectively, for Ctrl, ML-SA1-treated Ctrl and *PSEN1* KO). *$p < 0.05$, ** $p < 0.01$, Kruskal Wallis test with Dunn's multiple comparisons against Ctrl. **(G)** LC3-RFP-GFP was expressed in Ctrl and *PSEN1* KO neurons. Percentages of acidic LC3 vesicles (red only) in proximal-mid axons were quantified. n = 12 Ctrl, 11 *PSEN1* KO axons. **(H)** Cytosolic calcium was labeled with Oregon green 488-Bapta AM in live neurons grown in microfluidics devices. Average signal intensities were quantified within microgrooves. n = 51 Ctrl, 50 *PSEN1* KO axons. **(B, D, E, G, H)** Bars = mean + SEM. *$p < 0.05$, ** $p < 0.01$, Student's t-test against Ctrl. A.U., arbitrary unit. **(I)** Representative immunoblots of select gamma secretase components in PNS of Ctrl and *PSEN1* KO neurons. NCT, nicastrin. No significant changes were detected.

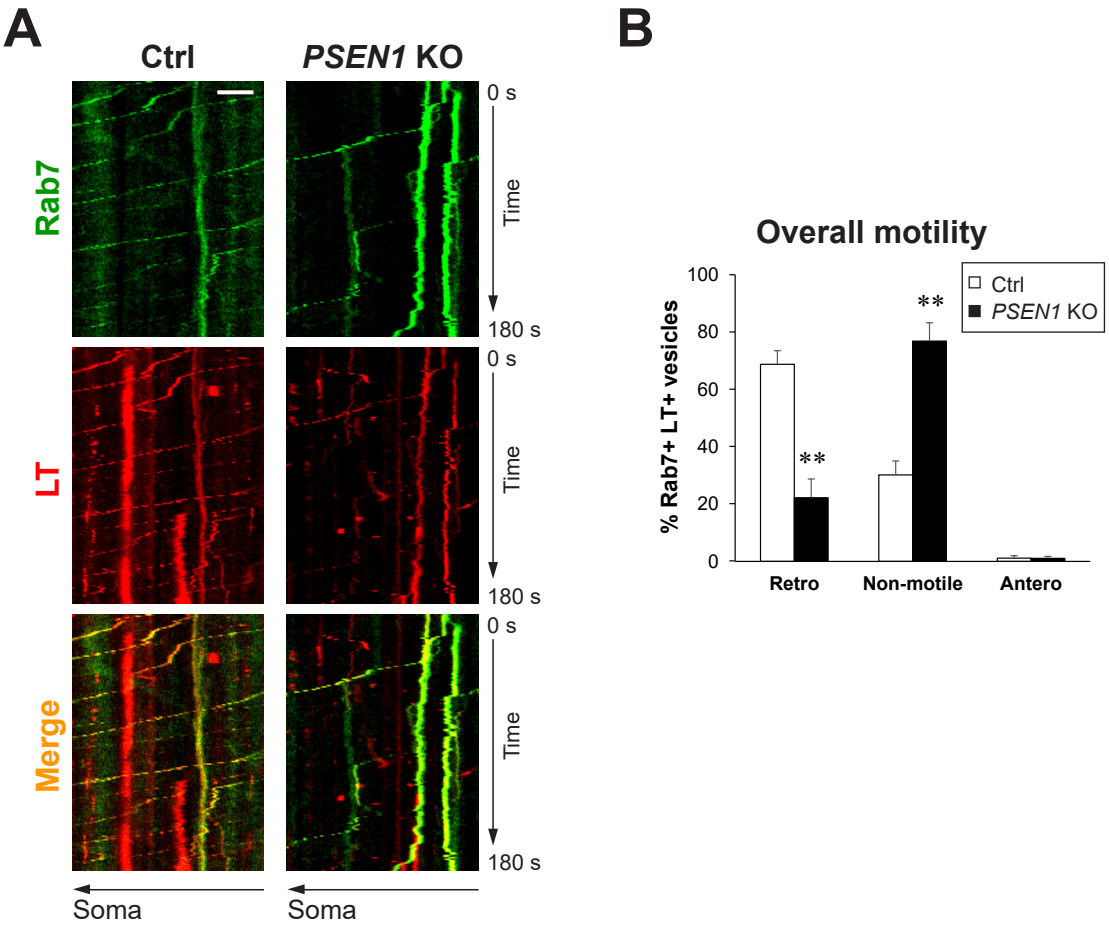
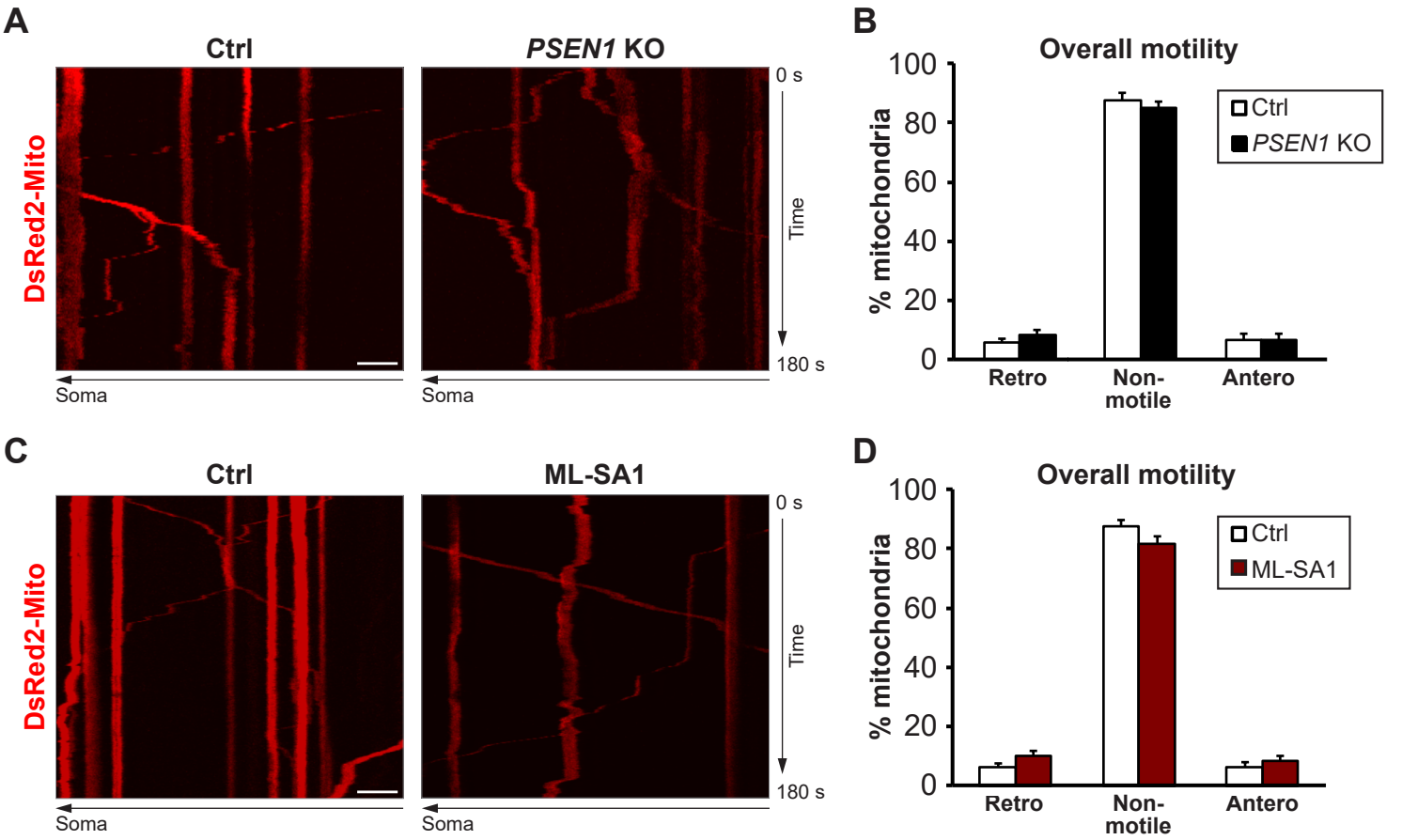
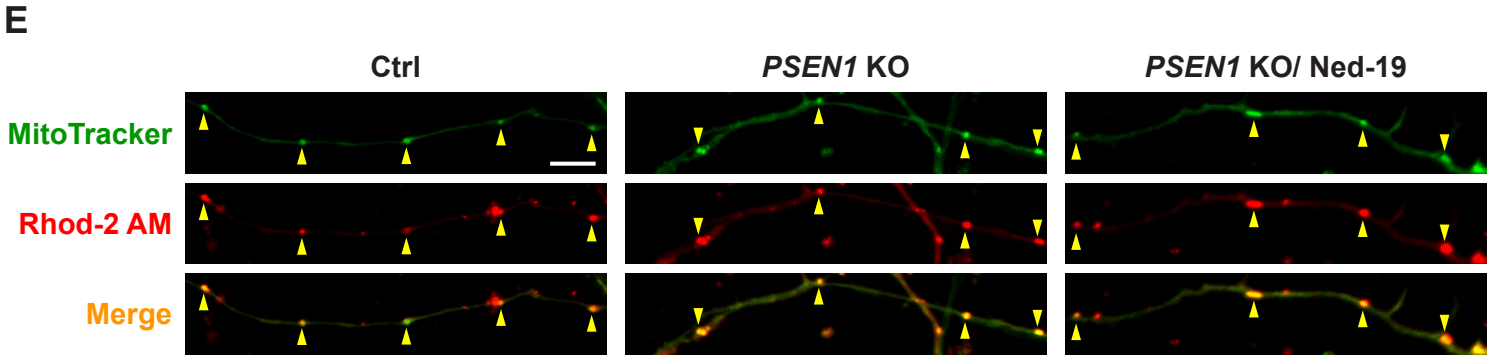

