The SAC1 domain in synaptojanin is required for autophagosome maturation at presynaptic terminals

Roeland Vanhauwaert\textsuperscript{1,2}, Sabine Kuenen\textsuperscript{1,2}, Roy Masius\textsuperscript{3}, Adekunle Bademosi\textsuperscript{4}, Julia Manetsberger\textsuperscript{1,2}, Nils Schoovaerts\textsuperscript{3,2}, Laura Bounti\textsuperscript{1,2}, Serguei Gontcharenko\textsuperscript{1,2}, Jef Swerts\textsuperscript{1,2}, Sven Vilain\textsuperscript{1,2}, Marina Picillo\textsuperscript{5}, Paolo Barone\textsuperscript{5}, Shashini T Munshi\textsuperscript{6}, Femke MS de Vrij\textsuperscript{6}, Steven A Kushner\textsuperscript{6}, Natalia V Gounko\textsuperscript{1,2,7}, Wim Mandemakers\textsuperscript{3}, Vincenzo Bonifati\textsuperscript{3}, Frederic A Meunier\textsuperscript{4}, Sandra-Fausia Soukup\textsuperscript{1,2,*} & Patrik Verstreken\textsuperscript{1,2,**}\textsuperscript{1}

Abstract

Presynaptic terminals are metabolically active and accrue damage through continuous vesicle cycling. How synapses locally regulate protein homeostasis is poorly understood. We show that the presynaptic lipid phosphatase synaptojanin is required for macroautophagy, and this role is inhibited by the Parkinson’s disease mutation R258Q. Synaptojanin drives synaptic endocytosis by dephosphorylating PI(4,5)P\textsubscript{2}, but this function appears normal in Synaptojanin\textsuperscript{RQ} knock-in flies. Instead, R258Q affects the synaptojanin SAC1 domain that dephosphorylates PI(3)P and PI(3,5)P\textsubscript{2}, two lipids found in autophagosomal membranes. Using advanced imaging, we show that Synaptojanin\textsuperscript{RQ} mutants accumulate the PI(3)P/PI(3,5)P\textsubscript{2}-binding protein Atg18a on nascent synaptic autophagosomes, blocking autophagosome maturation at fly synapses and in neurites of human patient induced pluripotent stem cell-derived neurons. Additionally, we observe neurodegeneration, including dopaminergic neuron loss, in Synaptojanin\textsuperscript{RQ} flies. Thus, synaptojanin is essential for macroautophagy within presynaptic terminals, coupling protein turnover with synaptic vesicle cycling and linking presynaptic-specific autophagy defects to Parkinson’s disease.

Keywords correlative light and electron microscopy; induced pluripotent stem cells; Parkinson’s disease; single-molecule tracking; synapse

Subject Categories Autophagy & Cell Death; Neuroscience

DOI 10.15252/embj.201695773 | Received 22 September 2016 | Revised 25 February 2017 | Accepted 1 March 2017 | Published online 22 March 2017
The EMBO Journal (2017) 36: 1392–1411

Introduction

Presynaptic terminals are often distantly located from the neuronal cell body, hampering rapid transport of components between these subcellular compartments (Paus et al, 2014). Despite this physical separation, synapses maintain a functional protein pool to support efficient information transfer. They must do so even under conditions of intense synaptic activity that place high metabolic energy demands on this area of the cell. However, the mechanisms that regulate and maintain protein homeostasis at synapses are poorly characterized. Furthermore, impaired synaptosomal protein homeostasis is implicated in neurodegeneration where combined failure of protein quality control systems and synaptic defects are recurrent themes (Selkoe, 2002; Burke & O’Malley, 2013; Soukup et al, 2013).

Parkinson’s disease (PD) is a common neurodegenerative condition characterized by the loss of substantia nigra neurons alongside broader pathology in most patients. While ~90% of PD cases are idiopathic, at least 10% have a family history of disease and several causative genes are identified (Thomas & Beal, 2007; Klein & Westenberger, 2012). While these PD genes encode a functionally diverse set of proteins, there is an emerging theme that several act on synaptic vesicle trafficking (Vanhauwaert & Verstreken, 2015; Lepeta et al, 2016; Schirinzi et al, 2016). However, the nature and degree of synaptic vesicle trafficking impairment that is caused by pathogenic mutations remains unclear.

Synaptojanin 1 (Synj1) is a lipid phosphatase that is essential for synaptic vesicle trafficking (McPherson et al, 1996; Cremona et al, 1999; Harris et al, 2000; Verstreken et al, 2003). It is also mutated in several different families with hereditary early-onset PD (Krebs...
et al., 2013; Quadri et al., 2013; Olgiati et al., 2014; Kirola et al., 2016). Synj1 is evolutionarily well conserved and enriched at synapses (Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003; Van Epps et al., 2004). The protein has two different lipid phosphatase domains, 5-phosphatase and SAC1, that target different phosphoinositide phosphate (PtdInsP) species. The 5-phosphatase domain specifically hydrolyzes phosphates on the 5′ position of the inositol ring and has a preference for PI(4,5)P₂ as a substrate (Whistock et al., 2000; Tsujishita et al., 2001). In contrast, the SAC1 domain hydrolyzes PI(3)P, PI(4)P, and PI(3,5)P₂ (Guo et al., 1999; Zhong et al., 2012).

Differently phosphorylated PtdInsP lipids concentrate on different organelles or membrane subdomains. Many proteins recognize particular PtdInsPs, and thus are recruited to a specific membrane. This underlies the concept that PtdInsPs lipids act as molecular tags that confer membrane identity (Wenk & De Camilli, 2004; Di Paolo & De Camilli, 2006; Ueda, 2014; Lauwers et al., 2016; Marat & Haucke, 2016). For example, patches of PI(4,5)P₂ at the synaptic membrane bind clathrin adaptors and other endocytic factors to promote synaptic vesicle formation adjacent to active zones. This PI(4,5)P₂-dependent binding underlies why Synj1 is essential for synaptic vesicle cycling, whereby the 5-phosphatase domain hydrolysis of PI(4,5)P₂ lowers the membrane-binding affinity of endocytic adaptors and uncouats newly formed vesicles (Cremona et al., 1999; Cad et al., 2000; Verstreken et al., 2003). Other PtdInsPs are critical as well, for example, PI(4)P in Golgi membranes and PI(3)P in endosomes recruit specific proteins to the surface of these organelles. In addition, PI(3)P and PI(3,5)P₂ are essential during the formation of autophagosomal vesicles where they are recognized by proteins like Atg18α/Atg18a that promote autophagosome biogenesis (Simonsen & Toooze, 2009; Noda et al., 2010; Dall’Armi et al., 2013; Proikas-Cezanne et al., 2015).

The PD-causing mutations (R258Q and the newly identified R459P) reside in the Synj1 SAC1 domain (Kreb et al., 2013; Quadri et al., 2013; Olgiati et al., 2014; Kirola et al., 2016). The R258Q mutation blocks the dephosphorylation of specific phosphoinositides including PI(3)P and to assess the functional consequences of this mutation, we used MiMIC technology and knocked in the R282Q mutation (corresponding to the human R285Q) into the endogenous Drosophila synj locus (synj[RO]) (Appendix Fig S1A) (Venken et al., 2011; Vilain et al., 2014). This synj[RO] knock-in allele expresses Synj1 at a comparable level to the wild-type synj locus, indicating that the mutation does not affect protein stability (Appendix Fig S1B, C, and H). We also developed a second system to study Synj1 function, where we use the UAS/Gal4 system to transgenically overexpress wild-type or R282Q Synj1 in neurons of synj1 null mutant flies that otherwise lack Synj expression (synj1−/−; nSyb > UAS synj1+ or synj1[RO]). We confirm similar expression of these transgenically expressed Synj1+ and Synj1[RO] proteins (~1.2-fold over the levels produced from the endogenous synj locus (Appendix Fig S1D, E, and F)). We compared the subcellular localization of endogenous Synj against that produced from the R282Q knock-in locus, or transgenically expressed wild-type Synj1+ and Synj1[RO] proteins. We find that all concentrate in the presynaptic terminals of the larval neuromuscular junction (NMJ) (Appendix Fig S1F–G* and J–M*), indicating the mutant protein localizes correctly and similar to the presynaptic protein EndoA (Verstreken et al., 2002).

