Autophagy promotes synapse development in Drosophila

Wei Shen and Barry Ganetzky

Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53706

Introduction

Regulation of synaptic growth and plasticity is essential for proper development and function of neural circuits that underlie behavior and its modification in response to experience and environment. The Drosophila melanogaster larval neuromuscular junction (NMJ) is a powerful system for dissecting molecular mechanisms that mediate synaptic growth (Collins and DiAntonio, 2007). Previous studies have identified key positive and negative regulators of synaptic growth, including proteins associated with cell adhesion, the cytoskeleton, endocytosis, and Wnt and bone morphogenic protein signaling (Schuster et al., 1996; Aberle et al., 2002; Packard et al., 2002; Coyle et al., 2004; Koh et al., 2004; Marie et al., 2004; Dickman et al., 2006; Pack-Chung et al., 2007; Liebl et al., 2008; O’Connor-Giles et al., 2008; Rodal et al., 2008). The balance of opposing forces and the multiple layers of regulation that are imposed reveal that complex mechanisms have evolved to ensure correct synaptic size.

Protein degradation via the ubiquitin–proteasome system (UPS) is an important negative regulatory mechanism of NMJ growth, as revealed by striking overgrowth in highwire (hiw) mutants (Wan et al., 2000). Hiw, an E3 ubiquitin ligase, restrains synaptic growth primarily by down-regulation of Wallenda (Wnd), a MAPK kinase kinase (Collins et al., 2006).

Another major protein degradation mechanism is autophagy, which involves activation of the induction complex, formation of autophagosomes that engulf cytoplasmic materials, subsequent fusion of autophagosomes with lysosomes, and finally, degradation of sequestered material by lysosomal enzymes (Levine and Klionsky, 2004). Various stress conditions trigger autophagy, including starvation, hypoxia, high temperature, accumulation of protein aggregates, and infection (Hara et al., 2006; Nakai et al., 2007). The association of autophagy with human diseases such as neurodegeneration and cancer reveals its importance as a cellular surveillance system (Levine and Klionsky, 2004; Levine and Kroemer, 2008).

Because the UPS is an important negative regulator of NMJ development, we examined whether autophagy also plays a role. We discovered that autophagy positively regulates NMJ development and, remarkably, does so by controlling Hiw levels. Thus, autophagy and the ubiquitin–proteasome system converge in regulating synaptic development. Because autophagy is triggered in response to many environmental cues, our findings suggest that it is perfectly positioned to link environmental conditions with synaptic growth and plasticity.

Autophagy, a lysosome-dependent degradation mechanism, mediates many biological processes, including cellular stress responses and neuroprotection. In this study, we demonstrate that autophagy positively regulates development of the Drosophila melanogaster larval neuromuscular junction (NMJ). Autophagy induces an NMJ overgrowth phenotype closely resembling that of highwire (hiw), an E3 ubiquitin ligase mutant. Moreover, like hiw, autophagy-induced NMJ overgrowth is suppressed by wallenda (wnd) and by a dominant-negative c-Jun NH2-terminal kinase (bskDN).

We show that autophagy promotes NMJ growth by reducing Hiw levels. Thus, autophagy and the ubiquitin–proteasome system converge in regulating synaptic development. Because autophagy is triggered in response to many environmental cues, our findings suggest that it is perfectly positioned to link environmental conditions with synaptic growth and plasticity.
Results and discussion

Autophagy promotes NMJ growth

Autophagy involves multiple steps, including induction, autophagosome formation, fusion of autophagosomes with lysosomes, and recycling of autophagy components. Disrupting any of these steps impairs autophagy. Several highly conserved ATG genes encoding core components of the autophagy machinery have been identified in yeast. Mutations in genes, including atg1, -2, -6, and -18, have also been isolated and characterized in Drosophila (Scott et al., 2004; Berry and Baehrecke, 2007). To assess the role of autophagy in NMJ development, we examined the effect of mutations in atg genes whose normal functions span the entire process: atg1 is defective in autophagy induction, atg6 is defective in autophagosome formation, and atg2 and -18 are defective in retrieval of other ATG proteins from autophagosomes (Levine and Kroemer, 2008). Regardless of the step impaired, all of these atg mutants exhibited significant reduction in NMJ size (Fig. 1, A and B). These results demonstrate that a basal level of autophagy is required to promote NMJ development.