A
Ctrl
PSEN1 KO
Rab7
LT
Merge
0 s
Time
180 s
Soma
B
Overall motility
% Rab7+ LT+ vesicles
100
80
60
40
20
0
Ctrl
PSEN1 KO
**
**
Retro
Non-motile
Antero

Fig. S2. Retrograde transport of axonal Rab7+ LT+ vesicles is impaired in *PSEN1* KO neurons (related to Fig. 1).

Time-lapse imaging in axonal segments of Ctrl and *PSEN1* KO neurons expressing Rab7-GFP and co-labeled with LysoTracker (LT)-Red. **(A)** Representative kymographs. Scale bar = 5 µm. **(B)** Quantitative analyses of (A). Overall motility is represented by % retrograde (retro), non-motile (<0.1 µm/s) and anterograde (antero) vesicles in Rab7+ LT+ vesicle subpopulation. Bars = mean + SEM (n = 10 Ctrl neurons and 10 *PSEN1* KO neurons). ** $p < 0.01$, Student's t-test.


A
Ctrl
PSEN1 KO
DsRed2-Mito
0 s
Time
180 s
Soma
Soma
B
Overall motility
% mitochondria
100
80
60
40
20
0
Ctrl
PSEN1 KO
Retro
Non-motile
Antero
C
Ctrl
ML-SA1
DsRed2-Mito
0 s
Time
180 s
Soma
Soma
D
Overall motility
% mitochondria
100
80
60
40
20
0
Ctrl
ML-SA1
Retro
Non-motile
Antero