Synj1 null mutant animals are not viable (Fig 1C), and deletion of synj specifically in the eye severely affects the efficiency of synaptic transmission between eye photoreceptors and the brain detected by electroretinogram (ERG) recordings (Fig 1D, arrowheads). As expected, these Synj1 null phenotypes are rescued by neuronal transgenic expression (nSyb-Gal4) of wild-type Synj1+. Surprisingly, however, transgenic expression of the Synj1[RO] mutant is as effective as the wild-type protein (Fig 1C and D). Since even a partially active mutant might result in rescue of the phenotype when overexpressed, we confirmed that synj[RO] knock-in flies are viable and that they too display normal ERGs (Fig 1D). Hence, it appears that the Synj1[RO] mutant protein retains core Synj activities that are essential for viability and synaptic communication in the eye.

Synj is best defined as the protein that uncoats newly endocytosed synaptic vesicles (Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003). To directly examine whether R282Q affects this endocytic function, we performed FM 1-43 fluorescent dye labeling at the excitatory glutamatergic Drosophila NMJ. FM 1-43 binds membranes and is incorporated into and labels newly formed synaptic vesicles, thereby labeling synaptic boutons proportional to the amount of vesicle recycling and thus synaptic endocytosis (Ramawami et al., 1994). As previously described, synj...
SYNJ1 mobilizes WIPI2/Atg18a in synaptic autophagy
Roeland Vanhauwaert et al

Figure 1.

The EMBO Journal
Published online: March 22, 2017
Vol 36 | No 10 | 2017
© 2017 The Authors
loss significantly reduces FM 1-43 labeling compared to controls (Verstreken et al., 2003). This labeling is restored by neuronal expression of wild-type synj, and again also by SynjRQ (Fig 1E–H and J). We confirmed that SynjRQ rescues the synj knockout phenotype independent of overexpression by finding normal FM 1-43 dye uptake in synjRQ knock-in animals (Fig 1I and J). Thus, FM 1-43 labeling suggests that SynjRQ supports presynaptic vesicle recycling and, under the conditions we tested, does so as efficiently as wild-type Synj.

The disruption of NMJ endocytosis often correlates with morphological defects. These also occur in the synj null mutants in the form of numerous satellite boutons that are small protrusions emanating from the primary axial branch (Dickman et al., 2006). We therefore examined whether SynjRQ possesses sufficient Synj activity to suppress this defect. We find a significant rescue of this synj null mutant-associated satellite bouton phenotype in synj null mutants that transgenically express wild-type Synj+ or mutant SynjRQ. We also observe no difference in satellite bouton number between synjRQ knock-in animals and controls (Fig 1K). Hence, SynjRQ appears to allow enough endocytosis to suppress this classic readout of NMJ endocytic defects, and is markedly different to the synj null allele.

We next directly examined how well SynjRQ supports neurotransmission at the larval NMJ. First, we measured basal release characteristics including miniature excitatory junctional currents (mEJCs) and EJCs. In controls and synj null mutants expressing Synj+ or SynjRQ, no difference is observed in mEJC and EJC amplitude nor in frequency (Fig 1L–N). We then stimulated the motor nerves for 10 min at 10 Hz to continuously drive synaptic vesicle exo- and endocytosis, and measured the post-synaptic excitatory junctional potentials (EJP). While control NMJs maintain neurotransmitter release under these conditions, synj null mutants fail to do so, and the block in endocytosis causes the EJP amplitude to drop to a low level in the first minutes of stimulation (Fig 1O) (Verstreken et al., 2003). This neurotransmission defect is rescued by transgenic neuronal expression of wild-type Synj+, or SynjRQ (Fig 1O). Here also, SynjRQ knock-in animals maintain release during this 10-Hz stimulation train at a level comparable to controls (Fig 1P). SynjRQ was previously shown to lack SAC1 domain phosphatase activity (Krebs et al., 2013) and our data now suggest that the SynjRQ mutant protein can support synaptic vesicle cycling. These results are consistent with data obtained in mouse cortical neurons using SAC1-dead Synj1 mutants that also supported vesicle recycling during persistent activity (Mani et al., 2007).

**Synaptojanin is required for autophagosome formation in presynaptic terminals**

Given that Synj is strongly enriched at presynaptic terminals (Verstreken et al., 2003), SynjRQ lacks PI(3)P phosphatase activity (Krebs et al., 2013), and the importance of PI(3)P on autophagosomes (Simonsen & Tooze, 2009; Burman & Ktistakis, 2010; Noda et al., 2010; Dall’Armi et al., 2013; Devereaux et al., 2013), we hypothesized that the Synj SAC1 domain acts in synaptic autophagy. We and others recently showed the presence of autophagic markers at presynaptic terminals (Williamson et al., 2010; Hernandez et al., 2012; Zhu et al., 2013; Maday & Holzbaur, 2014; Wang et al., 2015, 2016; Soukup et al., 2016; Stavoje et al., 2016). To determine whether Synj localizes on autophagic membranes, we examined the interaction between Synj and the transmembrane Atg9 marker of early pre-autophagosomal membranes (He et al., 2006; Tamura et al., 2010; Yamamoto et al., 2012; Stavoje et al., 2016).
2016). The distribution of a genomic Atg9HA construct where Atg9HA is expressed under endogenous promoter control is broad (Appendix Fig S2A–A′), consistent with the idea that Atg9 decorates early autophagic vesicles and autophagosomes. Next, we used anti-HA to immunoprecipitate neuronal autophagosome membranes from fly heads expressing Atg9HA at endogenous levels. We detect the co-immunoprecipitation of Synj with the Atg9-positive membrane fraction in both wild-type and synjRO knock-in animals expressing Atg9HA (Fig 2A). In contrast and indicating specificity, other synaptic proteins (alpha-SNAP and complexin) do not co-immunoprecipitate (Fig 2A).

Next, we assessed whether Synj is important for presynaptic autophagy. Such a role had recently been suggested in zebrafish cone photoreceptors (George et al., 2016). We expressed transgenes for the Atg8mCherry (fly LC-3) marker of mature autophagosomes (Kabeya et al., 2000) and Lamp1GFP, an autolysosomal marker (Marzella et al., 1982; Juhász et al., 2008), in motor neurons using D42-Gal4. In control animals, autophagy induced by starvation (amino acid deprivation) is marked by the presence of punctate dots of Atg8mCherry signal within presynaptic terminals (Fig 2B, B′, and G) that we previously characterized by correlative light and electron microscopy (CLEM) as synaptic autophagosome-like structures (Soukup et al., 2016). Moreover, boutons of starved control animals also accumulate the Lamp1GFP marker (Appendix Fig S2B, B′, and F). In contrast, in synj null mutants and synj null mutants that express SynjRQ, the Atg8mCherry and Lamp1GFP redistribution is blocked (Fig 2C–D′ and G, and Appendix Fig S2C–D′ and F). Unlike our findings with Synj-driven endocytosis, we observe that only wild-type Synj⁺⁺, and not Synj⁻⁻, can recover the Synj function in presynaptic autophagy (Fig 2F and G, and Appendix Fig S2E and F). Our results are also not unique to “starvation-induced autophagy” because when we induce the process by direct electrical motor neuron stimulation (30 min 20 Hz) (Soukup et al., 2016), the clear increase in the number of Atg8 dots observed in controls is absent in synjRO mutants (Fig 2H–J).

