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Presynaptic Biogenesis Requires Axonal Transport of Lysosome-Related Vesicles

Graphical Abstract



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In Brief

Synapse formation and maintenance require axonal transport of presynaptic cargoes. The identity of the precursor organelles involved has remained largely elusive. Vukoja et al. show that presynaptic vesicle and active zone proteins undergo anterograde axonal cotransport in lysosome-related vesicles.

Highlights

- SV and AZ proteins partially co-transport in *Drosophila* and mouse axons
- Presynaptic lysosome-related vesicles (PLVs) mediate axonal transport
- Genetic upregulation of PLV transport facilitates
 neurotransmission
- PLV packets represent precursor organelles for presynaptic biogenesis



Article

Presynaptic Biogenesis Requires Axonal Transport of Lysosome-Related Vesicles

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SUMMARY

Nervous system function relies on the polarized architecture of neurons, established by directional transport of pre- and postsynaptic cargoes. While delivery of postsynaptic components depends on the secretory pathway, the identity of the membrane compartment(s) supplying presynaptic active zone (AZ) and synaptic vesicle (SV) proteins is unclear. Live imaging in Drosophila larvae and mouse hippocampal neurons provides evidence that presynaptic biogenesis depends on axonal co-transport of SV and AZ proteins in presynaptic lysosome-related vesicles (PLVs). Loss of the lysosomal kinesin adaptor Arl8 results in the accumulation of SV- and AZ-protein-containing vesicles in neuronal cell bodies and a corresponding depletion of SV and AZ components from presynaptic sites, leading to impaired neurotransmission. Conversely, presynaptic function is facilitated upon overexpression of Arl8. Our data reveal an unexpected function for a lysosome-related organelle as an important building block for presynaptic biogenesis.

INTRODUCTION

The function of the nervous system requires the segregation of incoming from outgoing signals via the highly polarized architecture of neurons whose formation and maintenance depend on the tightly controlled directional transport of pre- and postsynaptic cargos (Hirokawa et al., 2010; Jin and Garner, 2008; Salinas et al., 2008). Disturbances of these processes are implicated in neurological diseases and can underlie neurodegeneration (Salinas et al., 2008) and neurodevelopmental defects such as micro- or macrocephaly (Parrini et al., 2016). The delivery of postsynaptic receptors and associated signaling components largely occurs through microtubule- and motor-protein-based

transport of elements of the conventional secretory pathwaymost notably, outposts of the Golgi complex present in dendrites (Horton et al., 2005; Ye et al., 2007), which are absent from axons.

In contrast, the cell biological origin and mechanism of biogenesis of the presynaptic compartment that is central to neurotransmission (Südhof, 2012) are incompletely understood. Presynaptic assembly requires the coordinated delivery of synaptic vesicle (SV) and active zone (AZ) proteins, multidomain scaffolds that define sites of neurotransmitter release (Owald and Sigrist, 2009; Schoch and Gundelfinger, 2006; Südhof, 2012), to nascent synapses. How AZ and SV protein transport are coordinated to ensure stoichiometric assembly of functional presynaptic units is uncertain (Goldstein et al., 2008; Jin and Garner, 2008; Murthy and De Camilli, 2003). Evidence from vertebrate and invertebrate models suggests the presence of SV and AZ proteins in vesicular structures in the axon (Goldstein et al., 2008). According to one model, AZ and SV components are transported on distinct organelles with (Shapira et al., 2003) and without (Maeder et al., 2014; Yonekawa et al., 1998), respectively, an electron-dense core and only assemble into functional presynaptic units after being delivered independently to nascent presynaptic sites. Alternatively, presynaptic AZ and SV proteins may largely be co-transported along the axon either via the same organelle or as pre-assembled clusters (Bury and Sabo, 2011; Tao-Cheng, 2007) that serve as axonal transport packets for assembly of the presynapse (Ahmari et al., 2000). The cotransport of both AZ and SV proteins in pre-assembled organelle clusters along axons would allow for the coordinated delivery of AZ and SV proteins to ensure the formation of functional presynaptic units for neurotransmitter release (Owald and Sigrist, 2009; Schoch and Gundelfinger, 2006; Südhof, 2012). Anterograde axonal transport of SV proteins has been shown to depend on kinesins, most notably on Unc104/KIF1A (also called Imac) (Hall and Hedgecock, 1991; Hirokawa et al., 2010; Jin and Garner, 2008; Pack-Chung et al., 2007; Yonekawa et al., 1998). In the absence of Unc104/KIF1A, the formation of mature presynaptic boutons is impaired (Hall and Hedgecock, 1991; Hirokawa et al., 2010; Jin and Garner, 2008; Pack-Chung et al., 2007; Yonekawa et al., 1998) and SV proteins are depleted from nerve

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terminals; instead, vesicles carrying SV cargo accumulate in neuronal cell bodies (Hall and Hedgecock, 1991; Yonekawa et al., 1998). Moreover, elegant genetic studies in *C. elegans* have shown that the balance between Unc104/KIF1A-mediated delivery of presynaptic cargos and their local assembly at presynaptic sites is regulated antagonistically by the small GTPase Arl8 (Klassen et al., 2010; Maeder et al., 2014) and the JNK kinase pathway in DA9 neurons (Wu et al., 2013).

An important yet unresolved question pertains to the cell biological identity of the transported presynaptic precursor organelle(s). Although AZ proteins initially associate with Golgi membranes (Maas et al., 2012), the supply of presynaptic components to axons is sustained surprisingly well under conditions of impaired secretory pathway activity (Ye et al., 2007) and blockade of post-Golgi trafficking rapidly suppresses dendritic growth with much less effect on axonal proteins (Horton et al., 2005). These observations suggest that presynaptic biogenesis must rely, at least in part, on other membrane sources that, in spite of their importance, have remained elusive.