E
Ctrl
PSEN1 KO
PSEN1 KO/ Ned-19
MitoTracker
Rhod-2 AM
Merge

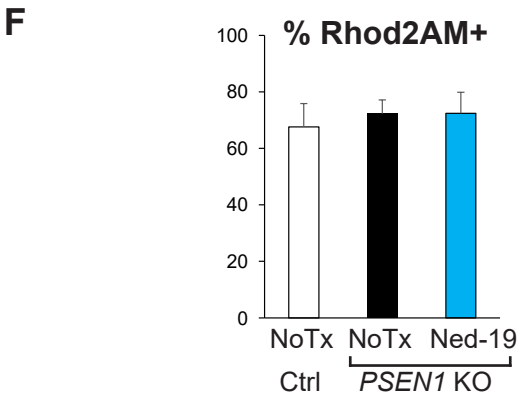
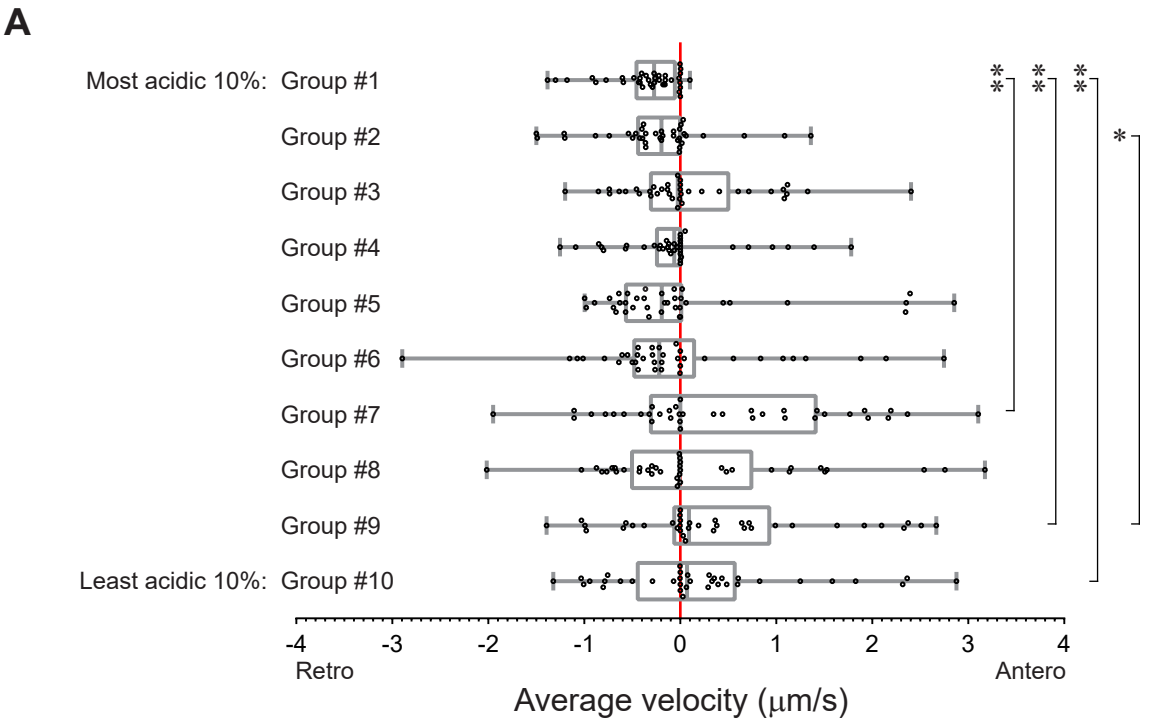

F
% Rhod2AM+
100
80
60
40
20
0
NoTx
NoTx
Ned-19
Ctrl
PSEN1 KO

Fig. S3. Axonal transport of mitochondria is not impaired in *PSEN1* KO or ML-SA1-treated Ctrl neurons and mitochondrial Ca^{2+} level is not affected by *PSEN1* KO and Ned-19 treatment (related to Fig. 1).

(A-D) Time-lapse imaging in axonal segments in Ctrl, *PSEN1* KO and ML-SA1-treated Ctrl neurons expressing DsRed2-Mito. **(A, C)** Representative kymographs. Scale bar = 5 μm. **(B, D)** Overall motility represented by the % retrograde (retro), non-motile (<0.1 μm/s) and anterograde (antero) mitochondria. Bars = mean + SEM. N = 22 Ctrl, 22 *PSEN1* KO neurons in (B); n = 25 Ctrl, 24 ML-SA1-treated Ctrl neurons in (D). **(E-F)** Quantitative analysis of mitochondrial Ca^{2+} level. Mitochondria were labeled with MitoTracker-green and mitochondrial Ca^{2+} was co-labeled with Rhod-2 AM. To prevent Rhod-2 AM accumulation in endosomes and lysosomes, labeling and imaging were performed at room temperature. Only MitoTracker+ vesicles were analyzed. **(E)** Representative images. Scale bar = 5 μm. **(F)** Graph showing the percentage of MitoTracker vesicles with detectable Rhod-2 AM signal. NoTx, untreated.


A
Most acidic 10%:
Group #1
Group #2
Group #3
Group #4
Group #5
Group #6
Group #7
Group #8
Group #9
Least acidic 10%:
Group #10
**
**
**
*
-4
-3
-2
-1
0
1
2
3
4
Retro
Antero
Average velocity (μm/s)