To scrutinize our results further and provide evidence that the block in synaptic autophagy is caused by the lack of SAC1 activity, we created flies that express SynjCS (SynjCS), a SAC1 enzymatic dead mutant (Mani et al., 2007). We expressed SynjCS in synj⁻⁻ null mutants under the same conditions as used to express SynjRO using D42-Gal4 driver. We then assessed Atg8mCherry localization and found that Atg8mCherry also does not concentrate in dots upon induction of starvation (Fig 2E, E′, and G). These data are in further support of the notion that Synj SAC1 domain function is required for synaptic autophagy and that the pathogenic PD mutant Synj behaves in this respect similar to a Synj–SAC1 domain mutant.

Next, to distinguish whether loss of Synj SAC1 function blocks autophagosome formation or promotes autophagosome degradation, we pharmacologically blocked the fusion of autophagosomes with late endosomes/lysosomes using chloroquine (Stauber et al., 1981; Chi et al., 2010). Subsequently, we monitored Atg8mCherry localization. As expected in wild-type controls, chloroquine under fed or starved conditions causes an increase in the number of Atg8mCherry dots. In contrast, in synjRO mutants, chloroquine does not result in such an increase in the number of Atg8mCherry dots (Fig 2K–M), suggesting autophagosome biogenesis is slowed. We provide further evidence for this by using the flux marker Atg8 tagged with GFP and RFP in tandem. Here, Atg8-positive organelles are green and red fluorescent, but when they fuse with lysosomes, the GFP is quenched and autolysosomes are only seen (DeVorkin & Gorski, 2014). We quantified the number of yellow and red Atg8-positive organelles in controls as well as the rare Atg8-positive organelles in synjRO mutants. We do not observe a significant difference in the ratio of red (mature) over total Atg8-labeled organelles between control and synjRO (Control: 55%; synjRO, 57%). The data indicate that once a rare Atg8-positive autophagosome manages to form in synjRO mutants, its further maturation is not impeded (Appendix Fig S2–I).

**Synj mutants accumulate Atg18a within synaptic boutons**

Members of the PROPPIN domain-containing protein family are key PI(3)P- and PI(3,5)P2-binding proteins with a role in autophagy (Baskaran et al., 2012). Given that SynjRO cannot dephosphorylate PI(3)P (and likely PI(3,5)P2), we hypothesized that in the synj null mutants that express SynjRO, autophagic PROPPIN-containing proteins would accumulate on synaptic pre-autophagosomal membranes. Therefore, we generated flies expressing N-terminal GFP-tagged Drosophila homologs of the PROPPIN WIP12 (Atg18a, Atg18b, and CG11975; 70.8, 56.5, and 32.0% identity to human WIP12 respectively). Both Atg18aGFP and
SYNJ1 mobilizes WIPI2/Atg18a in synaptic autophagy

Figure 2.

Chloroquine assay: Atg8mCherry> D42 Gal4

© 2017 The Authors

The EMBO Journal Vol 36 | No 10 | 2017 1397
SYNJ1 mobilizes WIPI2/Atg18a in synaptic autophagy

Roeland Vanhauwaert et al

Figure 3.
CG11975GFP localize in puncta at synaptic boutons when expressed in motor neurons (D42-Gal4) (Appendix Fig S3A, B, G, and H). In contrast, Atg18aGFP is retained in neuronal cell bodies (Appendix Fig S3E and F). After starvation, we observe a clear upregulation of the number of Atg18aGFP puncta, while the number of CG11975GFP dots does not change (Appendix Fig S3C, D, I, and J and Fig 3A, A', and E). Additionally, Atg18aGFP puncta sometimes colocalize with Atg8mCherry puncta (Appendix Fig S3K and L) and expression of Atg18aGFP using the ubiquitous Da-Gal4 driver in atg18a null mutants rescues their lethality. These data indicate that Atg18aGFP is a functional protein.

We then assessed Atg18aGFP localization in synj null mutants that express synjRO. Consistent with SynjRO not being able to dephosphorylate PI(3)P (Krebs et al., 2013), we find a significant increase in the number of Atg18aGFP dots in fed synj mutants in comparison with fed controls (Fig 3A–C and E). In addition, the number of Atg18aGFP dots is also increased in synj null mutant boutons that express synjRO compared to synj null mutant boutons that express synj+ (Fig 3C–E). Atg18a can bind PI(3)P and PI(3,5)P2 that are both dephosphorylated by the SAC1 domain of Synj1. To delineate which phosphoinositide is involved, we took a pharmacological approach. We used wortmannin (WM) that inhibits Vps34, a kinase that produces PI(3)P, or YM201636 (YM) that inhibits PIKfyve, a kinase that produces PI(3,5)P2. We applied these drugs during starvation to control and synjRO mutant animals that express Atg18aGFP. While Atg18aGFP accumulates in dots in starved controls, application of either drug blocks this effect and Atg18aGFP does not accumulate anymore, indicating that Atg18aGFP likely binds both PI(3)P and PI(3,5)P2 during autophagy at synapses (Appendix Fig S3M). In synjRO mutants, the number of Atg18aGFP dots is always upregulated. However, when we starve the larvae in the presence of either WM or YM, the number of Atg18aGFP is lower than in untreated starved synjRO mutants (Appendix Fig S3M). These data suggest that consistent with the previously established function of the Synj SAC1 domain, both PI(3)P and PI(3,5)P2 accumulate and bind Atg18a in synjRO mutants.

We tried to find further evidence for upregulation of PI(3)P in synjRO using expression of 2xFYVE-GFP, a probe that binds PI(3)P. We were however unsuccessful and did not detect obvious differences (not shown). Nonetheless, we do believe our results are consistent with the specific loss of SAC1 domain function in synjRO. We assessed 5-phosphatase domain activity of Synj using the PLCδ-PE-eGFP probe that detects PI(4,5)P2. We find that the synaptic labeling intensity of PLCδ-PE-eGFP is much less affected in synj null mutants that express SynjRO (where 5-phosphatase activity is not affected) compared to synj null mutants (where 5-phosphatase activity is affected) (Appendix Fig S3N–R), suggesting synjRO mutants support PI(4,5)P2 dephosphorylation. Taken together, the data are consistent with normal PI(4,5)P2 dephosphorylation activity of SynjRO measured in vitro (Krebs et al., 2013), but altered PI(3)P dephosphorylation, thereby leading to the accumulation of Atg18a at synaptic boutons in synjRO mutants.

Visualization of Atg18a-labeled structures at synaptic boutons

Synapses in synj mutants expressing SynjRO harbor increased synaptic Atg18aGFP accumulations and largely lack Atg8mCherry accumulations. To further visualize the Atg18aGFP-labeled structures, we resorted to CLEM, a methodology that allows us to directly determine the ultrastructural appearance of Atg18a-positive structures within synaptic boutons. Synj mutants expressing SynjRO and Atg18aGFP were dissected, fixed, and imaged. Subsequently, a near-infrared laser was used to produce burn marks in the vicinity of the imaged region. These marks are easily discernable at the light and EM level and help us when producing serial sections of the imaged boutons (Fig 3F–H). After alignment of the confocal images with the serial TEM images, we are able to identify the Atg18aGFP-positive structures (Fig 3I–K). As indicated in Fig 3L–P, Atg18aGFP marks profiles that have the appearance of cisternae that sometimes contain a few smaller vesicular structures in their lumen. Given that the flux of synaptic autophagy is blocked in synj mutants expressing SynjRO and that these mutants do not harbor Atg8mCherry dots, we

---

**Figure 3.** Atg18a-positive structures accumulate at synaptic boutons of synj mutants.

A–E Live imaging of fed (A–D) and 4-h-starved (A’–D’) NMJ boutons in controls (D42-Gal4 > Atg18aGFP) (A, A’) and synjRO–/– null mutants (B, B’) or synj+/– null mutants expressing either synjRO (C, C’) or synj+ (D, D’) and also expressing Atg18aGFP (D42-Gal4 > Atg18aGFP). Quantification of the number of Atg18aGFP dots (arrowheads) of indicated genotypes under fed and starved conditions (E). Full genotypes appear in the Appendix Supplementary Methods. Statistical analysis by t-test comparing control to individual genotypes separately in fed conditions and each genotype individually fed and starved. **P < 0.01, *P < 0.05, n ≥ 10 larvae and n ≥ 20 NMJs per genotype. Error bars represent SEM, scale bar, 5 μm. Note that loss of Synj increases the number of Atg18aGFP dots even under fed conditions.