Overexpression of atg1+ is sufficient to induce high levels of autophagy in larval fat bodies and salivary glands (Berry and Baehrecke, 2007; Scott et al., 2007). If autophagy is a positive regulator of NMJ development, an increase in autophagy might enhance synaptic growth. Consistent with previous studies, pan-neuronal overexpression of upstream activating sequence (UAS)-atg1+ under the control of C155-Gal4 or elav-Gal4 drivers induced high levels of autophagy in the nervous system, as indicated by increased staining with LysoTracker, an acidophilic dye which has been used to assess autophagy by labeling acidic structures, including lysosomes (Fig. 1 C; Berry and Baehrecke, 2007; Scott et al., 2007). Under these conditions, bouton number increased more than twofold (Fig. 1, D and E). To further verify that this NMJ overgrowth was caused by elevated autophagy rather than to some other effect of atg1+ overexpression, we tested whether mutations in other atg genes suppress this phenotype. For this purpose, we generated a null allele of atg18 (atg18DN). Removal of one copy of atg18DN had no effect on NMJ growth in an otherwise wild-type background but significantly suppressed NMJ overgrowth caused by atg1+ overexpression (Fig. 1, D and E). Removal of both copies of atg18DN conferred almost complete suppression (Fig. 1, D and E). Therefore, NMJ overgrowth caused by atg1+ overexpression is primarily caused by elevated levels of autophagy.

As a further test, we examined NMJ morphology after feeding larvae with rapamycin, which induces autophagy by inhibiting TOR (target of rapamycin), the key negative regulator of autophagy (Rubinsztein et al., 2007). Wild-type larvae fed rapamycin exhibited striking NMJ overgrowth similar to that caused by overexpressing atg1+ (Fig. 1, D and F), which is consistent with the results of Knox et al. (2007). Rapamycin-induced NMJ overgrowth was completely suppressed by mutations in atg18 (Fig. 1, D and F). Collectively, these results demonstrate that autophagy is a key positive regulator of NMJ growth.

Wairkar et al. (2009) observed NMJ undergrowth in atg1 mutants but did not see overgrowth with atg1+ overexpression. This discrepancy likely results from the use of different UAS-atg1+ transgenes. For example, Wairkar et al. (2009) were only able to obtain partial (~50%) rescue of NMJ undergrowth in atg1 mutants by overexpression of their UAS-atg1+rasV12 construct, whereas we obtained complete rescue of this phenotype (Fig. S2).

Atg1-mediated synaptic growth is independent of its nonautophagic functions

Atg1 has several functions unrelated to autophagy. We found that axonal transport is disrupted in atg1-null mutants (Fig. S1), which is a result also recently reported by Toda et al. (2008) and Wairkar et al. (2009). In addition, Atg1 suppresses translation by inhibiting the S6K kinase (Lee et al., 2007; Scott et al., 2007) and controls active zone density by inhibiting extracellular signal-regulated kinase (ERK) signaling (Wairkar et al., 2009). However, several lines of evidence indicate that these functions of Atg1 are not responsible for the NMJ phenotypes we observed when Atg1 activity was altered. First, atg2 or -18 mutants exhibited similar NMJ undergrowth but did not have defects in axonal transport (Fig. 1 A and Fig. S1). Thus, in agreement with Toda et al. (2008), we conclude that Atg1’s role in axonal transport is distinct from its function in autophagy and NMJ growth. Second, blocking or activating translation by overexpressing a dominant-negative S6K transgene or constitutively activated S6K transgenes by elav-Gal4 driver had little affect on NMJ growth (Fig. 2). Moreover, coexpression of any of the three constitutively activated S6K transgenes failed to suppress NMJ overgrowth caused by atg1+ overexpression (Fig. 2). Thus, the role of Atg1 in S6K-dependent translation does not contribute to the NMJ phenotypes associated with manipulations of Atg1. Third, an ERK mutation does not affect NMJ growth. Although this ERK mutation suppresses the deficit in active zone density in atg1 mutants, it does not suppress atg1’s NMJ undergrowth phenotype (Wairkar et al., 2009), indicating that it is not mediated by the ERK pathway. Collectively, these results demonstrate that altered levels of autophagy are primarily responsible for the effects of Atg1 on NMJ development.

