A dual function of V0-ATPase α1 provides an endolysosomal degradation mechanism in Drosophila melanogaster photoreceptors

W. Ryan Williamson,1 Dong Wang,1 Adam S. Haberman,1 and P. Robin Hiesinger1,2

1Department of Physiology and 2Green Center for Systems Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390

The vesicular adenosine triphosphatase (v-ATPase) is a proton pump that acidifies intracellular compartments. In addition, mutations in components of the membrane-bound v-ATPase V0 sector cause acidification-independent defects in yeast, worm, fly, zebrafish, and mouse. In this study, we present a dual function for the neuron-specific V0 subunit α1 orthologue v100 in Drosophila melanogaster. A v100 mutant that selectively disrupts proton translocation rescues a previously characterized synaptic vesicle fusion defect and vesicle fusion with early endosomes. Correspondingly, V100 selectively interacts with syntaxins on the respective target membranes, and neither synaptic vesicles nor early endosomes require v100 for their acidification. In contrast, V100 is required for acidification once endosomes mature into degradative compartments. As a consequence of the complete loss of this neuronal degradation mechanism, photoreceptors undergo slow neurodegeneration, whereas selective rescue of the acidification-independent function accelerates cell death by increasing accumulations in degradation-incompetent compartments. We propose that V100 exerts a temporally integrated dual function that increases neuronal degradative capacity.

Introduction

Neurons have highly specialized demands on intracellular trafficking during development and function (Sann et al., 2009). Although disruption of endosomal sorting typically leads to early developmental defects, aberrant late endosomal, lysosomal, or autophagic functions have been implicated in neurodegeneration (Mizushima et al., 2008; Nixon et al., 2008; Tooze and Schiavo, 2008). Many disease-causing mutations directly affect degradation pathways, and a common hallmark of neurodegenerative disorders are accumulations of undegraded proteins. Disruption of the autophagy pathway in postdevelopmental neurons in mice leads to neurodegeneration within weeks (Hara et al., 2006; Komatsu et al., 2006). Despite the apparent susceptibility of neurons in particular, the intracellular degradation machinery is thought to be shared with other cell types, and there is little evidence for a dedicated neuronal degradation mechanism.

Most intracellular compartments require acidification to function, and all require targeted membrane fusion to obtain and deliver intracellular cargo. The vesicular ATPase (v-ATPase) is a multisubunit complex that consists of the membrane-bound V0 sector and a cytosolic V1 sector. V0 and V1 assembly is reversible. The V0V1 holoenzyme acidifies intracellular compartments and is required for membrane protein sorting and degradation (Nishi and Forgac, 2002; Marshansky and Futai, 2008). Loss of v-ATPase-dependent acidification leads to signaling defects in early development in Drosophila melanogaster (Yan et al., 2009) and Caenorhabditis elegans (Kolotuev et al., 2009). In addition, several studies in yeast, worm, fly, zebrafish, and mouse suggest acidification-independent roles for the V0 complex in secretion or membrane fusion. These roles include yeast vacuolar fusion (Peters et al., 2001) and synaptic vesicle exocytosis in Drosophila (Hiesinger et al., 2005), Hedgehog secretion in C. elegans (Liégeois et al., 2006), insulin secretion (Sun-Wada et al., 2006)
and osteoclast fusion (Lee et al., 2006) in mouse, and phagosome–lysosome fusion in zebrafish (Peri and Nüsslein-Volhard, 2008). The v-ATPase thus represents a molecular machine that may regulate intracellular trafficking by integrating the two basic cellular functions of acidification and membrane fusion (Nishi and Forgac, 2002; Wada et al., 2008). The reversible assembly of the V0 and V1 sectors provides an elegant regulatory mechanism for V0V1 holoenzyme activity. V0V1 disassembly may also regulate the availability of the V0 sector in the membrane for acidification-independent functions (Nishi and Forgac, 2002; Marshansky and Futai, 2008). It is unclear how both functions could be coordinated and integrated to regulate intracellular trafficking.

The Drosophila gene vha100-1 (v100) encodes subunit a1 of the V0 sector. Mutations in v100 were originally identified in a genetic screen for synaptic malfunction (Hiesinger et al., 2005). The genomes of Drosophila, C. elegans, mouse, and human contain four subunit a1–a4 homologues (Marshansky and Futai, 2008). Mutations in subunit a2 have been shown to cause autosomal recessive cutis laxa type II (wrinkly skin syndrome), and mutations in subunit a3 cause osteopetrosis (Kornak et al., 2000, 2008). Drosophila and C. elegans subunit a1 orthologues exhibit neuron-specific expression, and a mammalian subunit a1 was originally cloned from a vesicle preparation from bovine brain (Perin et al., 1991). Neuronal expression of v100 in null mutant embryos is sufficient to rescue embryonic lethality to adulthood in Drosophila (Hiesinger et al., 2005). In addition to this cell specificity, subunit a1–a4 homologues confer intracellular compartment specificity. For example, in yeast, there are two subunit “a” homologues, whereas all other 13 core subunits are encoded by a single gene. Of these two, Vph1p localizes to the vacuole, and Stv1p localizes the v-ATPase to the Golgi and endosomal compartments (Manolson et al., 1994; Kane, 2007). A Torpedo a1 subunit was shown to be specifically sorted to nerve terminals (Morel et al., 2003). How this compartment-specific targeting of the v-ATPase is mediated by subunit “a” homologues is unknown. An active role of subunit a1–a4 homologues in targeting of vesicle populations or their cargo has, to our knowledge, not been shown.

In this study, we report a dual function of subunit a1 in Drosophila that provides an integrated neuronal degradation mechanism. First, V100 directs vesicle fusion independently of acidification. Second, V100 is required for the acidification of a subset of neuronal lysosomes and autophagosomes but not synaptic vesicles and early endosomes. Collectively, these findings show how two completely different molecular functions can be temporally and functionally integrated to provide a cell biological mechanism of “sort and degrade.” We propose that V100’s dual function provides a neuronal degradation mechanism that functions only after neuronal differentiation in addition to essential endolysosomal function and bestows neurons an increased degradative capacity.