This is not rescued by expression of synjRO.

F–P Correlative light and electron microscopy (CLEM) of 4-h-starved synj+/– null mutant NMJ boutons expressing Atg18aGFP and synjRO under the control of D42-Gal4. NMJ boutons with Atg18GFP fluorescence were imaged (F), and subsequently surrounded by burn marks using near-infrared branding (NIRB) (F–H). Branding marks are clearly visible in transmission and fluorescent mode due to autofluorescence of the marks (F, G). Branding marks allow subsequent detection of the same region of interest in electron micrographs (H). The marks then allow for the correlation of fluorescent and electron micrographs of Atg18aGFP-positive boutons (I–K). Arrows indicate Atg18aGFP-positive labeling (intensities were adjusted as to identify structures) in EM micrographs and boxes are enlarged in subsequent panels. Magnification of Atg18aGFP-positive structures identified in (I–K) is shown in (L) and further examples of Atg18aGFP-labeled structures are taken from independent CLEM experiments (M–P). Scale bars: 50 μm (F–H), 5 μm (I), 50 nm (J, K), and 200 nm (L–P).

Q–U Representative transmission electron micrographs of 4-h-starved synj+/– null mutants expressing either synj+ (Q) or synjRO (R) using D42-Gal4, as well as of 4-h-starved synj+/– null mutants (S), controls (μA128 (T)), and synjRO knock-in animals (U). Full genotypes appear in the Appendix Supplementary Methods. Scale bars, 1 μm.

V Quantification of the number of synaptic vesicles (diameter < 80 nm) and cisternae that resemble Atg18aGFP-positive structures identified using CLEM. Note that at boutons of synj+/– null mutants, synj−/– null mutants expressing synjRO and synjRO, there are cisternae that resemble the Atg18aGFP-positive structures identified in CLEM, in synj+/– null mutants expressing synjRO and in synjRO knock-in, there are numerous synaptic vesicles detectable. For statistical analysis of both synjRO and cisternae, t-test comparing genotypes individually, *P < 0.05, **P < 0.001, ***P < 0.0001, n ≥ 3 larvae and n ≥ 8 NMJs per genotype. Error bars represent SEM.
propose that these cisternae are early presynaptic autophagic intermediates.

Using the morphological features of Atg18aGFP-positive structures as a guide, we assessed the ultrastructural morphology of synapses in starved animals. We used TEM images to quantify the number of synaptic vesicles and the number of structures that have an appearance similar to the Atg18a-positive cisternae in our CLEM studies (Fig 3V). Compared to starved controls and synj null mutants expressing Synj+, starved synj null mutants show a strong reduction in the number of synaptic vesicles per bouton area, consistent with a defect in synaptic endocytosis. These mutants also show an increased number of cisternal structures (Fig 3Q, S, T, and V), correlating with an increased number of Atg18a-positive dots in synj null mutants. Starved synj null mutants that express synRQ and synRQ knock-in animals also show an increased number of cisternal structures at boutons (Fig 3R, U, and V). However, there is a large normal amount of synaptic vesicles compared to controls (Fig 3Q–V), consistent with the normal synaptic vesicle endocytosis that we measured in these mutants (see Fig 1).

**Synaptojanin regulates Atg18a mobility at autophagosomal membranes**

In synaptic vesicle endocytosis, the dephosphorylation of PI(4,5)P2 by Synj on nascent vesicles regulates the detachment of adaptors with affinity for this lipid. We reasoned that a similar mechanism may be at play during autophagosome formation and tested whether Synj regulates Atg18a attachment and detachment. We expressed Atg18aGFP and assessed the fluorescence recovery after bleaching one specific Atg18aGFP dot. Synj null mutants and synj null mutants expressing synRQ show significantly slower recovery of Atg18a fluorescence in the bleached area compared to synj null mutants expressing Synj+ or compared to wild-type controls (Fig 4A–E).

To further find evidence for our model, we also generated Atg18a\\textsuperscript{medos1-2}, expressing flies. mEos is a photoconvertible fluorescent protein that shifts from green to red fluorescence when activated (Zhang et al., 2012). We reasoned that Atg18a mobility would decrease after induction of autophagy, as more Atg18a would bind to newly forming autophagosomes. Therefore, we used single-particle tracking photoactivation localization (sptPalm) microscopy to track single molecules within synaptic boutons expressing Atg18a\\textsuperscript{medos3-2} (Bademosi et al., 2017). Tracking each fluorescent molecule (Video EV1) allows us to generate trajectory maps of Atg18a\\textsuperscript{medos3-2}, as well as intensity and instantaneous diffusion coefficient maps (Fig 4F–H). Moreover, we quantified Atg18a\\textsuperscript{medos3-2} mobility by analyzing the mean square displacement (MSD) of sptPalm trajectories of single fluorescent localizations (Fig 4G). Compared to fed larvae, Atg18a mobility significantly decreases in starved larvae (Fig 4H), in good agreement with recruitment of cytosolic molecules to presynaptic autophagosomes.

Analysis of the diffusion coefficient distribution reveals a decrease in the mobile population of Atg18a\\textsuperscript{medos3-2} in starved conditions (Fig 4I). This is most apparent in the ratio of the mobile-to-immobile fractions (Fig 4J). This change in mobility of Atg18a after induction of autophagy is consistent with the recruitment of Atg18a to presynaptic autophagosomes.

We subsequently followed the accumulation of Atg18a on synaptic autophagosomes by converting a boutonic pool of Atg18a\\textsuperscript{medos3-2}. We converted Atg18a\\textsuperscript{medos3-2} in one bouton and followed accumulation of red Atg18a\\textsuperscript{medos3-2} on (green Atg18a\\textsuperscript{medos3-2}, positive) autophagosomes in the neighboring boutons of control and synj\\textsuperscript{RQ} knock-in flies. While the amount of red photoconverted Atg18a in the cytoplasm of controls and synj\\textsuperscript{RQ} is similar, the red Atg18a accumulates significantly less quickly on nascent autophagosomes in synj\\textsuperscript{RQ} compared to controls (Fig 4K–M). This slowed acquisition of red fluorescence indicates reduced exchange between membrane bound and soluble pools of Atg18a, and thus indicates that the SAC1 domain of Synj normally promotes Atg18a mobility at synaptic autophagosomes.

**SynRQ reduces lifespan upon starvation and causes neurodegeneration**

To more broadly assess the physiological consequences of the SynRQ mutation, we examined whether this affects animal survival under starvation conditions, a readout that is strongly modulated by autophagy (Juhasz et al., 2007). While wild-type flies reared in these conditions can survive for ~4 days, we detect significantly reduced survival times for synj null mutants that...
Roeland Vanhauwaert et al. SYNJ1 mobilizes WIPI2/Atg18a in synaptic autophagy

The EMBO Journal

Figure 4.

FRAP Atg18a\textsuperscript{GFP}

Single molecule tracking Atg18a\textsuperscript{mEOS3.2}

Photoconversion Atg18a\textsuperscript{mEOS3.2}
express SynjRQ in neurons or synjRQ knock-in animals which display 100% lethality within 3 days (Fig 5A and A’). The results correlate with impaired autophagosome maturation in animals lacking Synj SAC1 activity, suggesting that presynaptic autophagy connects to an important survival mechanism during starvation in the fruit fly.

**Figure 5.** Defects in synaptic autophagy result in reduced lifespan upon starvation and neurodegeneration.

A, A’ Complete starvation (water-only diet) assay in synj null mutants expressing synjRQ (light green) or synj+ (blue) (expressed using nSyb-Gal4) (A) and synjRQ knock-in animals (dark green) as well as controls (w1118, black) (A’). Statistical analysis of survival curves by log-rank (Mantel–Cox) test, ****P < 0.0001, n ≥ 134 flies per genotype for (A) and n ≥ 292 flies per genotype for (A’), dotted lines surrounding the curves indicate SEM. Note, synj null mutants expressing synjRQ as well as synjRQ knock-in flies have a shorter life span under these starvation conditions.