wnd and bskDN suppress autophagy-dependent synaptic overgrowth

NMJ overgrowth induced by autophagy is distinctive and offers potential clues about pathways that may be involved. Formation of multiple long synaptic branches containing many small diameter boutons without any hyperbudding or satellite boutons most closely resembles the hiw phenotype (Wan et al., 2000), suggesting that autophagy and Hiw may function through the same pathway. Recent evidence indicates that Hiw inhibits NMJ growth by down-regulating Wnd, which in turn activates a Jun kinase encoded by bsk (basket). NMJ overgrowth in hiw is suppressed by mutations of wnd and by a dominant-negative mutation of bsk (bskDN, Collins et al., 2006). If the phenotypic similarity between hiw and increased autophagy reflects convergence on a common pathway, autophagy-induced NMJ overgrowth should also be suppressed by wnd and bskDN. Indeed, this is what we observed (Fig. 3). These results strongly support the idea that autophagy and Hiw converge on aWnd-dependent MAPK signaling pathway to regulate NMJ development.
Figure 1. **Autophagy promotes NMJ growth in Drosophila.** (A) Confocal images of NMJ 4 labeled with FITC-HRP. Compared with control (w^1118), atg1/Df, atg2/Df, and atg18/Df mutants exhibit smaller NMJs. (B) Quantification of bouton numbers at NMJ 4 in larvae of the genotypes shown. (C) Fluorescent images of larval brain hemisphere of control (C155-Gal4/+) and atg1^+/overexpressing larvae (C155-Gal4/UAS-atg1^+) stained with LysoTracker. Substantially increased staining is observed in the latter, indicating elevated levels of autophagy. (D) Confocal images of NMJ 4 labeled with FITC-HRP. Compared with control (elav-Gal4/+), neuronal overexpression of atg1^+ (UAS-atg1^+) results in strong NMJ overgrowth. Overgrowth is reduced by loss of one copy of atg18^+ (UAS-atg1^+; atg18/+), and almost completely suppressed by removing both copies of atg18^+ (UAS-atg1^+; atg18/Df). Larvae fed on 2 μM rapamycin (Rap) to induce autophagy exhibit NMJ overgrowth, which is completely suppressed in an atg18/Df background. (E and F) Quantification of bouton numbers at NMJ 4 in the indicated larvae. (B, E, and F) Error bars denote SEM. **, P < 0.01; ***, P < 0.001. CS, Canton-S; WT, wild type. Bars: (A and D) 15 μm; (C) 50 μm.
To further test whether autophagy promotes NMJ growth by limiting Hiw, we eliminated one copy of hiw+ to determine whether this further decrease in Hiw levels enhanced the effects of atg1+ overexpression. In an otherwise wild-type background, loss of one copy of hiw+ had no affect, but it significantly enhanced atg1+–induced NMJ overgrowth (Fig. 4 G). The phenotype of hiw homozygotes overexpressing atg1+ was no more extreme than hiw homozygote alone (Fig. 4, E–G). The absence of any additive or synergistic effects further supports the hypothesis that autophagy promotes NMJ development by down-regulating Hiw.

Because Hiw antibodies do not work for immunohistochemistry, we visualized Hiw using a fully functional GFP-tagged construct to test directly whether abundance of Hiw is affected by autophagy (Wu et al., 2005). In an otherwise wild-type background, Hiw-GFP was strongly expressed in neurons throughout the ventral ganglion and brain lobes driven by C155-Gal4, as detected by anti-GFP staining (Fig. 4, H1–H3). However, in larvae co-overexpressing atg1+, the GFP signal

**Figure 2.** Atg1-dependent changes in NMJ growth are independent of effects on translation. (A) Confocal images of NMJ 4 labeled with FITC-HRP. Blocking or activating translation by overexpressing a dominant-negative S6K (UAS-S6KQ) or a constitutively activated S6K transgene (UAS-S6KΔ, UAS-S6KΔE, or UAS-S6KΔETE) has little effect on NMJ growth. None of three constitutively activated S6K transgenes suppresses NMJ overgrowth when coexpressed with atg1+. (B) Quantification of bouton numbers at NMJ 4 in larvae of genotypes shown. Comparisons are made with elav-Gal4/UAS-atg1+. Error bars denote SEM. Bar, 15 µm.
was reduced by ~60% relative to anti-HRP staining (Fig. 4, I1–I3 and J). We confirmed this result by Western blot analysis (Fig. 4 K). Reduction of Hiw-GFP is not caused by the dilution of GAL4 by the presence of a second UAS element because coexpression of UAS-myr-RFP did not affect abundance of Hiw-GFP (Fig. 4 K). These results further indicate that autophagy promotes NMJ growth by down-regulating Hiw.