Results

A putative v100 acidification mutant that rescues neurotransmission

We previously reported synaptic transmission defects in neurons mutant for v100, the gene that encodes the v-ATPase V0 subunit a1 in Drosophila. Importantly, electrophysiological recordings revealed normal neurotransmitter content of synaptic vesicles in this mutant. Because neurotransmitter loading requires synaptic vesicle acidification, we concluded that V100 is not part of the proton pump that acidifies synaptic vesicles (Hiesinger et al., 2005). These findings not only suggest a second function for v100 but also raise the question of whether it has a v-ATPase–dependent acidification function elsewhere.

To genetically dissect the two putative functions of V100, we set out to generate a mutation that specifically disrupts the proton translocation function of V100. Such a mutation has been described previously for the yeast orthologue vph1; mutation of arginine 735 to alanine (R735A) in vph1 leads to the specific disruption of proton translocation without affecting assembly of the v-ATPase or its localization (Kawasaki-Nishi et al., 2001). This arginine lies in a 17–amino acid region that is 100% conserved in V100 homologues from yeast to humans and corresponds to R755 in V100 (Fig. 1 A). Neurotransmission in v100-null mutant photoreceptors can be rescued using the GAL4/UAS system (Brand and Perrimon, 1993) by exclusively expressing v100 presynaptically (Hiesinger et al., 2005). We generated and selected two independent UAS-v100R755A lines that exhibit indistinguishable expression levels of the synaptically localized protein compared with control UAS-v100WT (unpublished data). Remarkably, synaptic transmission, as indicated by the on transient of the electroretinogram (ERG), is significantly restored by v100R755A expression in v100 mutant photoreceptors (Fig. 1, B and C). Surprisingly, however, the rescue of the ERG on transient is accompanied by a significant decrease in ERG depolarization (Fig. 1 D). This reduction in response amplitude does not occur in v100-null mutants. Furthermore, the decrease in depolarization is more pronounced when v100R755A is expressed in mutant photoreceptors compared with its overexpression in wild-type (WT) neurons (Fig. 1, B and D). A reduction in response amplitude often indicates an unhealthy neuron. Indeed, even mildly elevated levels of v100R755A in mutant neurons leads to a reduced eye size and loss of photoreceptors (Fig. 1, E–I). In contrast, this phenotype is not observed when v100R735A is expressed at the same level in the presence of WT v100 (Fig. 1, compare G with I). Higher levels of expression (>24°C) lead to almost complete abolishment of the eye when v100R755A is expressed in v100-null mutant photoreceptors and a milder rough eye phenotype when v100R755A or WT v100 are expressed in WT photoreceptors (unpublished data). Thus, v100R755A has a strongly detrimental effect on photoreceptor viability only when WT v100 is absent. These observations raise two questions: first, how can expression of v100R755A rescue neurotransmitter release yet cause cell death when expressed at higher levels in mutant neurons? Second, why is v100R755A strongly detrimental to cell viability only in the absence of WT v100? v100R755A exhibits a genetically unusual behavior: it is not a dominant negative because it causes a phenotype that is very different from the null mutant and because it does not cause the same phenotype when expressed in a WT background. If V100R755A is selectively disrupted in proton translocation, a straightforward hypothesis would be that v100R755A rescues a second, acidification-independent function that is sufficient to restore neurotransmitter release but
A v100 mutant that rescues neurotransmission but causes cell death at high levels. [A] Alignment of 17 amino acids surrounding the arginine at position 755 (R755) shows 100% conservation across species. [B] Neurotransmitter release rescue experiments. Representative ERG traces from left to right show control (ctrl; yellow), v100-null mutant (gray with mosaic blue), v100R755A expression in WT photoreceptors (red), and v100R755A expression in v100 mutant photoreceptors (green). Note that rescue with v100R755A exhibits opposite phenotypes from the null mutant: rescue of the on transient (red circles) and a strong reduction of the response amplitude (depolarization). [C] Quantification of on transients for all four genotypes. [D] Quantification of depolarization for all four genotypes. [E] Eye pictures show dosage-dependent loss of photoreceptors upon v100R755A expression in the mutant (H and I) but not upon v100R755A expression in WT (G). Error bars indicate SEM. Asterisks denote statistical significance in pairwise comparisons with control (P < 0.001).

Figure 1.

V100 is required for the acidification of a subset of neuronal degradative compartments

First, we needed to know whether v100R755A is indeed acidification defective. No acidification role of V100 has so far been characterized (Hiesinger et al., 2005). Therefore, we performed live measurements of intracompartmental pH using the fluorescent dye Lysotracker in the null mutant, and control photoreceptors. Lysotracker is a membrane-permeable compound that can be added to the bath of a live Drosophila larval or pupal eye–brain culture and selectively labels strongly acidified compartments. To quantitatively assess even slight differences in Lysotracker labeling, we generated 50% fluorescently labeled mutant clones in developing photoreceptor neurons (mosaic analysis with a repressible cell marker [MARCM] technique; Lee and Luo, 1999). As a further control, we confirmed that WT MARCM clones cause no effect (unpublished data). As shown in Fig. 2 A, Lysotracker labeling of mutant and control cells is indistinguishable in L3 larval eye discs, i.e., at the time point when neuronal differentiation and v100 expression commence. Remarkably, however, in mutant cells at 20% of pupal development (P + 20%), the Lysotracker signal reduces slightly but significantly (unpublished data). At P + 40%, the Lysotracker signal is reduced to ∼50% (Fig. 2, B and D). A level of ∼50% Lysotracker labeling is maintained in 1-d adult photoreceptors (unpublished data). This phenotype is rescued by expression of WT v100 (Fig. 2 D). The change of Lysotracker fluorescence is reflected in a corresponding loss of Lysotracker-positive compartments in mutant compared with control clones from 100% in L3 to 67% at P + 20% and 47% at P + 40%. These observations indicate that developing photoreceptors exhibit a progressive reduction of strongly acidified compartments after neuronal differentiation.

Next, we tested whether expression of v100R755A under the same conditions that rescue neurotransmitter release also rescues this acidification defect. As shown in Fig. 2 (C and D), expression of v100R755A in v100 mutant photoreceptors does not rescue the Lysotracker defect observed in v100 mutant neurons. This result supports a direct acidification function of V100 for at least some Lysotracker-positive compartments in neurons.