B–E Representative electron micrographs of the retina after 7 days of continuous light exposure of control (nSyb-Gal4/+)(B) and synj null mutants expressing synjRQ (C) or synj+ (D) (expressed using nSyb-Gal4). Green highlighting in (C) shows disintegrating rhabdomeres as a sign of degeneration. Scale bar, 2 µm. (E) Quantification of number of intact ommatidia per indicated genotype when flies were kept in complete darkness or when exposed to light. Full genotypes are in the Appendix Supplementary Methods. Statistical analysis by t-test, ****P < 0.0001, n = 3–5 flies per genotype. Error bars represent SEM.

F–F″ Dopaminergic neuron degeneration assay in 30-day-old control (w1118) and synjRQ knock-in flies. Schematic representation (F) and image (z-stack, sum of slices) of posterior side of an anti-TH-labeled 30-day-old adult fly brain (F”) to show the location of the different dopaminergic neuron cell bodies in this part of the brain. Scale bar, 40 µm. (F’) Bar graph showing the number of dopaminergic neurons (TH-positive) in each cluster indicated in (F”) in 30-day-old flies of control (black bars) and synjRQ knock-in (dark green bars) flies. Statistical analysis per dopaminergic cluster by t-test, **P < 0.01, *P < 0.05, n ≥ 15 brains per genotype. Error bars represent SEM. Note at PPM and PPL dopaminergic neuron clusters the reduction of anti-TH-positive neuronal cell bodies in the of synjRQ mutant animals compared to controls.
Next, we tested neuronal survival, another parameter sensitive to autophagy (Komatsu et al., 2006; Juha´sz et al., 2007). To induce neurodegeneration, flies were kept 7 days in constant light challenging photoreceptor neurons of the fly eye. In synj null mutant flies that express Synj RQ, a significant reduction in the number of intact ommatidia and an accumulation of vacuoles was observed. In contrast to control animals, when synj null mutant animals expressing Synj+ or animals kept in dark for 7 days, these photoreceptors appear unaffected (Fig 5B–E). Finally, given the vulnerability of dopaminergic neurons in PD, we assessed the integrity of such neurons in fly brains of 30-day-old synjRQ knock-in animals using anti-TH labeling. In these mutants, anti-TH-positive neuronal cell bodies in the PPM3 and PPL1 dopaminergic neuron clusters were significantly reduced compared to controls (Fig 5F–F″). Hence, Synj SAC1 domain function is required for dopaminergic neuron survival in the fly brain.

WPII2/Atg18a accumulates in neurites of SYNJ1 R258Q patient-derived human induced neurons

To confirm our key findings in a disease-relevant condition, we resorted to human iPSC obtained from two patients with autosomal recessive, early-onset PD carrying the R258Q mutation in SYNJ1 as well as two age-matched controls (Appendix Table S1). Following
validation, including expression analysis of pluripotency and proliferation markers (Fig 6A–B’, Appendix Fig S4 and Appendix Table S1), we differentiated these iPSC into neurons. After 28 days of differentiation, we labeled the neurons with anti-WIPI2 (mammalian Atg18a) and imaged the neurites (Fig 6C–F). Similar to our observations at fly synapses, we find a significant increase in the number of WIPI2 dots in starved control neurons compared to fed control neurons (Fig 6C, D, and G). Moreover, in line with our observations made with fly synjRQ mutants, we find a significant increase in the number of WIPI2 dots in fed SYNJ1RQ neurons compared to fed controls and which is even further increased in starved SYNJ1RQ neurons (Fig 6C and E–G). These data indicate that also in human neurons, Synj1 is required for autophagy, and further supports our model that implicates SYNJ1-SAC1 function in removing Atg18a/WIPI2 from nascent autophagosomal membranes.

**Discussion**

The presynaptic protein SYNJ1 contains two PtdInsP lipid phosphatase domains and is critical for neurotransmission. In this work, we take advantage of both flies and human iPSC-derived neurons obtained from patients carrying the SYNJ1 R258Q mutation to examine the role of the Synj SAC1 phosphatase domain that is mutated in early-onset PD and that was previously shown to block PI(3)P dephosphorylation (Krebs et al., 2013). We show that Synj has a previously unrecognized role in promoting autophagosome maturation within presynaptic terminals and that this is distinct to the relatively well-described function in synaptic vesicle recycling. This predicts that defective synaptic autophagy and protein clearance contributes to SYNJ1-PD pathology. Furthermore, given that Synj is a presynaptic protein, these data show the existence of a synapse-specific autophagy machinery and suggest this process may be disrupted in PD.

We detected that Synj has dual presynaptic functions in endocytosis and macroautophagy when comparing the impact of the SAC1-domain mutation and a null mutation. While the Synj null mutation causes larval lethality, SynjRQ knock-in animals or synj null mutants that neurally express SynjRQ are viable. Similarly, synaptic endocytic mutants cause lethality in flies, while many of the mutants that disrupt macroautophagy are viable (Verstreken et al., 2002, 2003; Juhász et al., 2007; Soukop et al., 2016). Hence, our observation that synjRQ flies live is in line with the mutation not disrupting endocytosis. Furthermore, we provide several additional lines of evidence that SynjRQ mutants have largely normal synaptic endocytosis. First, SynjRQ mutants have a largely normal number of synaptic vesicles. Second, they display normal presynaptic FM1-43 dye uptake at NMJs. Third, they do not develop the NMJ morphological defects associated with impaired endocytosis. Fourth, we directly assessed how the SAC1 domain synjRQ PD mutation affects neurotransmission. Unlike endocytic mutants, we find that the SynjRQ mutant protein maintains NMJ neurotransmitter release during intense stimulation, as well as during normal neurotransmission between the fly eye and brain. These results all indicate that the SynjRQ protein can fulfill the endocytic function of Synj that is required for efficient synaptic vesicle cycling and is severely abnormal in synj null animals. Nevertheless, the RQ mutation in Synj does incapacitate aspects of Synj activity, as evidenced by loss of dopaminergic neurons and shorter lifespan of SynjRQ-expressing flies under starved conditions. We also find that synaptic boutons expressing SynjRQ fail to organize autophagy at presynaptic terminals. Instead, these boutons contain membrane structures positive for the early autophagosome marker Atg18a. Thus, given that under the used conditions, we see that synjRQ animals have severely abnormal autophagy alongside normal endocytosis, we conclude that Synj operates in both processes.

Our findings implicate Synj in autophagy at presynaptic terminals, which is surprising given the well-studied role of this protein at the synapse (McPherson et al., 1996; Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003). We therefore hypothesize that the severe endocytic defects that occur upon synj loss made it difficult to detect the importance in autophagy. The conclusion that Synj is a dual endocytic and autophagic protein is consistent with the evolutionarily conserved presence of two distinct Synj PtdInsP phosphatase domains, SAC1 and 5-phosphatase, that have different preferences for PtdInsP species. This suggests a cooperativity between the domains and it is interesting to consider the possibility that the dual roles of Synj allow this protein to couple autophagy to levels of synaptic vesicle cycling and thus ensure sufficient protein turnover possibly depending on the level of synaptic activity.

We provide evidence that SAC1-dependent PI(3)P and PI(3,5)P2 dephosphorylation is needed for normal autophagic flux at synapses using both SynjRQ and SynjC385S, in line with the activities of the PI(3)P phosphatases Ymr1, INP52, and INP53 in yeast cells (Parrish et al., 2004). These data in yeast and flies appear at odds with a study in zebrafish where expression of the SAC1 SynjC385S mutant results in a mild and partial rescue of autophagy in cone photoreceptors. While we currently do not understand the nature of this partial rescue, cone photoreceptors are tonically firing neurons (Van Epps et al., 2001; Heidelberger et al., 2005) and these cells may have different requirements for Synj activity than the spiking neurons at the fly NMJ or mouse hippocampal neurons. Nonetheless, the nature of the partial rescue of autophagy defects upon expression of SynjC385S in zebrafish suggests optimal SAC1 function is needed for normal autophagy. It will be interesting in this context to assess the effect of the Synj Parkinson mutations in zebrafish as well.