Hiw-dependent synaptic undergrowth in atg mutants

Our results indicate that NMJ overgrowth caused by elevated autophagy is primarily caused by reduction in Hiw. Is the converse also true? Is NMJ undergrowth in atg mutants caused by elevated levels of Hiw? To address these questions, we expressed Hiw-GFP in neurons using C155-Gal4 in various backgrounds. Hiw-GFP levels were significantly elevated in atg1 and -6 mutants compared with the controls, which is consistent with the idea that Hiw is down-regulated by autophagy (Fig. 5 A).

If this increase in Hiw is a primary cause of NMJ undergrowth in atg loss-of-function mutants, eliminating Hiw should prevent this undergrowth; i.e., mutations in hiw should be epistatic to atg mutations. Thus, we examined NMJ morphology in hiw; atg2 and hiw; atg18 double mutants and found that hiw was completely epistatic (Fig. 5, B and C), demonstrating the role of elevated levels of Hiw in NMJ undergrowth of atg mutants.

A more direct test is to determine whether overexpression of Hiw can reduce NMJ size. However, this experiment is complicated because overexpression of Hiw by a relatively weak neuronal driver (elav-Gal4) does not affect NMJ size (Fig. 4), whereas overexpression of Hiw by a strong neuronal driver (Elav-GeneSwitch) has a modest dominant-negative effect (Wu et al., 2005). To determine whether increased levels of Hiw can
Together, these results indicate that elevated levels of Hiw account for most of the NMJ undergrowth in atg mutants. However, excess Hiw cannot fully explain NMJ undergrowth in atg mutants because NMJ undergrowth caused by Hiw overexpression is less severe than that of atg1 and -18 mutants. Thus, when autophagy is impaired, additional negative regulators may accumulate to depress NMJ growth. It is also likely that elevated levels of Hiw target proteins other than Wnd to limit synaptic growth because loss-of-function mutations of wnd do not affect NMJ development (Collins et al., 2006).

Because autophagy is generally thought of as a nonselective bulk degradation process, the idea that autophagy regulates NMJ growth primarily through its effects on Hiw levels seems difficult to understand at first. However, recent studies demonstrate that autophagy can also operate in a substrate-selective mode in regulating specific developmental events (Rowland et al., 2006; Zhang et al., 2009). For example, in Caenorhabditis
Thus, one possibility is that Hiw is specifically targeted to autophagosomes via a mechanism that remains to be elucidated. It is also possible that many presynaptic proteins besides Hiw are degraded by autophagy, but it is the reduction in Hiw that primarily affects NMJ size. Moreover, although we favor the idea that autophagy regulates Hiw directly, we cannot
rule out the possibility that autophagy promotes degradation of Hwi through an indirect mechanism involving the proteasome or other pathway.

In principle, autophagy could be acting on either side of the NMJ to regulate its development. Because atg1+ overexpression in muscle results in lethality at the first larval instar, we are unable to assess whether this affects NMJ growth. Although we cannot rule out a postsynaptic role of autophagy in NMJ development, several results suggest that the effects of autophagy are primarily presynaptic: neuronal expression of UAS-atg1+ is sufficient to completely rescue the NMJ undergrowth in atg1 mutants (Fig. S2), the Hw–Wnd pathway functions presynaptically (Wu et al., 2005; Collins et al., 2006), and hiw is completely epistatic to autophagy for NMJ growth.

Autophagy is of particular interest as a regulator of synaptic growth because it is triggered in response to many environmental cues. Our results demonstrate that decreasing or increasing autophagy from basal levels results in corresponding effects on synaptic size. Thus, autophagy is perfectly positioned to link environmental conditions with synaptic growth and plasticity. As such, it is intriguing to speculate on a role for autophagy in learning and memory.