Because Lysotracker labeling indicates normal levels of acidification before neuronal differentiation, we wondered whether these compartments are acidified by other v-ATPase subunits. We tested this idea pharmacologically with the v-ATPase-specific blocker bafilomycin 1A. Indeed, 10-min preincubation with bafilomycin A1 fully prevents any Lysotracker labeling in both mutant and control cells, indicating that these Lysotracker-positive compartments are acidified in a v-ATPase–dependent manner (Fig. 2, F–H). The finding that strongly acidified compartments persist in v100 mutant neurons shows that v100 cannot be required equally for the acidification of all compartments.

We assessed the molecular nature of Lysotracker-positive compartments in the live preparation by expressing YFP-tagged versions of the early endosomal maker Rab5, the late endosomal marker Rab7, and the recycling endosomal marker Rab11 (Zhang et al., 2007) as well as the autophagosomal marker Atg8-GFP (Chang and Neufeld, 2009). As shown in Fig. 2 E and Fig. S1 (A and B), Lysotracker rarely marks Rab5- or Rab11-positive compartments. In contrast, 32% of Lysotracker-positive compartments are Rab7-positive, ring-shaped late endosomes. In addition, 20% of Lysotracker-positive compartments are Atg8-GFP positive (Fig. 2 E; and Fig. S1, C and D). This ratio of Rab7- and

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Syx7/Avl acts together with Rab5 in vesicular fusion with early endosomes (Lu and Bilder, 2005). Indeed, the second most colocalizing marker of V100-positive compartments is the early endosomal marker Rab5 (Fig. 3 B; and Fig. S3, E and F). This colocalization pattern is substantiated in a complementary experiment using photoreceptor-specific expression of GFP-Rab5 (Wucherpfennig et al., 2003). GFP-Rab5 exhibits strong labeling along the developing rhabdomeric structure in the cell bodies (Fig. 3 E, green arrowhead) as well as in photoreceptor axon terminals (Fig. 3 E, blue arrow). V100 immunolabeling colocalizes with GFP-Rab5 at the axon terminals (Fig. 3 E, blue arrow) but only little in the cell bodies (Fig. 3 E). These data suggest that V100 is enriched together with synaptic endosomes. In contrast, colabeling with the lysosomal marker Sun/CD63 (Xu et al., 2004) reveals little V100 colocalization and a converse distribution pattern (increased levels in the cell bodies [Fig. 3 E, red arrowhead] and very low levels in developing synaptic regions [Fig. 3 E]). Similarly, expression of fluorescently tagged V100 in v100−null mutant neurons (that rescues its function) reveals partial colocalization with the endosomal markers Hrs and Rab11 (Lloyd et al., 2002; Pelissier et al., 2003) but less colocalization with the lysosomal marker Spin/Bnch (Fig. S3, G–I; Sweeney and Davis, 2002; Dermaut et al., 2005). Collectively, our WT compartment characterization yields the surprising finding that V100 most strongly colocalizes with early endosome markers in addition to synaptic vesicles and exhibits comparably less colocalization with lysosomal markers.

Atg8-positive strongly acidified compartments is unaltered in the mutant (Fig. 2 E; and Fig. S1, E and F). Thus, neither compartment type is lost in the mutant. Instead, these findings indicate a loss of a neuronal subset of these compartment types. Collectively, our data suggest that V100 is required for the acidification of a neuronal subset of late endosomal and autophagosomal compartments and that this acidification function is not rescued by v100R755A.

**V100 is enriched on early endosomes**

To comprehensively characterize V100-positive compartments in WT, we used a panel of 16 markers for colocalization analyses in developing and adult photoreceptors. We performed these experiments using high resolution 3D confocal microscopy that allows the visualization of distinct subcellular compartments at the resolution limit of light (Hiesinger et al., 2001). To quantify compartment colocalization, we identified clearly discernible intracellular V100-positive compartments and counted how many of these were positive for a given other intracelluar compartment marker as shown in Fig. S2 (A–D) for the synaptic vesicle marker cystein string protein (CSP) and the late endosomal marker Rab7. As shown in Fig. 3 (A and B), WT V100 colocalizes to a varying degree with several markers of membrane compartments, including endosomes, lysosomes, and synaptic vesicles (Fig. 3 B, marked endo, lyso, and syn.ves.). Most strikingly, only immunolabeling of the early endosomal marker syntaxin7 (Syx7)/Avalanche (Avl) exhibits a localization pattern that is almost identical to V100 (Fig. 3, C and D).
Loss of v100 causes endosomal and autophagosomal accumulations

To understand the trafficking defects in v100 mutant photoreceptors, we examined changes of the 16 intracellular compartment markers presented in the previous section. We again engineered MARCM flies in which 50% of all photoreceptors are rendered mutant and positively labeled with a fluorescent marker, whereas the other 50% serve as control in the same brain. As shown in Fig. 4 (A and B), photoreceptor terminals of 1-d-old adults exhibit up-regulation specifically of endosomal and lysosomal compartment markers. The most prominently up-regulated markers include the early endosomal Syx7/Avl, the early and late endosomal rab GTPases Rab5 and Rab7, and the lysosomal marker Sun/CD63. In contrast, markers for the synaptic target membrane Syx1A, the Golgi/lysosomal Syx16, and several other membrane proteins remain largely unaltered. Thus, we find up-regulation of specific markers along the endolysosomal pathway that suggest accumulations of both early and late endosomal compartments. Correspondingly, Western blot analysis of total protein extract from 2-d-old v100 mutant eye–lamina complexes shows a clear increase of the endosomal proteins Syx7/Avl and V100 (Fig. S3, A–C). These data support the idea of a protein degradation defect.

We have previously performed transmission electron microscopy of 1-d adult v100 mutant photoreceptors. In our previous study, we found a significant increase of vesicular content
We did not observe any multilamellar structures that represent failed degradation in aberrant lysosomal structures, as are apparent for example in micrographs of photoreceptor terminals of the lysosomal storage mutant spinster/bnch (Dermaut et al., 2005). Instead, accumulations of both MVBs and AVs are indicative of compartments before lysosomal degradation.