While our data indicate an important role of the Synj SAC1 domain in synaptic autophagy, our data do not exclude that the Synj 5-phosphatase domain is—indirectly—needed for autophagy as well. The Synj 5-phosphatase domain dephosphorylates phosphoinositides critical for endocytosis and synj 5-phosphatase mutants strongly disrupt endocytosis in mouse neurons (Mani et al., 2007). Endocytosis itself is required for the maturation of autophagic markers, suggesting that ultimately, the 5-phosphatase domain may block autophagy as well (Jäger et al., 2004; Hyttinen et al., 2013; Puri et al., 2013). This idea is consistent with evidence from the zebrafish study where a Synj 5-phosphatase mutant (D732A) blocks autophagy in photoreceptor neurons (George et al., 2016). Further experiments to determine whether this mutant, similar to data in mouse neurons, also blocks endocytosis in zebrafish or fruit flies are awaited, but the data are consistent with endocytic defects to precede autophagy. Hence, we propose a direct function of the SAC1 domain in synaptic autophagosome flux by PI (3)P and PI(3,5)P2 dephosphorylation and a potential indirect role of the 5-phosphatase domain by mediating synaptic endocytosis.

PtdInsP phosphatase enzymes are classic drivers of organelle biogenesis and function since they alter membrane lipid
composition and thus which proteins interact with this membrane (Wenk & De Camilli, 2004; Lauwers et al., 2016). The biochemical basis of how Synj1 contributes to endocytosis is relatively well studied. The 5-phosphatase domain dephosphorylates PI(4,5)P₂, thereby lowering the affinity of clathrin adaptors for the membrane of newly formed vesicles and promoting synaptic vesicle uncoating (Cremona et al., 1999; Gad et al., 2000; Verstreken et al., 2003). Our data suggest that the Synj1 SAC1 domain that hydrolyzes PI(3)P, PI(4)P, and PI(3,5)P₂ may have a similar “uncoating” role, but acting during the early steps of autophagosome biogenesis. Indeed, it was previously established that the early-stage autophagosome membrane contains PI(3)P and PI(3,5)P₂ and that these phosphoinositides recruit PI(3)P/PI(3,5)P₂-binding proteins, like Atg18a (Simonsen & Tooze, 2009; Noda et al., 2010; Dall’Armi et al., 2013; Proikas-Cezanne et al., 2015). Here, we observe that synapses expressing Synj1 accumulate the Atg18a-GFP marker of early autophagosomes, but produce far fewer mature Atg8-positive structures. Furthermore, using in vivo CLEM for neurons expressing Atg18a-GFP, we ultrastructurally define that Atg18a is attached to cisternal-like membrane structures and these are overly abundant within Synj1RO synapses. Finally, live cell imaging shows that Atg18a-GFP accumulations are immobilized within presynaptic terminals expressing Synj1RO, suggesting that the Synj1 SAC1 domain normally promotes Atg18a cycling between soluble and membrane-bound pools. Note that these data are inconsistent with a direct involvement of the 5-phosphatase domain (George et al., 2016) as this domain is responsible for the dephosphorylation of phosphoinositide species that Atg18a does not bind to. We therefore propose a model, where Synj-mediated PI(3)P and PI(3,5)P₂ hydrolysis on nascent autophagosomes lowers the affinity for PI(3)P/PI(3,5)P₂-binding proteins, including Atg18a, to release these from the membrane. This mechanism, regulated by the synapse-enriched Synj, is similar to that thought to be mediated by myotubularin (Ymr1) during autophagy in yeast cells (Cebollero et al., 2012). Interestingly, WIP12 (the Atg18a homolog) cycling is known to recruit LC3/Atg8 to autophagosome of non-neuronal cell types (Polson et al., 2010; Dooley et al., 2014), and thus, Atg18a immobilization can explain why the Synj1 SAC1 activity is needed for Atg8 accumulation at synapses. Hence, we propose that cycles of Atg18a recruitment followed by Synj1 SAC1 domain-dependent shedding allow the recruitment and concentration of Atg8 on the synaptic autophagosomal membrane, explaining why the Synj1RO protein fails to support the production of mature autophagosomes.

This model of Synj1 function has parallels with the roles of the yeast myotubularin Ymr1 and INP52 and INP53 PtdInsP₅ phosphatases in autophagosome biogenesis (Parrish et al., 2004; Cebollero et al., 2012; Cheng et al., 2014), which provides conceptual support to our finding that the PI(3)P phosphatase activity of Synj1 operates in autophagy at synapses. Given that Synj1 is presynaptic, we hypothesize that a different PI(3)P/PI(3,5)P₂-hydrolyzing enzyme mediates autophagosome maturation in non-neuronal animal cells and neuronal cell bodies. In addition, our data do not preclude the possibility that PtdInsP dephosphorylation mediated by the Synj1 SAC1 domain also contributes to other cell biological processes, potentially including endocytosis under certain conditions, but thus far, we have found no evidence for this. It is intriguing to consider the evolutionary consequences of combining 5-phosphatase and SAC1 domains in Synj proteins throughout the animal kingdom. This is consistent with the idea that both domains contribute to a cell biological process, perhaps acting sequentially to drive multiple stages of organelle maturation. Alternatively, it is possible that the dual roles of Synj allow this protein to couple autophagy to levels of synaptic vesicle cycling, and thus ensure sufficient protein turnover depending on the level of synaptic activity.

Our data connect impaired presynaptic autophagy to PD pathology. This is consistent with the classical concept of abnormal protein homeostasis causing proteopathic stress and driving neurodegenerative disease. However, this role of Synj highlights the concept that protein homeostasis within presynaptic terminals has a particularly important role in these diseases. We demonstrate that the pathogenic R258Q mutation in the presynaptic PD protein SYNJ1 impairs synaptic macroautophagy, and we associate this with neurodegeneration including loss of dopaminergic neurons. Another presynaptic PD protein alpha-synuclein affects membrane trafficking (Chua & Tang, 2011; Soper et al., 2011), although its roles remain less well defined than those of Synj1. The PD proteins Parkin and Pink1 are thought to drive mitophagy, a specialized form of macroautophagy that targets mitochondria for degradation (Narendra et al., 2008; Kawaijiri et al., 2010; Vincow et al., 2013). In addition, LRRK2, the most commonly mutated protein in PD, also affects autophagy within synaptic terminals (Soukup et al., 2016). In the case of LRRK2, this appears to occur via the essential presynaptic membrane factor EndophilinA (Soukup et al., 2016) that, interestingly, is a key regulator of Synj activity (Schuske et al., 2003; Verstreken et al., 2003). Future work should continue to examine whether the presynaptic terminal has a unique network of proteins that control macroautophagy, a membrane-mediated mechanism to clear damaged proteins or organelles. It is important to discover whether mutations in different PD genes indeed converge onto this process and, if so, to consider synaptic autophagy as an important target for development of anti-PD therapies.

### Experimental Procedures

#### Fly stocks and genetics

Flies were grown on standard cornmeal and molasses medium supplemented with yeast paste at 25°C. The following Drosophila stocks were obtained from the Bloomington Stock Center: w; P[hs-I-CreI.R]1A Sb1/TM6, y[1] w*; Mi{y[+]=mDint2-MIC}Synj [MII1871]/SM6a (Venken et al., 2011). The following fly stocks were obtained from the laboratory where the stock was first described: w; UAS-mCherry-Atg8a (Chang & Neufeld, 2009), UAS GFP::Lamp1/Cyo;TM6B/Sb bos1 (Pulipparacharuvil et al., 2005), w; UAS-PLCL-PH-EGFP (Verstreken et al., 2009), yw,eyFLP GMR-LacZ, FRT42D syn1¹/Cyo, and yw,eyFLP GMR-LacZ,FRT42D syn1¹/CyoO (Verstreken et al., 2003), w; atg18a(W3)/TM6b, Hu,Tb and w; atg18a(W4)/TM6b,Hu,Tb (Shen & Ganetzky, 2009), genomic w; Atg9HA (G. Juhász, unpublished).