Here we show by live imaging in Drosophila larvae and mouse hippocampal neurons that during presynaptic biogenesis, SV proteins, such as Synaptotagmin 1 and the vesicular glutamate transporter 1 (VGlut 1), co-traffic with lysosomal markers and, albeit less efficiently, with the AZ scaffold proteins Bruchpilot (BRP) or Bassoon (Bsn). We demonstrate that loss or downregulation of Arl8, a small GTPase known to regulate axonal transport of SV proteins (Klassen et al., 2010; Maeder et al., 2014) and lysosome motility (Rosa-Ferreira and Munro, 2011), results in the accumulation of AZ- and SV-protein-containing vesicles in neuronal cell bodies. These results suggest that transport of AZ and SV proteins is mediated by presynaptic lysosomerelated vesicles (PLVs), distinct from mature lysosomes involved in protein turnover. Consistent with this hypothesis, we find that impaired axonal transport of PLVs in the absence of the kinesin adaptor Arl8 causes a profound depletion of AZ and SV components from presynaptic sites leading to impaired neurotransmission. Conversely, genetic upregulation of Arl8 increases the levels of AZ proteins at presynaptic terminals and facilitates neurotransmission. Collectively, our data identify PLVs as axonal transport packets required for the proper assembly of a functional presynapse (Ahmari et al., 2000). They further reveal an unexpected function for a lysosome-related organelle as a basic building block for presynaptic biogenesis that may help to explain the close relationship between neuronal defects and lysosomal dysfunction in human diseases (Luzio et al., 2014; Marks et al., 2013).

RESULTS

Axonal Co-transport of SV and AZ Proteins via Lysosome-Related Vesicles in Drosophila Larvae

AZ and SV proteins previously have been postulated to be axonally transported either as pre-assembled clusters (Bury and Sabo, 2011; Tao-Cheng, 2007) or in distinct vesicular structures with (Shapira et al., 2003) and without (Yonekawa et al., 1998) an electron-dense core of unknown cell biological identity. To resolve this important issue, we analyzed whether and to what extent select bona fide SV and AZ proteins are co-transported. We first monitored the anterograde axonal transport of the presynaptic AZ scaffold BRP, a large AZ multidomain protein of the ELKS/CAST AZ protein family that couples release-ready SVs to presynaptic calcium channels (Böhme et al., 2016; Kittel et al., 2006) with the essential SV calcium sensor Synaptotagmin 1 (Jahn and Fasshauer, 2012; Südhof, 2013; Zhou et al., 2017) in live Drosophila larvae. A principle problem is the fact that once synapses are formed, only a small fraction of the total number of AZ and SV proteins present in neurons is actually axonally transported at any given time to sustain presynaptic function and assembly. To allow sensitive detection of such rare anterograde transport events, we generated transgenic flies co-expressing red-fluorescent-protein-tagged truncated BRP (Petzoldt et al., 2014) together with Synaptotagmin 1-eGFP (Zhang et al., 2002). A similar approach has been used successfully to study axonal transport of SV proteins in C. elegans (Klassen et al., 2010) and of mitochondria in Drosophila (Goldstein et al., 2008; Stowers et al., 2002). Anterogradely moving BRP puncta in about 50% (i.e., 8 out of 16) of all events also contained Synaptotagmin 1 (Figure 1A), suggesting that SV and AZ proteins, at least in part, are co-transported in Drosophila motoneuron axons.

To address the cell biological nature of the precursor organelles for presynaptic biogenesis, we monitored the axonal transport of green- or red-fluorescent-protein-tagged Synaptotagmin 1 or BRP together with fluorescent marker proteins of the secretory or endolysosomal system. Overexpression of fluorescentprotein-tagged organellar markers, such as the lysosomal membrane protein Spinster tagged with mRFP, had no adverse effects on the levels or localization of BRP or the vesicular glutamate transporter (VGlut; a SV protein) at neuromuscular junction (NMJ) synapses (Figures S1A-S1C) or on BRP, VGlut, or mitochondrial ATP synthase in motoneuronal cell bodies within the ventral nerve cord (VNC) (Figures S1D-S1G). Quantitative imaging of axonal transport revealed that anterogradely targeted Synaptotagmin 1-eGFP-labeled presynaptic precursor organelles to a large degree (≥85%, i.e., 127 out of 142 events from multiple animals and experiments; Video S1) are co-transported with the lysosomal marker Spinster-mRFP (Figure 1B; Video S1). Spinster-mRFP (Figure 1C) or an eGFP-tagged lysosome-associated membrane protein (Lamp) 1 chimera (Rong et al., 2011; Sweeney and Davis, 2002) (Figure 1D) were also co-transported frequently with fluorescent-protein-tagged BRP (in about onethird of all events), supporting the notion that SV and AZ proteins are often co-transported on the same organelle. In contrast, we hardly ever (<15%) detected co-transport of BRP-mRFP with late endosomal Rab7 (Figure S1H). Large BRP puncta not colocalizing with either Synaptotagmin 1 or lysosomal markers often were immobile and possibly represented axonal aggregates previously seen by us and others (Siebert et al., 2015). We also detected retrograde BRP or Synaptotagmin 1 transport events (data not shown) that could either reflect continuous circulation of SVs throughout the axon driven by molecular motors to yield an even distribution of presynaptic cargo ("conveyor belt model"; Wong et al., 2012) or transport of degradative organelles to the neuronal soma, where the majority of lysosomes reside.