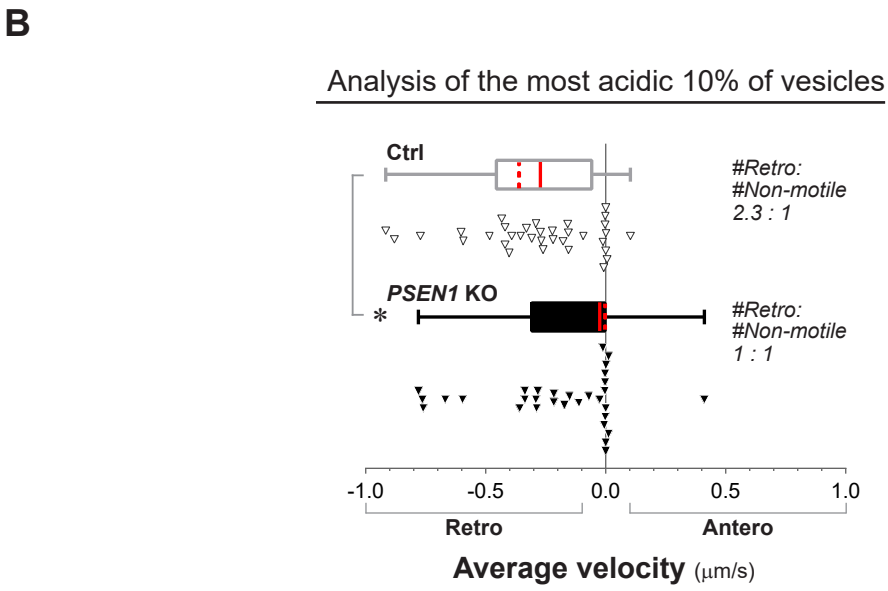

B
Analysis of the most acidic 10% of vesicles
Ctrl
#Retro:
#Non-motile
2.3 : 1
PSEN1 KO
*
#Retro:
#Non-motile
1 : 1
-1.0
-0.5
0.0
0.5
1.0
Retro
Antero
Average velocity (μm/s)

Fig. S4. Analysis of vesicle motility vs. acidification (related to Fig. 1).

Time-lapse imaging in axonal segments of LAMP1-mCherry-expressing Ctrl neurons loaded with equal amounts of dextrans conjugated with Alexa Fluor-647 (AF647) and Oregon green-488 (OG488) (see Figure 1E, F). **(A)** LAMP1+ AF647+ OG488+/− vesicles were sorted into 10 groups based on their acidification (reflected by OG488:AF647 ratio; low ratio indicates low pH). Box-and-whisker plots show the average velocity of vesicles in each group with individual datapoints represented by dots (whiskers = minimum and maximum datapoints). Group #1 (i.e. the most acidic 10% of vesicles) was determined to be exclusively retrograde (retro), with significant difference from the Groups #7, #8 and #10 that show substantial anterograde motility (antero) vesicles. *$p < 0.05$, **$P < 0.01$, Kruskal-Wallis test followed by Dunn's multiple comparisons (n = 36-37 vesicles per group). The most acidic 10% of vesicles were thus defined as the population of interest (i.e. acidic LEs/amphisomes with exclusive retrograde motility) in our analyses of Ctrl vs *PSEN1* KO (Figure 1E, F). **(B)** Same graph as in the bottom graph of Figure 1F supplemented with individual data points.

Figure S5

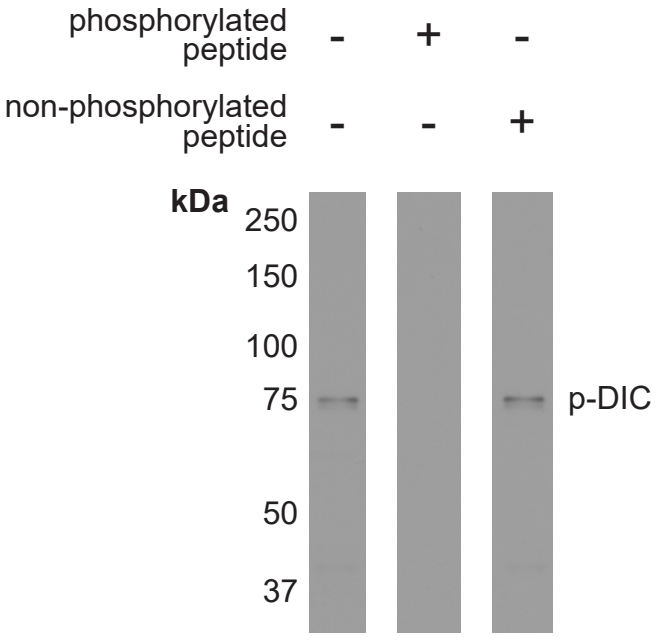

phosphorylated peptide
- + -
non-phosphorylated peptide
- - +
kDa
250
150
100
75
50
37
p-DIC

Fig. S5. Peptide competition assay to confirm specificity of p-DIC antibody (related to Fig. 4, 5, 6, 8).