Lastly, we performed a series of experiments using fluorescent reporters of the different trafficking and degradation compartments. As a marker for MVBs, we used YFP-Rab7, for AVs, but no alterations of active zones or mitochondria (Hiesinger et al., 2005). To identify ultrastructural correlates of the endolysosomal accumulations, we analyzed the transmission electron microscopy data for morphologically abnormal membranous compartments. As shown in Fig. 4 (C–E), quantitative analysis reveals a significant increase of highly heterogeneous enlarged structures, many of which appear morphologically as multivesicular bodies (MVBs; Fig. 4 D, arrowhead) or double-membrane autophagosomal vacuoles (AVs; Fig. 4, D [arrow] and E [quantification]).

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LC3/dATG8-GFP, and for early endosomes, YFP-Rab5 and 2xFYVE-GFP. Expression of any of these four markers results in large accumulations in v100 mutant 1-d adult photoreceptors (Fig. 4, F–M). Immunolabeling for Syx7 reveals substantial colocalization with endosomal Rab5, Rab7, and 2xFYVE accumulations (Fig. 4, F–I; and not depicted). In contrast, Atg8-positive autophagosomal compartments are mostly nonoverlapping with the Syx7-positive endosomal accumulations (Fig. 4, J and K). Similarly, both the early endosomal markers GFP-2xFYVE (Wucherpfennig et al., 2003) and the lysosomal marker Sun/CD63 (Xu et al., 2004) accumulate but do so in mutually exclusive compartments (Fig. 4, L and M). These data suggest that early endosomal markers, including Syx7, do not accumulate in a single type of Atg8-positive AV or Sun/CD63-positive lysosome. Instead, accumulations occur at the level of endosomes in addition to lysosomal and autophagosomal degradative compartments.

**V100 exerts an acidification-independent function on early endosomes**

The accumulation of late endosomes and autophagosomes is consistent with the acidification defects of late endosomal and autophagosomal compartments shown in Fig. 2. But why is V100 predominantly localized on early endosomes? Early endosomal compartments can mature into late endosomal compartments (Rink et al., 2005). V100 may thus exert an acidification-independent function on early endosomes that later mature into degradative compartments and require increased acidification. To assay how loss of v100 affects the pH of endosomal compartments, we expressed the membrane-tagged fluorescent probe synapto-pHluorin (hereafter referred to as pHluorin) in photoreceptors. pHluorin is a genetically encoded pH-sensitive GFP variant that can be used to measure the pH of synaptic vesicles and other intracellular compartments (Ng et al., 2002). Synaptic vesicles and endocytic vesicles can fuse with endosomal compartments, and pHluorin was previously shown to localize to endosomes (Machen et al., 2003).

At WT photoreceptor synapses, pHluorin labeling is very similar to the synaptic vesicle marker CSP (Fig. 5 A). In addition, pHluorin marks regions enriched for the early endosomal marker Rab5 (Fig. S3 D) but colocalizes very little with the late endosomal marker Rab7 and the recycling endosomal marker Rab11 (unpublished data). Remarkably, in v100 mutant terminals, the colocalization of pHluorin with the synaptic vesicle marker CSP is mostly lost (Fig. 5 B). Instead, accumulations of pHluorin partially colocalize with the early endosomal markers Syx7/Avl (Fig. 5 C) and Rab5, the late endosomal marker Rab7, and the recycling endosomal marker Rab11 (Fig. S3, E–G). Photoreceptor-specific expression of WT v100 fully rescues this phenotype (Fig. S3, H–K). These data indicate that pHluorin accumulates in v100 mutant terminals in compartments along the endosomal pathway, including Syx7/Avl-positive endosomes.

To measure the acidification of these compartments, we again used the v-ATPase–specific inhibitor bafilomycin 1 A. pHluorin has reduced fluorescence when exposed to acidic pH, and loss of acidification results in a fluorescence increase. In our photoreceptor preparation, the pharmacological block of all v-ATPase function leads to a 1.37-fold pHluorin fluorescence increase in the WT and a statistically not significantly different 1.42-fold increase in the mutant (Fig. 5, H–N). This finding indicates that pHluorin-positive compartments are acidified in v100 mutant neurons. We further confirmed that pHluorin accumulates in acidified compartments in cell bodies and at synapses using a calibrated alkalinization/acidification protocol based on NH4Cl washes as described in Materials and methods and shown in Fig. S4 (A–D). Collectively, with the WT localization data, these findings suggest that V100 is an endosomal protein that is not required for endosomal acidification.

Next, we tested how v100R755A expression affects the pHluorin localization and measurements in v100 mutant neurons. Surprisingly, fluorescence levels reveal a dramatic further increase of pHluorin accumulations in v100 mutant photoreceptors expressing v100R755A compared with the mutant alone (Fig. 5, D–F; and Fig. S4 E). This finding follows the previously observed pattern of phenotypes worsened by v100R755A expression, namely ERG amplitude and cell death (Fig. 1). Because v100R755A expression causes massive photoreceptor cell loss only when expressed in null mutant photoreceptors (Fig. 1, G–I), we wondered whether the same is true for pHluorin accumulations. Fig. 5 G shows a mosaic of synaptic terminals of 1-d-old photoreceptors in which WT terminals are marked with RFP (Fig. 5 G, magenta), whereas the v100-null mutant terminals are unmarked. In addition, we co-expressed both v100R755A and pHluorin at identical levels in all photoreceptors. Remarkably, only v100R755A expression in v100 mutant photoreceptors, but not in a WT control clone, causes dramatic pHluorin accumulations at the synaptic terminals (Fig. 5 G). This finding shows that the pHluorin accumulation phenotype indeed follows the same pattern as the reduction in ERG depolarization and eye size; in all cases, v100R755A causes more severe phenotypes in the absence of WT v100.

Finally, we performed acidification measurements using the aforementioned bafilomycin protocol. As shown in Fig. 5 (H–N), pharmacological block of all v-ATPase function in photoreceptors with v100R755A expression in a mutant background causes a 1.79-fold increase in fluorescence. This finding indicates that the increased accumulations occur in compartments that are at least as acidified as in WT and the null mutant (Fig. 5 N). Indeed, the 1.79-fold increase is significantly higher than in the null mutant, which is likely a result of the dramatic increase in the amount of pHluorin. The accumulation of pHluorin in acidified compartments was again confirmed using calibrated NH4Cl washes (Fig. S4 E). Our findings support the notion that selective rescue of an acidification-independent function causes protein accumulations only when no WT protein provides acidification.