Microtubule-based anterograde transport of lysosomes in various cell types, including neurons (Farías et al., 2017), is

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Figure 1. Axonal Co-transport of AZ and SV Proteins in Presynaptic Lysosome-Related Vesicles

(A–E) Anterograde transport of Bruchpilot (BRP) and Synaptotagmin-1 (Syt-1) with lysosomal markers and synaptic vesicle markers in *Drosophila* motoneuron axons *in vivo*. Live confocal imaging of motoneuron axons of intact *Drosophila* third-instar larvae co-expressing fluorescent-protein-tagged truncated BRP (hereafter only referred to as BRP) with lysosomal markers. Single frames and kymographs of (A) co-transport of BRP-mRFP (red) and Syt-1-eGFP (green), (B) Spinster-mRFP (red) and Syt-1-eGFP (green), (C) BRP-eGFP (red) and Spinster-mRFP (green), (D) BRP-mRFP (red) and Lamp1-eGFP (green), and (E) BRP-mRFP (red) and Arl8-eGFP (green). Scale bars, single frames: 2 µm; kymographs: 2 µm and 2 s. See also Figure S1 and Video S1.

mediated by the small GTPase Arl8, which connects lysosomes to kinesin motors (Farías et al., 2017; Rosa-Ferreira and Munro, 2011) such as Unc104/ KIF1A (Klassen et al., 2010). We confirmed that, in *Drosophila*, Arl8-eGFP is co-transported anterogradely with the lysosomal marker Spinster-mRFP (100%, i.e., 20 out of 20 events monitored) in axons (Figure S1I). Strikingly, BRP-mRFP in most (i.e., 9 out of 10) events monitored co-localized and was co-transported with Arl8-eGFP in axons (Figure 1E). Furthermore, BRP-mRFP and Lamp1-GFP-positive transport vesicles effectively reached NMJ nerve terminals (Figures S1J and S1K), consistent with the imaged cargos being physiologically transported. Hence, anterograde axonal transport of SV and AZ proteins may involve lysosome-related vesicles.

To be delivered to larval NMJ synapses, SV and AZ proteins need to be transported from the neuronal soma in the ventral nerve cord via the motoneuronal axon to presynaptic boutons. Consistent with their transport in lysosome-related vesicles, we found endogenous BRP or VGlut to partially co-localize with the lysosomal membrane markers Spinster or Lamp1 in neuronal somata (Figures 2A and 2C; Figures S2A and S2C) and axons (Figures 2F and 2H) of wild-type (WT) Drosophila larvae. Furthermore, endogenous BRP or VGlut puncta partially overlapped with Arl8-GFP (Figures 2B, 2D, 2G, and 2I; Figures S2B and S2D), which itself displayed extensive co-localization with Spinster and Lamp1 in somata (Figures S2F and S2G) and axons (Figures S2J and S2K). Endogenous Synaptotagmin 1 was also observed to partially co-localize with Arl8-GFP in the neuronal soma and in axons (Figures S2H and S2I). Finally, we found Arl8-GFP to be enriched at BRP- and VGlut-containing presynaptic nerve terminals of larval NMJs (Figures 2J and 2K).

If lysosome-related transport vesicles are indeed involved in the delivery of presynaptic cargo, they should be distinct from mature degradative lysosomes characterized by proteolytic cathepsin enzymes and a highly acidic pH. We found that SVand AZ-protein-containing lysosome-related vesicles present in neuronal somata were indeed distinct from mature degradative lysosomes marked by the lysosomal protease cathepsin L (Figure 2E; Figure S2E). This conclusion was further supported by the fact that anterogradely moving lysosome-related vesicles tagged on their lumenal side with GFP, whose fluorescence is quenched at the highly acidic pH characteristic for mature lysosomes (Johnson et al., 2016), could not be de-quenched by externally added ammonium chloride (Figures S2L and S2M).

Collectively, these observations suggest that lysosomerelated vesicles (hereafter referred to as PLVs) are involved in the anterograde axonal transport of SV and AZ proteins. These PLVs are distinct from mature degradative lysosomes present in the neuronal soma. This hypothesis was further tested and corroborated below.

Arl8-Mediated Anterograde Transport of PLVs Is Required for Presynaptic Biogenesis and Neurotransmission

To further explore the mechanisms of axonal co-transport of AZ and SV proteins via PLVs, we focused on the functional analysis of Arl8. Arl8 conceivably may mediate anterograde PLV transport (see Figure 1) and delivery to nerve terminals analogous to the transport of secretory lysosomal vesicles described in other cell types (e.g., melanocytes and cytotoxic T cells) (Marks et al., 2013).

We therefore first analyzed whether the observed co-transport of lysosomal markers and motile BRP puncta was dependent on Arl8 function. Indeed, we found that genetic loss of *arl8* (Figure 3A) greatly reduced the anterograde motility of Lamp1eGFP (Figure 3B) and, importantly, of BRP-mRFP puncta (Figure 3C). By contrast, axonal transport of mitochondria proceeded unperturbed in the absence of *arl8* (Figures S1L and S1M). Arl8, thus, specifically mediates axonal transport of lysosomes and PLVs.

If PLVs indeed represented precursor organelles for the presynaptic co-assembly of SV and AZ components, stalling their axonal transport and delivery should result in the depletion of SV and AZ proteins from presynaptic nerve terminals. If, however, loss of arl8 would render synapses degradation defective (e.g., if lysosomal proteolysis was perturbed), presynaptic cargo would be expected to locally accumulate at synapses. To probe these hypotheses, we analyzed NMJs from WT and arl8 mutant larvae. In comparison to WT synapses, NMJs from arl8 mutant larvae always appeared atypically thin with anomalously small or no presynaptic boutons (Figures 3D and 3E), a phenotype reminiscent to the loss of the Unc104/KIF1A kinesin Imac (Pack-Chung et al., 2007). Furthermore, the number of AZs and the total intensity of BRP were dramatically reduced at arl8 mutant NMJs (Figures 3D, 3F, and 3G). Thus, inhibition of axonal transport of PLVs in the absence of Arl8 results in severe defects in presynaptic biogenesis, most notably a near complete loss of AZ proteins such as BRP from presynaptic boutons, eventually resulting in the death of arl8 mutant animals at the late larval stage (L3, data not shown). This phenotype is in marked contrast to defects in lysosomal proteolysis and is expected to result in the accumulation of presynaptic components.