A peptide competition assay was performed to verify that this antibody specifically recognizes p-DIC S80, but not the non-phosphorylated epitope. Equal amounts of neuronal lysate were immunoblotted with the p-DIC antibody only (left lane), or in the presence of 400-fold molar excess of peptides containing either phosphorylated (middle lane) or non-phosphorylated DIC S80 (right lane). These blots were processed and exposed simultaneously. The p-DIC-S80-containing peptide completely abolished the signal, but the non-phosphorylated peptide had no effect, thus clearly illustrating the specificity of this antibody against the phosphorylated epitope.

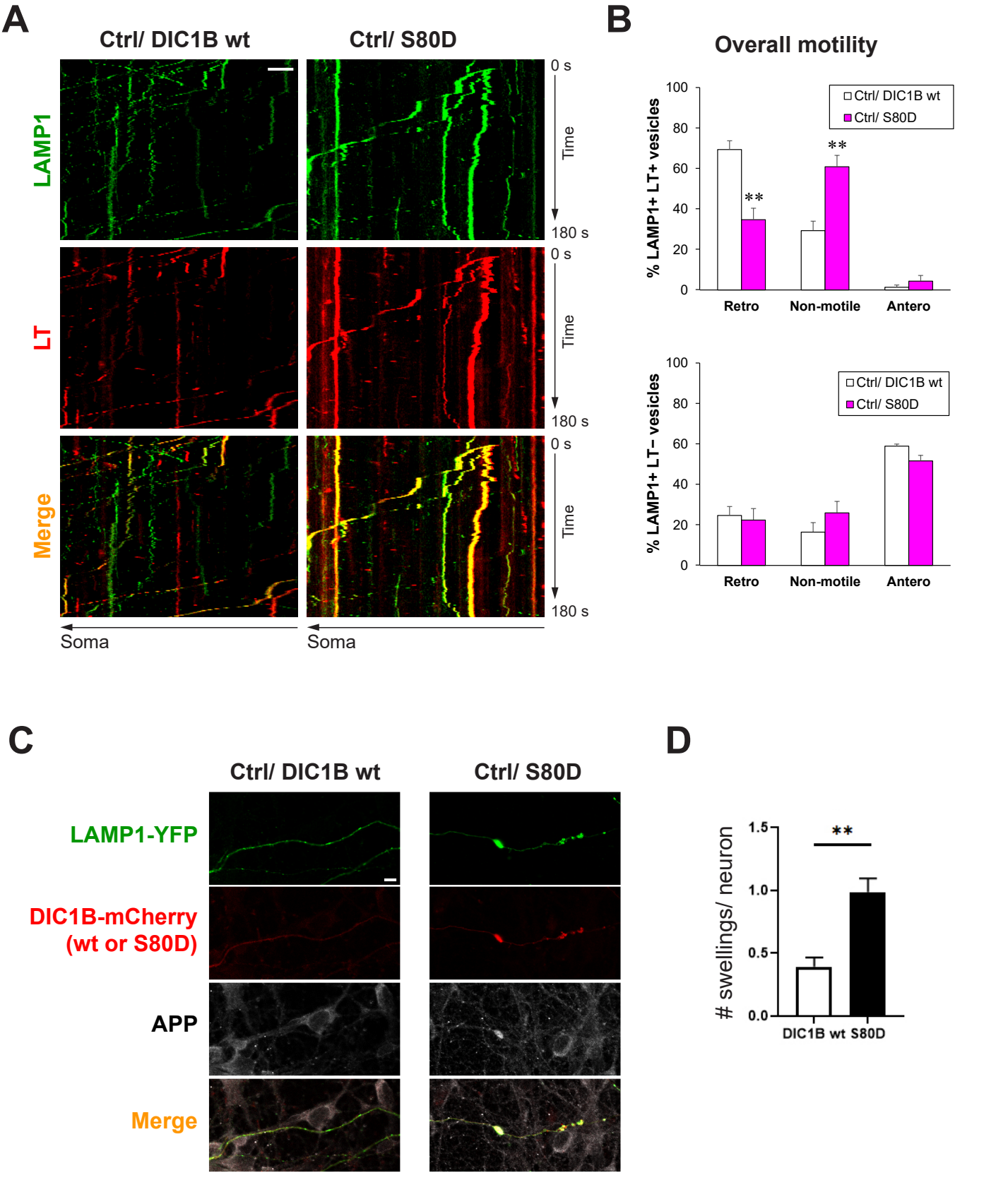

A
Ctrl/ DIC1B wt
Ctrl/ S80D
LAMP1
LT
Merge
0 s
Time
180 s
Soma
B
Overall motility
% LAMP1+ LT+ vesicles
% LAMP1+ LT− vesicles
Ctrl/ DIC1B wt
Ctrl/ S80D
Retro
Non-motile
Antero
**
C
Ctrl/ DIC1B wt
Ctrl/ S80D
LAMP1-YFP
DIC1B-mCherry
(wt or S80D)
APP
Merge
D
swellings/ neuron
DIC1B wt S80D