**V100 interacts with the early endosomal Syx7/Avl but not the Golgi/lysosomal Syx16**

Our findings raise the question about the molecular mechanism of the acidification-independent function. An important hint comes from the observation that V100 is an endosomal protein that is not required for endosomal acidification. We were intrigued by the Syx7 colocalization with V100 in WT as well as the Syx7 accumulation in the v100 mutant because previous studies suggested acidification-independent functions of V100 and its homologues in
Syx16, we performed coimmunoprecipitations (co-IPs) using the V100 antibody from fly heads. As shown in Fig. 6 A, some Syx7/Avl, but not Syx16, coimmunoprecipitates with V100. These results indicate that V100 exists in a complex with Syx7/Avl but not Syx16 in the brain. Conversely, V100 is coimmunoprecipitated with Syx7/Avl from brain extracts (Fig. 6 B). The amount of coimmunoprecipitated Syx7 was low compared with Syx1A (Fig. 6 A). To test the strength and specificity of these interactions, we performed a series of pull-down assays. First, we performed pull-downs from fly head extracts with His-tagged V100N (the N-terminal half of V100 that is unaffected by the R755A mutations) or His-tagged Syx7 and probed for Syx7 and V100.

**Figure 5.** V100 exerts an acidification-independent function on early endosomes. ([A–C]) High resolution section of WT (A) and v100 mutant (B and C) 1-d adult photoreceptor terminals expressing pHluorin (green) and immunolabeled for CSP or Syx7/Avl (magenta). Note that pHluorin forms accumulations that exclude CSP (arrowheads) but partially colocalize with Syx7 (arrows) in the mutant. ([D–F]) Levels of pHluorin accumulation in live adult photoreceptor terminals at equal pH for the genotypes indicated. (G and G’) Negatively marked v100 mutant clone (magenta, WT terminals). Both mutant and control (ctrl) clones equally overexpress pHluorin and v100R755A. Note that v100R755A expression only in the mutant terminals leads to pHluorin accumulations. The dotted line approximates the clonal boundary. ([H–M]) Live confocal scans of P + 20% eye discs before and after 10-min incubation with the v-ATPase inhibitor bafilomycin 1A. ([H and I]) Control before and after bafilomycin treatment. ([J and K]) v100 mutant before and after bafilomycin. ([L and M]) v100 mutant with v100R755A rescue. (N) Quantification of fluorescence increases after bafilomycin treatment shows that pHluorin accumulates in compartments that are acidified in a v-ATPase–dependent manner. Error bars indicate SEM. Bars, 10 µm.
depolarization becomes apparent after 3 wk (Fig. 7, E and F) and progressively worsens by 5 wk (Fig. 7, I and J). All flies were raised in a 12-h light/12-h dark cycle. We also dark reared a set of control and v100 mutant flies to investigate whether this reduction of photoreceptor responses is stimulus dependent. Dark-reared control flies exhibit a significantly increased depolarization (Fig. 7 M). The dark-reared mutants do not show this increase, but a depolarization that is not significantly different from 4–5-wk-old control flies raised in a normal light/dark cycle (Fig. 7 N). Fig. 7 Q summarizes these results quantitatively. Next, we analyzed the morphology of these eyes by immunolabeling with a monoclonal antibody against the transmembrane protein chaoptin that labels the rhabdomeres, i.e., the light-sensitive membrane organelles. In contrast to control flies, v100 mutant photoreceptor cell bodies exhibit accumulations of chaoptin-positive structures while losing most discernible rhabdomeric structure by 5 wk (Fig. 7, K and L). Again, these phenotypes are progressive (compare with Fig. 7, C–L). Traditional plastic sections of these eyes further reveal the extent of the degenerative phenotypes with little discernible structures after 5 wk (Fig. S4, F and G). In 5-wk-old dark-reared mutants, aberrant chaoptin-positive accumulations are also apparent, but the rhabdomeric and overall structure are significantly less disrupted than in mutants after a 5-wk light/dark cycle (Fig. 7 N). Fig. 7 Q summarizes these results quantitatively. Next, we analyzed the morphology of these eyes by immunolabeling with a monoclonal antibody against the transmembrane protein chaoptin that labels the rhabdomeres, i.e., the light-sensitive membrane organelles. In contrast to control flies, v100 mutant photoreceptor cell bodies exhibit accumulations of chaoptin-positive structures while losing most discernible rhabdomeric structure by 5 wk (Fig. 7, K and L). Again, these phenotypes are progressive (compare with Fig. 7, C–L). Traditional plastic sections of these eyes further reveal the extent of the degenerative phenotypes with little discernible structures after 5 wk (Fig. S4, F and G). In 5-wk-old dark-reared mutants, aberrant chaoptin-positive accumulations are also apparent, but the rhabdomeric and overall structure are significantly less disrupted than in mutants after a 5-wk light/dark cycle (Fig. 7, O and P). Together, these data show a degenerative phenotype in v100 mutant neurons that coincides with transmembrane protein accumulation, progresses slowly, and can be attenuated by absence of stimulation.

Selective rescue of the acidification-independent function accelerates degeneration by increasing accumulations in Syx7-positive, degradation-incompetent compartments

Our combined data support a model in which the acidification-defective V100R755A rescues vesicle fusion with the presynaptic...
Finally, our model predicts a dominant function of V100R755A that is different from the loss of function phenotype but consistent with the ERG defects shown in Fig. 1 (B–D). Therefore, we analyzed the effect of v100R755A overexpression on photoreceptor morphology and degeneration in 2-wk-old flies. As shown in the previous section, loss of v100 causes slow neurodegeneration that is accompanied by accumulations of chaoptin (Fig. 7 and Fig. S5 A). This phenotype is rescued by v100 expression in mutant photoreceptors (Fig. S5 B). Overexpression of WT v100 causes a slight reduction in depolarization. Therefore, we analyzed the effect of v100R755A overexpression on photoreceptor morphology and degeneration in 2-wk-old flies. As shown in Fig. 8 (B–D), in 1-d adult synaptic terminals of the null mutant, chaoptin exhibits small accumulations that are partially associated with Syx7 accumulations but mostly Syx7 negative. In contrast, v100R755A rescue of the null mutant at the same time point exhibits a dramatic increase of chaoptin accumulations in large vesicles that are invariably enclosed by large Syx7-positive compartments (Fig. 8 D, arrows; and Fig. S5, F–I, schematic). This observation supports the idea that V100R755A promotes the fusion of cargo vesicles with Syx7-positive endosomes.