Given that SVs undergo exo-endocytic cycling at the AZ and that the AZ protein BRP and the SV protein Synaptotagmin 1 are co-transported in PLVs along the axon to the presynapse (see Figure 1), we probed whether loss of arl8 affects the delivery of SV proteins. We found arl8 mutant NMJs to contain strongly reduced amounts of SV proteins such as Synaptotagmin 1 (Figures 3H and 3J), VGlut (Figures 3I and 3K), cysteine string protein, and synapsin (Figures S3D and S3E). The levels of endocytic proteins that orchestrate SV recycling and associate with SV and AZ proteins (Haucke et al., 2011; Kononenko and Haucke, 2015; Murthy and De Camilli, 2003; Podufall et al., 2014), including dynamin, Dap160/intersectin, and Stoned B (StnB), were also reduced (Figures S3A-S3C and S3F). Importantly, the loss of SV and AZ proteins in arl8 mutant animals was rescued by presynaptic re-expression of native Arl8 (Figures 3L and 3M; Figure S3G), indicating that it is a cell-autonomous and neuron-specific phenotype. Consistently, defective presynaptic biogenesis, including depletion of BRP, was also observed if Arl8 expression was downregulated specifically in presynaptic motoneurons by RNA interference (RNAi) (Figures 3N-3P). A phenotype similar to downregulation of Arl8 was elicited by motoneuron-specific depletion of VPS39, a subunit of the HOPS complex (Figures 3N-3P) that associates with Arl8 (Khatter et al., 2015), further confirming the lysosome-related nature of PLVs. As seen for arl8 mutants, downregulation of Arl8

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Figure 2. Lysosomal-Marker-Positive PLVs Transport Endogenous Presynaptic Scaffold and SV Proteins

(A–E) Confocal images of neuronal soma in the ventral nerve cord (VNC) of third-instar *Drosophila* larvae immunostained for endogenous presynaptic proteins and endogenous lysosomal markers. (A) BRP (red) signals are partly co-positive for the lysosomal marker Lamp1 (green) and (B) Arl8-GFP (green). (C) VGlut (red) signals are also partly co-positive for Spinster (green) and (D) Arl8-GFP (green). (E) On the contrary, BRP (red) signals are not overlapping with the degradative lysosomal marker Cathepsin-L (green). Scale bar, 2 µm.

(F–I) Confocal images of PLVs in the proximal axon of third-instar larvae. (F) Several endogenous BRP-positive PLVs are co-labeled with Lamp1 (green) and (G) Arl8-GFP (green). (H) Furthermore, several endogenous VGlut-positive PLVs are co-positive for Spinster (green) and (I) Arl8-GFP (green). Scale bar, 2 μm.

(J) Confocal images of the neuromuscular junction of third-instar larvae expressing Arl8-GFP. Arl8 reaches the synaptic terminal and surrounds the active zone (AZ) marked by BRP (red) and partly co-localizes with VGlut (magenta). Scale bar, 3 μ m.

(K) Zoom of one terminal bouton. Scale bar, 1 μ m.

See also Figure S2.

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Figure 3. Arl8 Mediates Presynaptic Delivery of PLVs Required for Presynaptic Biogenesis and Defective HOPS Complex Phenocopies Arl8 Knockdown

(A) Loss of Arl8 expression in *arl8* mutant *Drosophila*. RT-PCR analysis of Arl8 transcription. Total mRNA isolated from wild-type (WT) and homozygous *arl8* mutant larvae was reverse transcribed into cDNA and probed by PCR for the presence of Arl8 or AP-2\sigma2 (ctrl) transcripts.

(B and C) Loss of Arl8 in arl8 null mutant flies abolishes anterograde transport of PLVs containing (B) Lamp1-eGFP and (C) BRP-mRFP. Kymographs with control larvae (left) and arl8 null mutant (right). Scale bars, 2 μ m and 4 s.

(D–K) Defective presynaptic biogenesis in absence of Arl8-mediated PLV delivery. (D) Reduced AZ count and BRP levels at *arl8* mutant NMJs. Confocal images of WT and *arl8* mutant NMJ synapses of *Drosophila* third-instar larvae stained for BRP (green) and HRP as axonal membrane marker (magenta). Top: overview. Bottom and insets on the right represent zooms. *arl8* mutant NMJs display small boutons with drastically reduced numbers of active zones (AZs). (E–K) Quantification of representative data shown in (D). (E) In *arl8* mutants, the NMJ area is reduced to 57% of that in WT (µm²; HRP area of the maximum projection).

expression by RNAi did not result in defects in mitochondrial motility (Figure S1N). Importantly, these defects were not caused by effects of loss of *arl8* on the microtubule-based cytoskeleton marked by the microtubule-associated protein Futsch (Figure S3H) or defects in motoneuron outgrowth caused by absence of *arl8* (Figures S3I and S3J).

Analysis of arl8 mutant NMJs by electron microscopy and morphometry confirmed the severe reduction in presynaptic bouton size (and consequently NMJ size) that correlated with a proportionate reduction in the number of SVs per bouton area (Figures 4A and 4B). While there was a remarkable reduction of overall NMJ size and the total numbers of AZs and SVs (Figure 4C), AZ size (Figures S4A and S4B) and SV density (Figure 4D) within the remaining "skinny terminals" were grossly unaltered. Thus, Arl8 and, by extension, PLVs do not play a direct role in the local assembly of the AZ and the associated SV population but instead control the overall amount of presynaptic material available for assembly at NMJs. Defects in SV and AZ protein transport and presynaptic biogenesis likely impair neurotransmission. Indeed, we found the recycling SV pool size to be drastically decreased in optophysiological assays (FM1-43 labeling) (Figure 4E). Furthermore, electrophysiological recordings from WT or arl8 null larvae revealed reduced evoked excitatory junctional currents (eEJCs) in arl8 mutants (Figures 4F and 4G) and a corresponding decrease in eEJC charge (Figure 4H). In contrast, no significant alterations in eEJC kinetics (Figure 4I) and in the paired-pulse ratio (Figure 4J), a parameter for shortterm plasticity, were observed. These results indicate that the profound reduction in delivery of presynaptic SV and AZ proteins in the absence of arl8 limits neurotransmitter release, while the residual SVs and AZs remain functional.