#### Transgenic flies

A list of fly genotypes used in this study can be found in the Appendix Supplementary Methods section. All constructs were
generated using a seamless cloning method (Gibson Assembly, NEB). 
UAS synR², UAS synF², and UAS syn¹ were created using gBlocks (Integrated DNA Technologies, Inc.) and PCR products generated using primers listed in the Appendix Supplementary Methods section from CH322-135G1 [pacman] plasmid [BACPAC Resources Center (BPRC)] (Venken et al., 2006, 2009), cloned into the EcoRI site of pUAST-attB, sequence-verified, and inserted into attP site VK37 (22A3) using PhiC31-mediated integration at BestGene Inc.

synR² knock-in flies were created as described in Vilain et al. (2014). Targeting vector MiMIC 1322 pBS-KS-attB1-2 I-SceI SynR² was generated using primers listed in the Appendix Supplementary Methods section from CH322-135G21 [pacman] plasmid (BPRC) and cloned first into Xhol and PacI sites of pSV004 (containing I-SceI and I-CreI sites, see Appendix Supplementary Methods section) followed by restriction with SmaI, gel purification, and cloning into BbsI sites of MiMIC 1322 pBS-KS-attB1-2 vector with an additional linker sequence containing BbsI sites (see Appendix Supplementary Methods section) into XbaI and EcoRI sites.

Next, the construct was inserted into flies with Mi(MIC)Syn[MI1871] using PhiC31-mediated integration by in-house injection. Efficiencies were 30% for integration into the Mi(MIC)Syn[MI1871], 15% for desired orientation, and 37% of I-CreI double-strand break/repair. After genomic manipulation, the whole locus was sequence-verified.

UAS GFP::Atg18a, UAS GFP::Atg18b, UAS GFP::CG11975, UAS mEOS3.2::Atg18a, UAS eGFP-mCherry::Atg8a were created using gBlocks (Integrated DNA Technologies, Inc.) and PCR products generated using primers listed in the Appendix Supplementary Methods section, all Atg18 homologues cDNAs as template were obtained from the Proteomic ORF Collection (Yu et al., 2011) (respectively, LD38705, GH07816, and LD32381), mEOS3.2 was PCR from Addgene plasmid #54550 and cloned into the XhoI site of pUAST-attB, and Atg8 was cloned in between the EcoRI and XbaI sites of pUAST-attB. All constructs were sequence-verified and inserted into attP site VK20 (99F8) using PhiC31-mediated integration at BestGene Inc or by in-house injection.

Induction of autophagy by starvation, electrical stimulation, and chloroquine assay

Autophagy induction and flux assay were performed by placing early third-instar larvae on petri dishes containing 20% sucrose and 1% agarose for 3–4 h prior to experimentation (Scott et al., 2004). Electrical stimulation, 20 Hz for 30 min, to induce autophagy was conducted as described before (Soukup et al., 2016). For the chloroquine assay, we fed third-instar larvae 4 h on standard and starvation food containing 10 mg/ml chloroquine diphosphate salt (Sigma).

Correlative light and electron microscopic studies (CLEM and TEM)

Correlative light and electron microscopy studies were performed as described in Soukup et al. (2016), Before dissection in HL3, larvae were starved for 3–4 h. The samples were directly fixed for 2 h at room temperature in the dark (0.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.2), followed by washing steps in 0.1 M phosphate buffer, pH = 7.2. near-infrared branding (NIRB) was performed on a LSM 710 upright confocal microscope using the bleaching function as described (Urwyler et al., 2015). Before and after NIRB, z-stacks of the region of interest were acquired. Next, larvae were post-fixed in 1% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.2 at 4°C overnight or until further processing as described in Fernandes et al. (2014). NIRB introduced branding marks were used as a guide to identify Drosophila NMJ boutons previously imaged with the confocal microscope. Electron and fluorescent micrographs overlay, and identification of Atg18aGFP-positive ultrastructural features and processing of micrographs using brightness and contrast were performed with NIH ImageJ and Adobe Photoshop Elements 14.

TEM studies of larval boutons were performed as described in Kasprowicz et al. (2008). Larvae were starved for 3–4 h prior to dissection and fixation. TEM of the adult fly head was performed as described previously in Slabbaert et al. (2016).

Imaging and quantification

For live imaging of Atg8mCherry, Atg8GFP-mCherry, Atg18aGFP, Atg18bGFP, CG11975GFP, or Lamp1GFP, larvae expressing these markers were dissected in HL3 on sylgard plates and imaged on a Nikon A1 confocal microscope with a 60× NA 1.0 water-dipping lens. All images were collected with a pinhole of 1 airy unit and a resolution of 1,024 × 1,024. Imaging settings between experiments with the same marker were identical. Images are shown either in grayscale or using a 16-color lookup table (the LUT is shown in the figure).

Quantification of number of puncta or intensity was performed in NIH ImageJ. Atg8mCherry, Atg8GFP-mCherry, Atg18aGFP, or CG11975GFP dots were counted using particle analysis of a binary image combined with applying a threshold mask on the cytoplasmic signal to calculate the bouton area. Lamp1GFP fluorescence intensities were measured by first applying a threshold to mark the boutonic areas followed by measuring the average pixel intensities in boutonic areas. The different conditions were blinded before imaging and quantifying the data.

Fluorescence recovery after photobleaching (FRAP) was performed on third-instar larval fillets dissected in HL3 on sylgard-coated plates using a protocol adapted from Seabrooke et al. (2010). Experiments were conducted on a Nikon A1 confocal microscope with a 60× NA 1.0 water-dipping lens. Before bleaching, 10 images were acquired to set the pre-bleach intensity. Next, bleaching (minimum 50% of the pre-bleach intensity, by two rapidly iterated laser bursts), using a 488-nm laser, was focused on an Atg18aGFP dot, directly followed by image acquisition for 120 s (60 scans with a 2.12-s interval). The fluorescence intensity of the selected Atg18aGFP dot was analyzed in NIH ImageJ.

Photoconversion of mEOS3.2 was performed on third-instar larval fillets dissected in HL3 on sylgard-coated plates; next, nerves were cut in HL3 with NAS (100 µM) followed by mounting the larval prep on a glass slide in HL3 with NAS. Experiments were conducted on a Nikon A1 confocal microscope with a 60× NA1.4 oil lens; all images were collected with an open pinhole and a resolution of 1,024 × 1,024. Images of the Atg18a[mdosS]² dot of interest were taken before, directly after, and 5 and 10 min after photoconversion (conducted using a 405-nm laser) of the neighboring bouton. The fluorescence intensity of the selected Atg18a[mdosS]² dot was analyzed in NIH ImageJ.
Single-particle tracking photoactivation localization microscopy (sptPALM)

SptPALM was carried out on transgenic Atg18a^mEOS3.2 third-instar *Drosophila* larvae dissected on sylgard base as previously described (Bademosi et al., 2017). Briefly, Atg18a^mEOS3.2 was imaged at the larva NMJ on muscles 6 and 7 of the second abdominal segments, using total internal reflection (TIRF) microscopy under oblique illumination. Dissected larvae on the sylgard base were inverted onto glass-bottomed imaging dishes (In Vitro Scientific). Localization and tracking of single Atg18a^mEOS3.2 molecules were carried out using a 63× water-immersion objective (1.2 numerical aperture, Achromat) on the ELYRA PS.1 microscope (Zeiss). NMJs were located using 488-nm laser (18.72 mW) illumination. A 405-nm laser (0.6 mW) was used for photoconversion, while imaging of photoconverted Atg18a^mEOS3.2 molecules was done using 561-nm laser (20.6 mW) to spatially distinguish individual molecules (405-nm laser was used at 0.009–0.3% power, while 75% power was used for 561 nm). Single-molecule fluorescence was collected by a sensitive electron-multiplying charge-coupled device (EMCCD) camera (Evolve, Photometric). Zen Black acquisition software (2012 version, Carl Zeiss) was used for movie acquisition. Images were captured at 33 Hz and 15,000 frames were obtained per NMJ. sptPALM analysis is described in Appendix Supplementary Methods section.