Figure 7. Loss of v100 causes slow, adult-onset neurodegeneration. (A and B) ERG recordings of 1-wk-old flies. Note that mutant ERGs lack the on transient (arrows) but display normal depolarization. (C and D) Eyes from 1-wk-old WT and v100 mutant flies labeled with the photoreceptor-specific rhabdomere marker chaoptin (green) and the nuclear marker Toto-3 (blue). (E and F) ERG recordings from 2–3-wk-old flies show a slight reduction in depolarization. (G and H) 2–3-wk-old WT and v100 mutant eyes. (I–L) ERGs and immunolabeling of 5-wk-old WT and mutant eyes reveal progressive degeneration. (M–P) ERG recordings and immunolabelings from 5-wk-old flies kept in the dark show attenuated mutant phenotypes. (Q) Quantification for ERG depolarizations. Ctrl, control. Error bars indicate SEM. Bar, 10 µm.

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ERG Depolarization

1 week 3 weeks light 5 weeks dark

Ctrl v100 Ctrl v100 Ctrl v100 Ctrl v100

p<0.001 p<0.001

Finally, our model predicts a dominant function of V100R755A that is different from the loss of function phenotype but consistent with the ERG defects shown in Fig. 1 (B–D). Therefore, we analyzed the effect of v100R755A overexpression on photoreceptor morphology and degeneration in 2-wk-old flies. As shown in the previous section, loss of v100 causes slow neurodegeneration that is accompanied by accumulations of chaoptin (Fig. 7 and Fig. S5 A). This phenotype is rescued by v100 expression in mutant photoreceptors (Fig. S5 B). Overexpression of WT v100 causes a slight reduction in depolarization. As shown in Fig. 8 (B–D), in 1-d adult synaptic terminals of the null mutant, chaoptin exhibits small accumulations that are partially associated with Syx7 accumulations but mostly Syx7 negative. In contrast, v100R755A rescue of the null mutant at the same time point exhibits a dramatic increase of chaoptin accumulations in large vesicles that are invariably enclosed by large Syx7-positive compartments (Fig. 8 D, arrows; and Fig. S5, F–I, schematic). This observation supports the idea that V100R755A promotes the fusion of cargo vesicles with Syx7-positive endosomes.
The genetic dissection of two molecularly distinct functions of v100 suggests one role of V100 in endosomal vesicle fusion, and a second role in acidification downstream of endosomal sorting. Our data support an acidification-independent function in promoting vesicle fusion with target membranes marked by specific syntaxins that explains both V100’s role in endosomes presented in this study as well as in synaptic vesicle exocytosis reported previously (Fig. 8 A; Hiesinger et al., 2005). Importantly, v100 is only required in neurons after neuronal differentiation; general cellular functions that require essential endolysosomal trafficking are unaffected. We conclude that V100 provides a neuronal degradation mechanism whose loss leads to neurodegeneration.

**Discussion**

In this study, we characterize an integrated dual function of V100, the neuron-specific V0-ATPase subunit a1 in *Drosophila*. The mutant phenotype (compare with Fig. S5, G and H). Finally, expression of v100R755A in null mutant photoreceptors causes dramatically “blown-up” accumulations that appear like exacerbated versions of those in the null mutant (Fig. S5, E and I). These data suggest that v100R755A in WT photoreceptors causes mild intracellular accumulations and no degeneration, whereas v100R755A in mutant photoreceptors greatly exacerbates the null mutant phenotype.

**Figure 8.** Selective rescue of the acidification-independent function accelerates degeneration by increasing accumulations in Syx7-positive, degradation-incompetent compartments. (A) Summary of V100 functions and v100R755A rescue data. Our findings indicate two acidification-independent functions that are rescued by v100R755A, synaptic vesicle (SV) fusion through Syx1A interaction and early endosomal (EE) fusion through Syx7 interaction. In contrast, V100 has a function as part of the V0V1 holoenzyme once endosomes mature into degradative compartments that are not rescued by v100R755A. Consequently, selective rescue of the acidification-independent function leads to increased accumulations in Syx7-positive, degradation-incompetent compartments. (B, C, and D) 1-d adult photoreceptor terminals for control (B), v100-null mutant (C), and v100R755A rescue in the v100 mutant (D). In the v100 mutant (D), Chaoptin alone (green channel) is shown. (B”, C”, and D”) Syx7 alone (magenta channel) is shown. In the v100R755A rescue, most chaoptin accumulates in large, Syx7-encircled MVB-like structures [arrows]. RE, recycling endosome; RV, recycling vesicle. Bar, 5 µm.
Genetic dissection of the dual function
We propose the following model based on V100’s dual function (Fig. 8 A and Fig. S5). In the absence of V100, vesicles with heterogeneous cargo, either endocytosed or ER/Golgi derived, fail to fuse with Syx7-positive endosomes, resulting in intracellular accumulations (Fig. S5, F and G). Over time, many of these accumulations are engulfed by AVs. V100-dependent acidification is required, and possibly acts as a trigger, for the subsequent maturation and degradation of MVBs and AVs. Expression of v100F255A does not rescue acidification-dependent degradation in v100-null mutant neurons but promotes fusion of cargo vesicles with endosomes that thereby contain acidification-defective v-ATPases. In a WT background, the presence of WT V100 provides some acidification for the degradation of cargo (Fig. S5 H), whereas in a mutant background, this rerouting of cargo leads to dramatically increased MVB and AV formation (Fig. S5 I).