Elevated Arl8 Function Facilitates Neurotransmission

In light of the unexpected nature of our findings, we decided to challenge our results by an independent approach. We hypothesized that if Arl8 was a limiting factor for presynaptic biogenesis, Arl8 gain of function might increase the levels of AZ proteins (i.e., BRP) and facilitate neurotransmission. To test this, we selectively overexpressed Arl8 in motoneurons. As hypothesized, elevation of Arl8 function increased the levels of BRP at presynaptic boutons (Figures 5A and 5B), resulting in elevated evoked neurotransmission (Figures 5C–5F), whereas the size of BRP-containing AZs (Figures S4A and S4B) and the paired-pulse ratio remained unchanged (Figure 5G). Arl8 gain of function, hence, is sufficient to boost neurotransmission, possibly by facilitating presynaptic biogenesis, although other mechanisms cannot be ruled out at this point.

Arl8 Mediates Anterograde Co-transport of AZ and SV Proteins on PLVs in Hippocampal Neurons

The machinery for presynaptic neurotransmitter release is evolutionary highly conserved from invertebrates to mammals (Haucke et al., 2011; Schoch and Gundelfinger, 2006; Südhof, 2012). We therefore asked whether the role of Arl8 in presynaptic biogenesis in Drosophila is conserved in the mammalian nervous system. We monitored the axonal transport of presynaptic AZ proteins in developing mouse hippocampal neurons (DIV4) coexpressing eGFP-, mCherry-, and/or iRFP-tagged variants of the AZ scaffold Bsn (Dresbach et al., 2006; Maas et al., 2012) and the SV protein VGlut1 together with the lysosomal marker Lamp1. Triple-color live imaging in fact revealed anterograde co-transport of VGlut1-mCherry with eGFP-Bsn and Lamp1iRFP (Figure 6A). Quantitative analysis of multicolor live-imaging experiments showed that more than 85% (i.e., 23 out of 27 monitored) of all anterogradely transported VGlut1 vesicles also contained Lamp1. About half of all VGlut1 vesicles were cotransported with eGFP-Bsn (>50%, i.e., 13 out of 22 events), while about 40% of all anterogradely moving eGFP-Bsn puncta contained Lamp1 (i.e., 15 out of 37 events) (Figures 6B-6D). We also observed cotransport of eGFP-Bsn with Arl8B-mCherry (Figures S5A and S5B), but not with Rab7 (as in Drosophila, see Figure S1H), which almost exclusively moved retrogradely in axons (Figure S5C). Consistently, endogenous Bsn partially colocalized with Arl8B-mCherry in axons (Figure 6E). Moreover, Arl8-mCherry co-localized nearly perfectly with Lamp1-iRFP, akin to endogenous Arl8A/B and endogenous Lamp1 (Figures S5D and S5E). Collectively, these data indicate that presynaptic biogenesis in developing hippocampal neurons involves the axonal transport of SV- and AZ-protein-containing PLVs, similar to what is observed in Drosophila (compare Figure 1). Consistent with this hypothesis, fluorescence recovery after photobleaching (FRAP) experiments revealed the delivery of Lamp1-, Arl8B-, and

See also Figure S3.

⁽F) Absolute numbers of presynaptic AZs/NMJ (WT 147.38 \pm 12.6; *ar/8* mutant 48.13 \pm 7.4) and (G) total BRP sum intensity/NMJ (WT 100 \pm 12.8; *ar/8* mutant 27.6 \pm 3.5) are severely reduced. n = 16 (WT) and 15 (*ar/8* mutant) NMJs. (H and J) Reduced levels of SV proteins synaptotagmin 1 (Syt-1) (WT 100 \pm 16.8; *ar/8* mutant 37.05 \pm 8.1; n = 4 NMJs) and (I and K) the vesicular glutamate transporter (VGlut) (WT 100 \pm 18.8, n = 10; *ar/8* mutant 34.6 \pm 5.4, n = 6 *ar/8*). Scale bars, 5 μ m (upper), 4 μ m (lower), and 1 μ m (zoom). Data in (J) and (K) are quantifications of representative examples displayed in (H) and (I).

⁽L and M) Re-expression of Arl8 in motoneurons rescues defective presynaptic biogenesis in *arl8* mutants. Quantification of representative data shown in Figure S3G. (L) The BRP total intensity is reduced in the *arl8* mutants compared to WT (WT 100 \pm 18.63, n = 25; *arl8* mutant 38.85 \pm 6.21, n = 16, while the presynaptic rescue show WT levels (99.17 \pm 21.81, n = 19) and is increased compared to the rescue-control (31.09 \pm 7.11, n = 20). (M) Similar results were obtained for VGlut (WT 100 \pm 42.92, n = 8; *arl8* mutant 63.67 \pm 10.09, n = 9; rescue 124.51 \pm 34.68, n = 8; rescue-control 54.71 \pm 16.127, n = 13). Statistics: all graphs show mean \pm SEM. Mann-Whitney test for two groups; one-way ANOVA for more than two. *p < 0.05, **p < 0.01, ***p < 0.001, n.s. for not significant.