Fig. S6. Retrograde transport of axonal LAMP+ LT+ vesicles is impaired by DIC1B-S80D overexpression, causing neuritic dystrophy (related to Fig. 5).

(A-B) Time-lapse imaging in axonal segments of Ctrl neurons expressing LAMP1-YFP and co-labeled with LysoTracker (LT)-Red, co-expressing either flag-tagged wild-type DIC1B or flag-tagged DIC1B-S80D. **(A)** Representative kymographs. Scale bar = 5 μm. **(B)** Quantitative analyses of (A). Overall motility is represented by % retrograde (retro), non-motile (<0.1 μm/s) and anterograde (antero) vesicles in each LAMP1 vesicle subpopulation. Bars = mean + SEM (n = 10 neurons in each group). ** $p < 0.01$, Student's *t*-test. **(C-D)** Quantitative analysis of dystrophic neurites in Ctrl neurons expressing either mCherry-tagged wild-type DIC1B (red) or mCherry-tagged DIC1B-S80D (red), and co-expressing LAMP1-YFP (green). Dystrophic swellings were identified by an apparent thickness of a least 1.3 μm and positivity in both LAMP1-YFP and APP (immunostained, shown in gray scale). **(C)** Scale bar = 5 μm. **(D)** Graph showing the number of swellings identified per neuron, Bar = mean + SEM (n = 79 neurons in DICwt group and 77 neurons in S80D group). **$p < 0.01$, Student's t test.

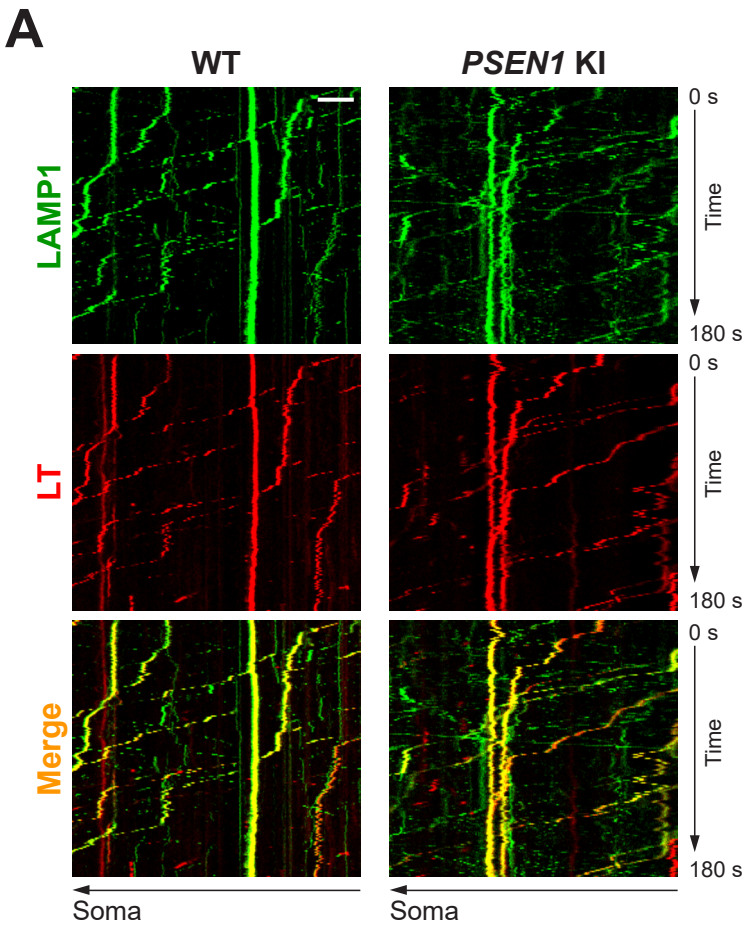

A
WT
PSEN1 KI
LAMP1
LT
Merge
0 s
Time
180 s
Soma
B
Overall motility
% LAMP1+ LT+ vesicles
100
80
60
40
20
0
WT
PSEN1 KI
Retro
Non-motile
Antero

Fig. S7. Retrograde transport of axonal LAMP+ LT+ vesicles is not affected in embryonic *PSEN1* KI neurons (related to Fig. 8).