Materials and methods

Fly stocks

Canton-S was used as a wild-type control. w^{1118} was used as a control for genetic background. atg1^{1A20}, UAS-atg1^{1A6}, and UAS-atg1^{1A8} (Scott et al., 2007) were provided by T. Neufeld (University of Minnesota, Minneapolis, MN). hiw^{ATM}, UAS-hiw^{ATM}, UAS-hiw^{GFP}, wnd^{ATM}, wnd^{Δ} (Wan et al., 2000; Wu et al., 2005; Collins et al., 2006), and elav-Gal4 were provided by A. DiAntonio (Washington University, St. Louis, MO). The following fly lines were obtained from the Bloomington Stock Center: atg2^{P{EP}607}, atg1^{1B}G03090, atg1^{G03090}0006, UAS-bak^{ATM}, UAS-S6K^{ATM}, UAS-S6K^{G1227E}, UAS-S6K^{E1714}, UAS-S6K^{E1716}, UAS-S6K^{E1727}, (Spradling et al., 1999; Weber et al., 2000; Barcelo and Stewart, 2002; Scott et al., 2004; Berry and Baehrecke, 2007), Df(3R)Exel112, Df(3R)Exel6091, Df(3R)Exel6197, and Df(3R)USBC10.

Generation of atg1^{18} mutant in Drosophila

P element KG03090 was inserted within the first exon of the atg1^{18} gene, 127 bp upstream of the ATG start codon. atg1^{18} was generated by precise excision, which deletes 585 bp downstream of the KG03090 insertion site, removing the second exon, including the start codon, and part of the third exon. The atg1^{BP}/Df mutant exhibited an NMJ undergrowth phenotype identical to atg1^{G03090}/Df.

Immunohistochemistry

Wandering third instar larvae were dissected in Ca^{2+}-free saline and fixed in 4% paraformaldehyde for 20 min. Larvae were incubated in primary antibodies overnight at 4°C or for 2 h at room temperature and then mounted in Vectashield (Vector Laboratories) for microscopy. For bouton quantification, we used rabbit anti-NWak at 1:1,000 (Coyle et al., 2004) or rabbit anti–synaptotagmin I at 1:1,000 (H. Bellen, Baylor College of Medicine, Houston, TX). To detect Hiw-GFP fusion protein, mouse anti-GFP was used at 1:1,000. Specific secondary antibodies conjugated to Alexa Fluor 488, 568, and 647 (Invitrogen) were used at 1:200 (Invitrogen). Western blots were probed with rabbit anti-GFP at 1:500 (Invitrogen) or goat antia at 1:1,000 (Santa Cruz Biotechnology, Inc.). Immunoreactive bands were detected with an enhanced chemiluminescence (ECL) kit (Amersham). Films were analyzed using a暴s Kodak image analysis system.

Imaging and quantification

We focused on NMJ 4 because of its relative simplicity; however, comparable results were also observed at NMJ 6/7 as well as at other NMJs we examined in mutant larvae. Quantification of bouton number was performed at NMJ 4 in segments A2–A4 as described previously (Coyle et al., 2004). Bouton numbers for each genotype are summarized in Table S1. The muscle size of all larvae analyzed was similar. Samples for each experiment were processed simultaneously with corresponding controls and imaged using the same confocal gain settings. Confocal images were obtained on a microscope (LSM 510; Carl Zeiss, Inc.) with Plan-Apochromat 20× NA 1.0, EC Plan-Neofluar 40× NA 1.3 oil differential interference contrast, or Plan-Apochromat 63× NA 1.4 oil differential interference contrast objectives and the accompanying software. Images were processed in LSM browser software (Carl Zeiss, Inc.) or with corresponding controls and imaged using the same confocal gain settings. Confocal images were obtained on a microscope (LSM 510; Carl Zeiss, Inc.) with Plan-Apochromat 20× NA 1.0, EC Plan-Neofluar 40× NA 1.3 oil differential interference contrast, or Plan-Apochromat 63× NA 1.4 oil differential interference contrast objectives and the accompanying software. Images were processed in LSM browser software (Carl Zeiss, Inc.) or with corresponding controls and imaged using the same confocal gain settings. Confocal images were obtained on a microscope (LSM 510; Carl Zeiss, Inc.) with Plan-Apochromat 20× NA 1.0, EC Plan-Neofluar 40× NA 1.3 oil differential interference contrast, or Plan-Apochromat 63× NA 1.4 oil differential interference contrast objectives and the accompanying software. Images were processed in LSM browser software (Carl Zeiss, Inc.) or with corresponding controls and imaged using the same confocal gain settings. Confocal images were obtained on a microscope (LSM 510; Carl Zeiss, Inc.) with Plan-Apochromat 20× NA 1.0, EC Plan-Neofluar 40× NA 1.3 oil differential interference contrast, or Plan-Apochromat 63× NA 1.4 oil differential interference contrast objectives and the accompanying software. Images were processed in LSM browser software (Carl Zeiss, Inc.) or with corresponding controls and imaged using the same confocal gain settings.