⁽N–P) Specific presynaptic downregulation of Arl8 by RNAi also causes defective synaptic biogenesis, and downregulation of a subunit of the HOPS complex VPS39 leads to a similar phenotype as the Arl8 knockdown. (N and O) Quantification of representative data shown in (P). (N) Total BRP intensity/NMJ (100 ± 22.91 , n = 10; Arl8-RNAi 29.95 \pm 6.51, n = 9; VPS 39-RNAi 41.43 \pm 6.606, n = 9) and (O) absolute numbers of presynaptic AZs normalized to the NMJ area (control $100.0 \pm$ 6.16, n = 10; Arl8-RNAi 71.86 \pm 7.61, n = 9; VPS39-RNAi 67.57 \pm 3.47, n = 9) are severely reduced, with an equal reduction level for VPS39 knockdown compared to the Arl8-RNAi. (P) Confocal images of control (driver-line only, left), Arl8-RNAi (middle), and VPS39-RNAi (right) NMJ synapses of *Drosophila* third-instar larvae stained for BRP (green, top, and HRP, magenta, central panel). Scale bar, 10 μ m. Both Arl8 and VPS39 knockdown display thinner NMJs with small boutons and reduced numbers of AZ (see B).





Figure 4. Arl8-Mediated Delivery of PLVs Is Required for Presynaptic Synaptic Function

(A) Electron micrographs of wild-type (WT) and *arl8* mutant boutons of *Drosophila* third-instar larval NMJs depicting an entire bouton (left panels) and a zoom (right panels). Presynaptic ultrastructure and SV accumulation are not affected in the *arl8* mutant. Scale bars, 70 nm in overview and 20 nm in zoom. (B–D) Quantification of representative data shown in (A). (B) Bouton area is severely reduced in the *arl8* mutant (WT 100 ± 31.46 with n = 8; *arl8* mutant 38.24 ±

7.47 μ m², n = 12) with a proportional reduction in (C) SV numbers (WT 99.50 ± 27.62 μ m²; *arl8* mutant 44.50 ± 12.20 μ m²), although the (**D**) SV density was not affected (WT 100 ± 28.56 μ m²; *arl8* mutant 103.2 ± 20.88).

(E) FM dye uptake 6 assay at NMJs of third-instar *Drosophila* larvae for WT, *arl8* mutant, and rescue (WT 100, n = 10; *arl8* mutant 0.37 \pm 0.07, n = 6; rescue 1.04 \pm 0.05, n = 20). The recycling SV pool is strongly reduced in *arl8* mutants, and presynaptic Arl8 overexpression can fully rescue this phenotype.

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Figure 5. Arl8 Gain of Function Facilitates Presynaptic Biogenesis and Neurotransmission

(A and B) Arl8-overexpression in motoneurons facilitates presynaptic biogenesis. (A) Confocal images of NMJs from control and transgenic *Drosophila* larvae overexpressing Arl8 (Arl8 OE) reveal increased BRP levels. Scale bars, 4 and 1 μ m (zoom). (B) Quantification of representative data shown in (A). BRP total intensity: WT 100 ± 7.5, n = 22; Arl8-OE 177 ± 24.18, n = 24.

(C–G) Arl8 overexpression facilitates neurotransmission. Two electrode voltage clamp electrophysiological recordings show increased eEJC amplitudes at Arl8overexpressing synapses. (C) Example traces of evoked EJC. (D) eEJC amplitudes (control -78.24 ± 4.61 nA, n = 10; Arl8-OE -101.0 ± 8.85 nA, n = 9). (E) Evoked excitatory junctional charge (WT -873.6 pC ± 56.29 , n = 10; *arl8* mutant -1062 pC ± 92.10 , n = 9). (F) eEJC tau (WT 6.354 ms ± 0.2080 , n = 10; *arl8* mutant 5.734 ms ± 0.1505 , n = 9) as well as (G) the 10 ms paired-pulse ratio (WT 0.9145 ± 0.06574 , n = 10; *arl8* mutant 0.7493 ± 0.07202 , n = 9). Statistics: all graphs show mean \pm SEM. Mann-Whitney test. *p < 0.05.

Bsn-containing PLVs to presynaptic boutons immunopositive for Synapsin (Figures S5F–S5H). Moreover, overexpression of Arl8 facilitated axonal transport of eGFP-Bsn, as evidenced by its depletion from the neuronal soma and axon initial segment (AIS) when compared to control neurons (Figure 6F; Figure S5I). Conversely, when the expression level of Arl8A, one of the two mammalian isoforms of Arl8 (Rosa-Ferreira and Munro, 2011) expressed in the brain, was reduced, we observed a partial accumulation of eGFP-Bsn in the neuronal soma and the AIS (Figure 6G; Figure S5J). These results suggest that the role of Arl8-mediated anterograde transport of PLVs in presynaptic biogenesis is evolutionary conserved from flies to mammals.

Somatic Accumulation of "PLV Packets" Containing AZ and SV Proteins under Conditions of Impaired Axonal Transport

The observed accumulation of AZ proteins in the neuronal soma and AIS of hippocampal neurons depleted of Arl8A (Figure 6G) and their corresponding depletion from the soma and AIS of Arl8-overexpressing hippocampal neurons (Figure 6F) suggests that in the absence of Arl8 function, AZ proteins contained in PLVs may accumulate in the cell bodies of motoneurons from arl8 mutant animals. Analysis of larval brains from WT and arl8 mutants indeed revealed a profound accumulation and co-localization of AZ and SV proteins (Figure S6L), such as BRP (Figures 7A, 7B, and 7D; Figure S6L) and Synaptotagmin 1 (Figures 7B and 7F; Figure S6B), in distinctive puncta within neuronal cell bodies (Figures 7A and 7B). A similar accumulation and somatic co-localization was observed for the SV protein VGlut (Figures 7B and 7G; Figure S6C), the AZ scaffold-associated release factor Unc13A (Figure 7E; Figure S6A), and the endocytic protein Dap160/intersectin (Figures S6D and S6I). Downregulation of Arl8 expression by RNAi also caused BRP to accumulate in neuronal somata (Figure S6M). Interestingly, the peripheral AZ proteins Syd-1 and Liprin- α , factors that instruct presynaptic biogenesis prior to core-AZ assembly (Owald et al., 2010; Patel