Time-lapse imaging in axonal segments of WT and *PSEN1* KI neurons expressing LAMP1-YFP and co-labeled with LysoTracker (LT)-Red. **(A)** Representative kymographs. Scale bar = 5 µm. **(B)** Quantitative analyses of (A). Overall motility is represented by % retrograde (retro), non-motile (<0.1 µm/s) and anterograde (antero) vesicles in LAMP1+ LT+ vesicle subpopulation. Bars = mean + SEM (n = 10 neurons in each group).

Table S1. Antibodies used in this study.

Antibodies against dynein- & kinesin-related proteins	**Source (Catalog no.)**
DHC (Dynein heavy chain)	Santa Cruz (sc-9115)
DIC1/2 (Dynein intermediate chain 1/2)	Millipore (MAB1618)
DIC1 (Dynein intermediate chain 1)	GeneTex (GTX112418)
p-DIC (phospho-dynein intermediate chain)	Biomatik (custom made)
LIC1 (Dynein light intermediate chain 1)	GeneTex (GTX120114)
DYNLL1 (Dynein light chain LC8-type 1)	BD Biosciences (610726)
DYNLT1 (Dynein light chain Tctex-type 1)	Proteintech (11954-1-AP)
DYNLRB2 (Dynein light chain Roadblock-type 2)	Abcam (ab107721)
p150 (Dynactin subunit 1)	Abcam (ab11806)
p50 (Dynactin subunit 2)	Millipore (AB5869)
Arp1 (Actin-related protein 1)	Millipore (AB6058)
Snapin (SNAP-associated protein)	Proteintech (10055-1-AP)
LIS1 (Lissencephaly 1)	Abcam (ab2607)
Nudel (NudE-like)	Abcam (ab25959)
KIF3A (Kinesin family member 3A)	Covance (MMS-198P)
KIF5A (Kinesin family member 5A)	Santa Cruz (sc-13353)
KIF5C (Kinesin family member 5C)	GeneTex (GTX45387)
KLC1 (Kinesin light chain 1)	Santa Cruz (sc-13362)
Antibodies against MAPKs	**Source (Catalog no.)**
JNK (c-Jun N-terminal kinase)	Cell Signaling (9252)
p-JNK (phospho-JNK Thr183/Tyr185)	Cell Signaling (9255)
ERK (extracellular signal-regulated kinase 1/2)	Cell Signaling (9107)
p-ERK (phospho-ERK 1/2 Thr202/Tyr204)	Cell Signaling (9101)
Antibodies against organelle markers/ loading controls	**Source (Catalog no.)**
LIMP2 (Lysosomal integral membrane protein type-2)	Novus Biologicals (NB400-129)
LAMP1 (Lysosomal associated membrane protein 1)	Developmental Studies Hybridoma Bank (1D4B)
LAMP2 (Lysosomal associated membrane protein 2)	Developmental Studies Hybridoma Bank (ABL-93)
Rab7 (Ras-related protein Rab7)	Abcam (ab50533)
CatD (Cathepsin D)	Generated in house
MPR (cation-independent mannose-6-phosphate receptor)	Sigma (HPA011332)
LC3 (Microtubule-associated protein 1 light chain 3)	Novus Biologicals (NB100-2220)
EEA1 (Early endosome antigen 1)	Santa Cruz (sc-33585)
Calnexin	Enzo (ADI-SPA-860)
TOM20 (Translocase of outer membrane 20)	Santa Cruz (sc-11415)
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Santa Cruz (sc-25778)
β-actin	Novus Biologicals (NB110-67828)
APP	gift from Dr. Paul Mathews (C1/6.1)
Secondary antibodies for immunocytochemistry	**Source (Catalog no.)**
Alexa Fluor 488 AffiniPure goat anti-rat IgG	Jackson ImmunoResearch (112-545-167)
Alexa Fluor 568 goat anti-rabbit IgG, highly cross adsorbed	Thermo Fisher (A11036)
Alexa Fluor 633 AffiniPure goat anti-mouse IgG2b	Jackson ImmunoResearch (115-605-207)

Data S1. (separate file)
Excel file containing source data for all figures, tables, and supplementary figures.