Western blotting

Approximately 20 larval brains of each genotype were collected and homogenized on ice with 20 μl of homogenization buffer [67 mM Tris-HCl, pH 8.0, 67 mM NaCl, 2 M urea, 1.3% SDS, 2 mM DTT, and 1 mM EDTA; Wu et al., 2005]. After adding SDS loading buffer, the homogenate was boiled for 10 min. The volume of six brains was loaded for each sample onto a two-layer SDS-PAGE gel with 5% upper layer and 12% lower layer. Western blots were probed with rabbit anti-GFP at 1:500 (Invitrogen) or goat antia at 1:1,000 (Santa Cruz Biotechnology, Inc.)

Online supplemental material

Fig. S1 shows that atg1 mutants have axonal transport defects not seen in other autophagy mutants. Fig. S2 shows that NMJ undergrowth in atg1 mutants is rescued by presynaptic expression of an atg1+ transgene. Table S1 presents quantification of bouton numbers for each genotype. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200907109/DC1.

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References

Aberle, H., A.P. Haghhighi, R.D. McCabe, B.D. Magalhães, and C.S. Goodman. 2002. wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila. Neuron. 33:545–558. doi:10.1016/S0896-6273(02)00559-5

Barcelo, H., and M.J. Stewart. 2002. Altering Drosophila S6 kinase activity is consistent with a role for S6 kinase in growth. Genesis. 34:83–85. doi:10.1002/gene.10132

Berry, D.L., and E.H. Baehrecke. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell. 131:1137–1148. doi:10.1016/j.cell.2007.10.048

Collins, A., and A. DiAntonio. 2007. Synthetic development: insights from Drosophila. Curr. Opin. Neurobiol. 17:35–42. doi:10.1016/j.conb.2007.01.001

Downloaded from jcb.rupress.org on August 8, 2017
Collins, C.A., Y.P. Wairkar, S.L. Johnson, and A. DiAntonio. 2006. Highwire regulates synaptic growth by attenuating a MAP kinase signal. *Neuron*. 51:57–69. doi:10.1016/j.neuron.2006.05.026

Coyle, I.P., Y.H. Koh, W.C. Lee, J. Slind, T. Fergestad, J.T. Littleton, and B. Scott, R.C., O. Schuldiner, and T.P. Neufeld. 2004. Nervous wreck, an SH3 adaptor protein that interacts with Wsp, regulates synaptic growth in *Drosophila*. *Neuron*. 41:521–534. doi:10.1016/S0896-6273(04)00169-9

Dickman, D.K., Z. Lu, I.A. Meierzhange, and T.L. Schwarz. 2006. Altered synaptic development and active zone spacing in endocytosis mutants. *Curr. Biol.* 16:591–598. doi:10.1016/j.cub.2006.02.058

Hara, T., K. Nakamura, M. Matsu, A. Yamamoto, Y. Nakahara, R. Suzuki-Miyashima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushita. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 441:885–889. doi:10.1038/nature04724

Knox, S., H. Ge, B.D. Dimitroff, Y. Ren, K.A. Howe, A.M. Arsham, M.C. Emery, T.P. Neufeld, M.B. O’Connor, and S.B. Selleck. 2007. Mechanisms of TSC-mediated control of synapse assembly and axon guidance. *PLoS One*. 2:e375. doi:10.1371/journal.pone.0000375