(F–J) Defective presynaptic biogenesis in absence of Arl8 impairs evoked neurotransmission. Two electrode voltage-clamp electrophysiological recordings are shown. (F) Example traces of evoked excitatory junctional current (eEJC). (G) Reduced eEJC amplitudes at *arl8* mutant synapses (WT – 101.09 ± 9.4 nA, n = 12; *arl8* mutant -58.79 ± 3.1 nA, n = 11) and (H) reduced eEJC charge (WT –761.7 pC ± 85.50, n = 12; *arl8* mutant -407.8 pC ± 26.69, n = 11). (I) eEJC tau was not altered (WT 3.963 ms ± 0.1840, n = 12; *arl8* mutant 3.488 ms ± 0.1403, n = 11) as well as (J) the 10 ms paired-pulse ratio (WT 0.8299 ± 0.05414, n = 12; *arl8* mutant 0.7993 ± 0.04332, n = 10). All graphs show mean ± SEM. Mann-Whitney test. **p < 0.01, ***p < 0.001. See also Figure S4.



Bsn intensity Bsn intensity Bsn intensity 100 50 200 Bsn intensity 150 100 merge 50 60 60 75 75 0. 0-ANBIHA 0 SIANDA 0 SIANDA control A18-HA control sisc' sisci

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et al., 2006), did not significantly accumulate in neuronal cell bodies of *arl8* mutant larvae (Figure 7H; Figures S6E, S6G, and S6J). Syd-1 also did not co-localize with BRP-positive puncta in neuronal somata (Figure 7C), suggesting that early scaffold components may follow a distinct pathway of presynaptic biogenesis. No accumulation of p62 (Figure 7I; Figure S6F), a common substrate and reporter for the functioning of the autophagy-lysosomal pathway for protein degradation, or of ATG8a-containing autophagosomes (Figures S6H and S6K) was observed in neuronal cell bodies of *arl8* mutants. Moreover, somatic BRP clusters were devoid of p62 (Figure 7C; Figure S6L).

In contrast to SV and AZ proteins such as BRP, loss of Arl8 did not alter the levels or distribution of endogenous mitochondrial ATP synthase in the VNC of larval brains (Figures S6N and S6O), consistent with the observation that axonal transport of mitochondria proceeds unperturbed in absence of *arl8* (Figures S1L–S1N). These findings indicate that AZ and SV protein accumulations in neuronal somata of *arl8* mutant larvae are not caused by defective protein turnover via the autophagy-lysosomal pathway but rather result from impaired anterograde axonal transport of PLVs in the absence of the kinesin adaptor Arl8.

If this hypothesis was correct, one would expect the SV and AZ protein accumulations in neuronal cell bodies to correlate with the accumulation of morphologically identifiable vesicular organelles in *arl8* mutant neurons at the ultrastructural level. To characterize such putative organelles associated with the accumulation of presynaptic components, i.e., PLVs, at the molecular and ultrastructural levels, we turned to electron microscopy. SV and AZ protein accumulations seen in the soma of motoneurons by light microscopy at the ultrastructural level correlated with a

striking accumulation of homogeneously sized (about 70 nm in diameter, Figures 7J-7L and 7N) vesicular structures of varying electron densities (Figure 7K), consistent with prior reports of both clear (Yonekawa et al., 1998) and electron-dense (Shapira et al., 2003; Zhai et al., 2001) vesicles as presynaptic transport carriers. Electron tomography analysis of PLV clusters in arl8 mutant somata demonstrated that PLV profiles observed in electron micrographs in 2D correspond to a closely packed perinuclear array of comparably uniform vesicles (Figure 70), consistent with recent light microscopy data (Dresbach et al., 2006; Maas et al., 2012). PLVs were clearly distinct from other organelles such as SVs (Figures 7J-7L; Figure S6Q), degradative lysosomes, and multivesicular endosomes that accumulate in neuronal somata of arl8 mutant larvae (Figure S6, compare Q and R) as a result of their impaired anterograde transport into neurites (Farías et al., 2017) or the endoplasmic reticulum (Figures 7J, 7K, and 7O). Rarely, PLV-like organelles were detected in control animals (Figures 7J and 7N), often as either single vesicles (Figure S6P) or small clusters (Figure 7J). No change in the number or distribution of somatic mitochondria was observed in arl8 mutants (Figure 7M), further confirming the specific role of Arl8 in the anterograde transport of PLVs.

Given that AZ and SV cargo are partially co-transported along the axon in *Drosophila* and mammalian neurons, we wanted to explore the content of PLVs with respect to these presynaptic components. Immunogold labeling of PLVs in Tokuyasu cryosections (Tokuyasu, 1973), indeed, confirmed the presence of BRP and Synaptotagmin 1 (Figure 7P), in agreement with our observations in live *Drosophila* larvae (compare Figure 1). Dualcolor super-resolution imaging by gSTED microscopy showed Synaptotagmin-1-containing vesicles to be clustered around BRP puncta (Figure 7Q). These data further support the view

Figure 6. Axonal Co-transport of AZ and SV Proteins in PLVs in Mouse Hippocampal Neurons

(C) Images on the left show the position of the trafficking organelle at time point 0 of vGlut1-VENUS co-transported with Lamp1-mCherry (upper panels), eGFP-Bsn (95-3938) and Lamp1-mCherry (middle panels), and eGFP-Bsn (95-3938) and vGlut1-mCherry (lower panels). Images on the right mark the position of the trafficking organelle after 12 s. Scale bar, 2 μm.

(D) Kymographs depict the trajectories of vGlut1-VENUS co-transported with Lamp1-mCherry (upper panels), eGFP-Bsn (95-3938) and Lamp1-mCherry (middle panels), and eGFP-Bsn (95-3938) and vGlut1-mCherry (lower panels). Scale bars, 2 μ m, 10 s.

(E) Arl8B-mCherry co-localizes with endogenous Bsn in axons of hippocampal neurons (DIV8) (indicated by white arrow heads). Scale bar, 5 µm.