V0 and V1 sector assembly is thought to be a regulatory mechanism that initiates acidification (Nishi and Forgac, 2002; Marshansky and Futai, 2008). The reversible assembly of the V0/V1 holoenzyme provides an elegant mechanism for the integration of V100’s dual function. V100 is the largest component of the V0 membrane complex, and its N terminus is required for neurotransmitter release. In fact, neurotransmitter degradation or increasing the fusion probability of vesicles marked with pHluorin or chaoptin) accumulates. The observation that selective rescue of an acidification-independent sorting function causes more accumulation than the null mutant alone implies that degradation in v100 mutant neurons is only partly abolished. In other words, loss of v100 does not affect at least one other v-ATPase (i.e., bafilomycin sensitive and a2–a4 dependent) degradation pathway. The presence of at least one other degradation pathway is further supported by the presence of a normal number of strongly acidified degradative (Lysotracker positive) compartments in the mutant cells before neuronal differentiation. Together with the WT expression data, our results indicate that v100 only comes into play after a cell has adopted the neuronal cell fate. Finally, the idea that essential endolysosomal function is unaffected in v100 mutant neurons is also supported by the observation that v100 mutant neurons lack several hallmarks of general lysosomal degradation mutants, including accumulations of autofluorescent lipofuscin or ceroid (Futerman and van Meer, 2004). Together, these data suggest that V100 is not required for all endolysosomal degradation in neurons. The v100-dependent mechanism need not be different from the essential endolysosomal pathway in any component other than v100 itself; V100 promotes fusion with endosomes, which it later acidifies for degradation. Collectively, these data suggest that sorting and degradation are tightly balanced.

v100 is required only in neurons in Drosophila and is at least strongly enriched in the nervous system of all animals investigated for it. However, the temporally integrated mechanism of sort and degrade that we describe in this study could be more general. All a1–a4 homologues are highly conserved, and acidification-independent roles in secretion have been suggested for a2 in C. elegans (Liégeois et al., 2006) and a3 in mouse (Sun-Wada et al., 2006). The zebrafish a1 orthologue has an acidification-independent function in phagosome–lysosome fusion in microglial cells (Peri and Nüsslein-Volhard, 2008). Thus, it is possible that the mechanism we describe in this study for neurons may be used by other cell types.

Neuronal endolysosomal trafficking, autophagy, and neurodegeneration
Loss of v100 leads to heterogeneous accumulations of endosomal compartments, MVBs, and AVs. Autophagy intersects at many levels with the classical endolysosomal pathway: fusion of AVs with lysosomes, MVBs, and early endosomes are known to form amphisolam compartments and have been shown to be required for autophagic function (Eskelinen, 2005; Filimonenko et al., 2007; Razi et al., 2009). Numerous mutants in endolysosomal and autophagic pathways lead to late onset neurodegeneration (Futerman and van Meer, 2004; Hara et al., 2006; Komatsu et al., 2006). Conversely, induction of autophagy can alleviate neurodegenerative effects (Mizushima et al., 2008; Wang et al., 2009). Similarly, our findings are consistent with the idea that loss of degradation capacity sensitizes neurons to cargo overload. In support of this idea, overexpression of human disease proteins (including Aβ and τ) exhibit significantly accelerated neurodegeneration in v100 mutant photoreceptors (unpublished data). The recognition of a neuronal mechanism that operates in addition to essential degradation mechanisms may be an important parameter to consider in neuronal diseases associated with undegraded protein accumulations.

A neuronal “sort and degrade” mechanism
We show that v100 is not required for the acidification of some of the compartments in which cargo (e.g., pHluorin or chaoptin) accumulates. The observation that selective rescue of an acidification-independent sorting function causes more
Materials and methods

Genetics, molecular biology, and generation of transgenic flies

v100-null mutant and overexpression lines have been described previously (Hiesinger et al., 2005). Allele v100\(^{-}\) was the mutant allele used in all experiments. v100 was subjected to site-directed mutagenesis using full-length v100 cDNA in a pOT2 vector (BDGP clone LD121248) and the primer 5′-CACCCCTTCTTATCTGGCATTGTGGGCGCTTTCCT3′ and its reverse complement. Introduction of the mutation R755A was verified by sequencing. The resulting v100\(_{755A}\) cDNA was cloned into pUAST using EcoRI and XhoI restriction cutting sites as reported previously (Hiesinger et al., 2005). The UAS-Dendra2-v100 construct was generated by cloning the PCR-amplified Dendra2 open reading frame into the ePENTR vector and generating the fusion using the Gateway system. DNA injection for the generation of transgenic flies was performed by Rainbow Transgenics, Inc.

At least two independent transgene insertions per chromosome were isolated. Two independent insertions for both v100\(_{755A}\) and Dendra2-v100 on the second chromosome were tested and selected for comparable expression levels with the UAS-v100 WT insertion when expressed in photoreceptors. The two transgenic lines chosen for each construct behaved identically in all assays.

Immunohistochemistry, microscopy, and image processing

Adult brains, eyes, and eye–lamina complexes as well as pupal brains and eye–brain complexes were dissected as reported previously [Williamson and Hiesinger, 2010]. The tissues were fixed in PBS with 3.5% formaldehyde for 15 min and washed in PBS with 0.4% Triton X-100. High resolution light microscopy was performed using a resonance-scanning confocal microscope (Leica). Imaging data were processed and quantified using Amira 5.2 (Indeck) and Photoshop (CS4; Adobe). Fluorescence data and ERG data were quantified using Prism (version 4; GraphPad Software, Inc.). Eye pictures were captured on a stereoscope (MIX16F; Leica) using In-Focus (version 1.60; Meyer Instruments) and were processed for extended depth of field using ImagePro Plus (version 6.0; Media Cybernetics). The following antibodies were used at 1:1,000 dilution: Syx7/Avl, Rab5, Rab7, Rab11, Hrs, Syx16, Hook, Vps16, Car, Dor, Sun, Spin, and Syt. Anti-chiapthin, anti-CSP, and anti-Syx were used at 1:50, and anti-Sec15 and anti-V100 were used at 1:2,000.

ERGs

ERGs were performed as described previously (Fabian-Fine et al., 2003) with the following modifications: flies were fixed using non-toxic Glue-All (Elmer’s). We used 2 M NaCl in the recording and reference electrodes. Electrode voltage was amplified by a Digidata 1440A (MDS Analytical Technologies), filtered through an electrometer (IE-210; Warner Instruments), and recorded using Clampex (version 10.1; Axon Instruments). A postrecording filter was also provided by the Clampex software. Light stimulus was provided in 1-s pulses by a computer-controlled white light-emitting diode system (MC1500; Schott).