Complete starvation

Complete starvation assay was performed as described in Juhász et al. (2007), briefly 1-day-old fed flies were transferred to vials containing a gel of water (1% agarose in water) and transferred to a new vial every day. Dead flies were counted manually multiple times a day.

Light-induced degeneration

Light-induced retinal degeneration studies were performed by rearing flies for 7 days in constant illumination at 1,300 lux and 25°C (Soukup et al., 2013).

Visualization of TH-positive neuronal clusters after aging adult flies

Aging and visualization of dopaminergic clusters was performed by aging 1- to 3-day-old adult flies for an additional 30 days at 25°C (flies were placed on fresh medium every 3 days). At day 30, fly brains were dissected and stained (see immunohistochemistry). Images of dopaminergic clusters (labeled by the anti-TH antibody) were captured with a Nikon A1R confocal microscope using a 20× objective 0.75 NA (Nikon). The total number of TH-positive neurons throughout the brain and in both hemispheres for the PPM1, PPM2, PPM3, and PPL1 clusters was counted manually.

Neural progenitor differentiation

The iPSCs (generation and karyotyping described in Appendix Supplementary Methods and Appendix Table S1) were grown on irradiated MEFs and cultured in normal iPSC medium (DMEM/F12 Advanced (Gibco Life Technologies) supplemented with 20% KOSR, 2 mM L-glutamine, 0.1 mM MEM-NEAA, 0.1 mM 2-mercaptoethanol, 100 units/ml penicillin/streptomycin (all from Life Technologies)) and 10 ng/ml bFGF (Perprotech) at 37°C/5% CO₂. Medium was changed daily and cells were passaged every 4–6 days either mechanically or enzymatically with collagenase type IV (100 U/ml) (Life Technologies), and addition of 10 μM ROCK inhibitor (Sigma) to the medium after passaging.

Neural progenitor cells (NPCs) were created according to published protocols (de Esch et al., 2014; Gunhanlar et al., 2017) with minor modifications. Briefly, for embryoid body (EB) generation, iPSCs were dissociated and transferred to non-adherent plates in normal iPSC medium without bFGF and cultured on a shaker at 37°C/5% CO₂. After 2 days in vitro (DIV), medium was replaced by neural induction medium (DMEM/F12 Advanced, 1:100 N2 supplement, 100 units/ml penicillin/streptomycin (all from Life Technologies) and 2 μg/ml heparin (Sigma)). After 7 DIV, the EBs were partially dissociated and plated on laminin-coated culture dishes in neural induction medium.

Neural progenitor cells growing out of the attached EB fragments were cultured for 8 DIV at 37°C/5% CO₂, and medium was changed every other day. Subsequently, the NPCs were split between 1:2 and 1:6 with collagenase every 5–7 days and cultured on laminin (Sigma)-coated plates. The NPCs were maintained in NPC medium [DMEM/F12 Advanced, 1:100 N2 supplement, 1:50 B27-RA supplement, 100 units/ml penicillin/streptomycin (all from Life Technologies), 1 μg/ml laminin (Sigma), and 20 ng/ml bFGF (Preprotech)].

Neural differentiation

Neural progenitor cells were passaged 1:1 to poly-D-lysine (PDL) (Sigma)- and laminin (Sigma)-coated coverslips and cultured in neural differentiation medium [Neurobasal medium supplemented with 1:100 N2 supplement, 1:50 B27 supplement, 0.1 mM MEM-NEAA, 100 units/ml penicillin/streptomycin (all from Life Technologies), 20 ng/ml BDNF (ProspechBio), 20 ng/ml GDNF (ProspechBio), 1 μM db-cAMP (Sigma), 200 μM ascorbic acid (Sigma), and 2 μg/ml laminin (Sigma)]. The differentiating cells were cultured for 28 DIV at 37°C/5% CO₂, and medium was changed every other day.

Neuron starvation

At 28 DIV, neurons were either incubated for 3 h in neural differentiation medium supplemented with 100 μM leupeptin (Roche) for the control condition, or in preconditioned EBSS (Life Technologies) supplemented with 100 μM leupeptin (Roche) for the starved condition.

Statistics

GraphPad Prism 6 (San Diego, USA.) was used to determine statistical significance. Prior to this, datasets were tested for normal distribution using a D’Agostino-Pearson Omnibus test. Next, normally distributed data were tested with parametric tests: Student’s t-test was used when two datasets were compared and we used a one-way analysis of variance test (ANOVA) followed by a
**post hoc** Tukey test when more than two datasets were compared. Non-normally distributed data were tested using non-parametric tests; when comparing two datasets, we used a Mann–Whitney test, and when comparing more than two datasets, we used an ANOVA Kruskal–Wallis test followed by a Dunn’s **post hoc** test. Significance levels are defined as ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, ns (not significant) = P > 0.05. “n” in the legends indicates either the number of animals used or the number of NMJs analyzed.

Expanded View for this article is available online.

Acknowledgements
We thank T. Neufeld, H. Krämer, G. Juhász, and B. Gunetzky, the Bloomington *Drosophila* Stock Center the Developmental Studies Hybridoma bank for reagents and the VIB Bio-imaging core Leuven for help and BestGene Inc for *Drosophila* embryo injections. We thank Mehrazh Ghavzini and Joost Grnbau from the Erasmus MC iPSC core facility for generation of the iPSC lines, Bruno Van Swinderen (Queensland Brain Institute, The University of Queensland) for use of his fly facility and insightful discussions. We especially thank Rose Goodchild for excellent discussions throughout the study, Willem Van den Bergh for additional in-house injections, and Liesbeth Deaumiere and the Verstreken laboratory members for help and consultation. Research support was provided by a Flanders Innovation Agency (IWV) Fellowship to RV (121324), KU Leuven (CREA, GOA), ERC CoG (646671), the Research Foundation Flanders (FWO grants G08851N, G09491N, G00317N, G003417N), the Hercules Foundation, the Interuniversity Attraction Pole program by BELSPO (P7/16), a Methusalem grant of the Flemish government, VIB, the Vlaamse Parkinson Liga, the Stichting ParkinsonFonds (the Netherlands), the Dorpmans–Wigmans Stichting, an Erasmus MC MIRace promotie grant to VB and WM, and the EU Joint Programme—Neurodegenerative Disease Research (JPND) Memorabel (733051065) supported by the Nederlandse organisatie voor gezondheidszorg (ZonMW) under the aegis of JPND to VB and an Hersenstichting Fellowship to FdV, the National Health and Medical Research Council (NHMRC) project grant (GNT1120381 to FAM) and Australian Research Council LIEF Grants (LE0882864 and LE130100078 to FAM). FAM is a National Health and Medical Research Council Senior Research Fellow (GNT1060075).

Author contributions
RV, SFS, and PV conceived the study. RV, SFS, and PV analyzed data and wrote the paper. RV, SK, RM, AB, JM, NS, LB, SG, JS, SV, MP, PB, STM, FMSdV, SAK, NVG, WM, VB, FAM, SFS, and PV performed experiments and/or provided reagents, and NS performed the in-house injection. All the authors approved the final version of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References
SYNJ mobilizes WIPI2/Atg18a in synaptic autophagy

The EMBO Journal


Marat AI, Haucke V (2016) Phosphatidylinositol 3-phosphates—at the interface between cell signalling and membrane traffic. EMBO J 35: 561 – 579


SYNJ1 mobilizes WPI2/Atg18a in synaptic autophagy
Roeland Vanhauwaert et al.