Koh, T.W., P. Verstreken, and H.J. Bellen. 2004. Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. *Neuron*. 43:193–205. doi:10.1016/j.neuron.2004.06.029

Lee, S.B., S. Kim, J. Lee, G. Lee, Y. Kim, J.M. Kim, and J. Chung. 2007. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. *EMBO Rep.* 8:360–365. doi:10.1038/embor.7400917

Levine, B., and D.J. Klionsky. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell*. 6:463–477. doi:10.1016/S1534-5807(04)00009-1

Levine, B., and G. Kroemer. 2008. Autophagy in the pathogenesis of disease. *Cell*. 132:27–42. doi:10.1016/j.cell.2007.12.018

Lieb, F.L., Y. Wu, D.E. Featherstone, J.N. Noordermeer, L. Fradkin, and H. Hing. 2006. Derailed regulates development of the *Drosophila* neuromuscular junction. *Dev. Neurobiol.* 68:152–165. doi:10.1002/dneu.20562

Long, X., and L.C. Griffith. 2000. Identification and characterization of a SUMO-1 conjugation system that modifies neuronal calcium/calmodulin-dependent protein kinase II in *Drosophila melanogaster*. *J. Biol. Chem.* 275:40765–40776. doi:10.1074/jbc.M003949200

Marie, B., S.T. Sweeney, K.E. Poskanzer, J. Roos, R.B. Kelly, and G.W. Davis. 2004. Dap160/intersectin scaffolds the permissive zone to achieve high-fidelity endocytosis and normal synaptic growth. *Neuron*. 43:207–219. doi:10.1016/j.neuron.2004.07.001

Nakai, A., O. Yamaguchi, T. Takeda, Y. Higuchi, S. Hikosu, M. Taniike, S. Omiya, I. Mizote, Y. Matsumura, M. Asahi, et al. 2007. The role of autophagy in cardiomycocytes in the basal state and in response to hemo-dynamic stress. *Nat. Med.* 13:619–624. doi:10.1038/nm1574

O’Connor-Giles, K.M., L.L. Ho, and B. Ganetzky. 2008. Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth. *Neuron*. 58:507–518. doi:10.1016/j.neuron.2008.03.007

Pack-Chung, E., P.T. Kurshan, D.K. Dickman, and T.L. Schwarz. 2007. A *Drosophila* kinesin required for synaptic bouton formation and synaptic vesicle transport. *Nat. Neurosci.* 10:980–989. doi:10.1038/nn1936

Packard, M., E.S. Koo, M. Gorczyca, J. Sharpe, S. Cumberledge, and V. Budnik. 2002. The *Drosophila* Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell*. 111:319–330. doi:10.1016/S0092-8674(02)01047-4

Rodal, A.A., R.N. Motola-Barnes, and J.T. Littleton. 2008. Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. *J. Neurosci*. 28:8316–8325. doi:10.1523/JNEUROSCI.2304-08.2008

Rowland, A.M., J.E. Richmond, J.G. Olsen, D.H. Hall, and B.A. Bamber. 2006. Presynaptic terminals independently regulate synaptic clustering and autophagy of GABA receptors in *Caenorhabditis elegans*. *J. Neurosci*. 26:1711–1720. doi:10.1523/JNEUROSCI.2279-05.2006

Rubinsztein, D.C., J.E. Gestwicki, L.O. Murphy, and D.J. Klionsky. 2007. Potential therapeutic applications of autophagy. *Nat. Rev. Drug Discov.* 6:304–312. doi:10.1038/nrd2272

Schuster, C.M., G.W. Davis, R.D. Fetter, and C.S. Goodman. 1996. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron*. 17:641–654. doi:10.1016/S0896-6273(00)80197-X

Scott, R.C., O. Schuldiner, and T.P. Neufeld. 2004. Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. *Dev. Cell*. 7:167–178. doi:10.1016/j.devcel.2004.07.009

Scott, R.C., G. Hubáš, and T.P. Neufeld. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr. Biol*. 17:1–11. doi:10.1016/j.cub.2006.10.053

Spradling, A.C., D. Stern, A. Beaton, E.J. Rhem, T. Laverty, N. Mozden, S. Misra, and G.M. Rubin. 1999. The Berkeley *Drosophila* Genome Project