Protein interaction

Total proteins were extracted from adult fly heads in IP buffer containing 20 mM Tris, 150 mM NaCl, 1 mM PMSF, and 1x complete protease inhibitors (Roche), pH 7.4. The fly head extract was mixed well in 1% Triton X-100 (Bio-Rad Laboratories) and incubated for 1 h at 4°C. Samples were centrifuged at 16,000 g for 15 min at 4°C to remove cell debris. The resulting supernatant was incubated with 20 µl anti-V100 antibody (Hiesinger et al., 2005) coupled to protein A/G beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. After removing the supernatant, the beads were washed four times with IP buffer. The immunoprecipitates were eluted by boiling the beads in 50 µl SDS sample buffer and analyzed by Western blotting. A non-specific preimmune serum was used as control. Pull-down from adult fly head protein extract was performed using His-V100-N terminus or His-Syx7 as baits that were immobilized on Ni-charged beads (EMD). Bound proteins were eluted with SDS sample buffer and analyzed by Western blotting. Pull-down of bacterially produced proteins was performed using immobilized GST-V100-N terminus fusion protein as bait, which was then incubated with His-Syx7 for 1 h at 4°C in binding buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, and 0.2% Triton X-100, pH 7.4). After washing with binding buffer four times, the bound proteins were eluted with SDS sample buffer and analyzed by Western blotting.

Western blots of mutant eye–lamina complexes

For Western blots of adult eye–lamina complexes, 2-day-old eyFLP; FRT82B v100 and eyFLP; FRT82B WT eyes were dissected in HL3 as described previously (Williamson and Hiesinger, 2010). Tissues were washed twice in ice-cold lysis buffer (as used for IP experiments) and crushed with a pestle in lysis buffer. The solution was incubated on ice for 20 min and centrifuged at 4°C to remove solid material. 10 eye–lamina complexes per lane were run on a 12% SDS-PAGE gel.

Live imaging

Brains were dissected from either pharate adults or P + 20% pupae in HL3 and were immobilized on a coverslip coated with syldarg using glue stick as described previously (Williamson and Hiesinger, 2010). The tissue was mounted in an orientation that allows imaging of the desired region. The coverslip was placed in a perfusion chamber (RC-30; Harvard) with slow perfusion of HL3 solution. Images were captured using resonance-scanning confocal microscopy. For each scan from individual experiments, the same x/y region was captured.

*pHluorin.* We generated a calibration curve by first alkalizing intracellular compartments using NH\(_4\)Cl, pH 7, and subsequently progressively acidifying intracellular compartments using an acidification protocol modified from Boron and De Weer (1976) by reducing the amount of free extracellular NH\(_3\) using NH\(_4\)Cl washes, pH 6.5, 6.0, and 5.5. Using this method, the calibrated initial live measurements report intracompartamental pH, independent of the total amount of pHluorin. The raw data were processed in Amira (version 5.2; Visage Imaging GmbH) by first generating a maximum projection to eliminate the problem that differences in fluorescence vary among scans from a single experiment simply as a result of a slight shifting of the prep in the z direction resulting from perfusion of fluids over living tissue. Total fluorescence for each scan within a single experiment was calculated, and the scans from NH\(_4\)Cl (pH controlled) scans were plotted in Prism on a Kaplan–Meier survival curve and analyzed by the four-parameter logistic equation. Next, the live fluorescence measurement was mathematically fitted onto the curve to reveal the mean live pH of the environment to which pHluorin was exposed. This was performed for several animals, and the results were compiled to generate bar graphs representing the mean pH and error from the specified genotypes, tissues, and ages.

Lysotracker. For Lysotracker (Invitrogen) experiments, brains were removed from the animal and immobilized on a Sylgard-coated microscope slide using glue stick. Lysotracker red was added to HL3 at 50 nM. 200 µl of this solution was placed onto the prepared tissue, and an image was acquired within 5 min, as recommended by the manufacturer to prevent alkalizing effects.

Quantification of compartment marker analyses

Up-/down-regulation in mutant versus WT photoreceptor terminals. 50% mutant photoreceptor mosaics were created with all mutant photoreceptors expressing pHluorin just to mark the mutant cells. 3D confocal stacks of three to five specimens were quantified for the ratio of total fluorescence in 3 µm\(^3\) mutant terminals divided by total fluorescence in 3 µm\(^3\) WT terminals for each of the 20 markers individually.

WT colocalization. Anti-V100 immunolabeling was analyzed for at least three specimens per colocalization experiment. Colabelings with guinea pig antibodies (anti-Hrs, -Dor, and -Sec15) were generated using GMR-GAL4–driven fluorescently tagged and functionally rescuing V100 in v100 mutant neurons. For each colabeling experiment, clear and distinct V100-positive compartment was subsequently manually analyzed for colocalization with each of the 16 markers individually.

Plastic eye sections

1-µm plastic sections of adult eyes were made as described previously (Van Vactor et al., 1991). In brief, samples were fixed using standard electron microscopy fixatives containing 2% paraformaldehyde and 2% glutaraldehyde. Samples were subsequently dehydrated and embedded in epon/pararoldic plastic medium. Thick sections were stained with a combination of methylene blue and toluidine blue for light microscopy.

Online supplemental material

Fig. S1 shows Lysotracker labels late endosomal and autophagosomal compartments that are acidified in a v-ATPase–dependent manner. Fig. S2 shows high resolution 3D colocalization experiments of intracellular compartment marker colocalization. V100 colocalizes with endosomal markers but not the lysosomal protein Spin/Bnc1. Fig. S3 shows endosomal defects: the endosomal proteins Syx7 and Hrs exhibit elevated protein levels in v100 mutant eye–lamina complexes, and pHluorin accumulates in endosomal compartments. Fig. S4 shows eye defects: calibrated NH\(_4\)Cl washes reveal pHfluorin accumulations in acidified endosomes.
display almost complete loss of discernible structure. Fig. S5 shows that V100SSA expression causes a dominant defect that is different from the null mutant. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201003062/DC1.

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