(F) Arl8 overexpression in hippocampal neurons facilitates PLV-mediated transport of eGFP-Bsn (95-3938) from soma and proximal axons. Quantification of representative data shown in Figure S5I. Levels of eGFP-Bsn (95-3938) in soma of control neurons were set to 100. eGFP-Bsn (95-3938) levels were decreased in the soma (control 100 ± 8 , Arl8 OE 25 ± 4 each N = 60 neurons from n = 3 independent experiments) and proximal axon (control 100 ± 10 , Arl8 OE 46 ± 8 each N = 60 neurons from n = 3 independent experiments) of Arl8-overexpressing neurons compared to control transfected neurons. Mann-Whitney test was used for statistical analysis, ****p < 0.0001.

(G) Reduced PLV transport in hippocampal neurons partially depleted of Arl8A results in accumulation of eGFP-Bsn (95-3938) in neuronal somata and the proximal axon. Quantification of representative data shown in Figure S5J. Depletion of Arl8A led to increased eGFP-Bsn (95-3938) levels in the soma (siScr 100 \pm 12, N = 75 neurons from n = 4 independent experiments; siArl8A 147 \pm 14, N = 79 neurons from n = 4 independent experiments) and proximal axon (siScr 100 \pm 10, N = 75 neurons from n = 4 independent experiments; siArl8A 181 \pm 17, N = 79 neurons from n = 4 independent experiments) compared to scrambled siRNA-treated control neurons set to 100). Mann-Whitney test was used for statistical analysis, ****p < 0.0001, **p = 0.0021. Quantifications presented as mean \pm SEM. See also Figure S5.

⁽A–D) Anterograde co-transport of the AZ scaffold Bassoon (Bsn) with the lysosomal marker Lamp1 and the SV protein vGlut1 in proximal axons of hippocampal neurons.

⁽A) Live confocal imaging of mouse hippocampal neurons (DIV4) co-expressing eGFP-Bsn (95-3938) together with vGlut1-mCherry and Lamp1-miRFP703. Images on the left show the position of the trafficking organelle at time point 0; images on the right show the position of the trafficking organelle after 16 s. Scale bar, 2 µm.

⁽B) Percentages of anterograde co-transport in the proximal axon of hippocampal neurons (DIV4) transfected with either (1) eGFP-Bsn (95-3938) and vGlut1-mCherry, (2) vGlut1-VENUS and Lamp1-mCherry, or (3) eGFP-Bsn (95-3938) and Lamp1-mCherry (anterograde transport events analyzed for eGFP-Bsn (95-3938) and vGlut1-VENUS and vGlut1-mCherry showed 53.6% co-transport, for vGlut1-VENUS and Lamp1-mCherry 85.5% co-transport, and for eGFP-Bsn (95-3938) and Lamp1-mCherry 39.0% co-transport; mean percentages \pm SEM presented from n = 3-4 independent experiments, numbers of total events analyzed are indicated in each bar).

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that AZ and SV proteins are partially co-transported as vesicle aggregates (Tao-Cheng, 2007), possibly held together via the associated AZ scaffolds located on the cytoplasmic face of PLVs. These results suggest that SV- and AZ-protein-containing PLV packets represent a major type of precursor organelles for presynaptic biogenesis (Ahmari et al., 2000).

DISCUSSION

Our collective data based on live imaging of Drosophila larvae and hippocampal neurons, genetic analysis, and super-resolution light and electron microscopy reveal an unexpected role for presynaptic PLVs and, thus, for lysosome-related organelles in presynaptic biogenesis by anterograde transport of packets of AZ and SV proteins in developing neurons. Several lines of evidence support this model: first, live imaging of anterograde transport of AZ and SV proteins in Drosophila larvae and hippocampal neurons in culture provide evidence for the co-transport of SV and AZ proteins with lysosomal membrane proteins and, partially, with each other. Second, loss of Arl8, a Ras-related GTPase implicated in the anterograde transport of lysosomerelated organelles (Khatter et al., 2015; Rosa-Ferreira and Munro, 2011) and of SV proteins (Klassen et al., 2010) and present on PLVs (see Figures 1, 2, S1, S2, and S5), stalls anterograde PLV transport and causes the depletion of SV and AZ proteins from presynaptic nerve terminals and a corresponding striking accumulation of these factors in stacks of vesicles in neuronal somata. Third, Arl8 gain of function increases AZ protein levels and facilitates evoked neurotransmission, suggesting that Arl8 is a limiting factor for presynaptic biogenesis. Finally, we provide ultrastructural evidence that PLVs contain SV proteins,

as well as cytoplasmically associated BRP, and are morphologically and biochemically distinct from mature degradative lysosomes (see Figure 7; Figure S6) and SVs. Consistent with this view, we find PLVs to be devoid of degradative cathepsin (Figure 2E; Figure S2E) and to lack the highly acidic pH characteristic for perinuclear degradative lysosomes (Figures S2L and S2M) (Johnson et al., 2016). Although Arl8, in addition to its function as a kinesin adaptor and recruiter, conceivably may also regulate protein turnover via the autophagy/lysosomal pathway, we find no evidence that alterations in this pathway—expected to result in the synaptic accumulation of presynaptic cargo—underlie the phenotypic alterations observed in *arl8* mutant animals.

Instead, our data are consistent with work from C. elegans that has unraveled a physical and functional connection of Arl8 with Unc104/KIF1A, an anterograde kinesin motor previously implicated in the axonal transport of SV precursors in C. elegans, Drosophila, and mice (Pack-Chung et al., 2007; Rivière et al., 2011; Wu et al., 2013; Yonekawa et al., 1998; Zhang et al., 2016). Consistently, we find Drosophila Arl8 and Unc104/KIF1A to co-localize and associate with each other when expressed in cells (Figures S7A and S7B). Moreover, partial loss of unc104/Imac function in the hypomorphic bris allele (Zhang et al., 2016, 2017) causes BRP depletion from NMJs, similar to loss of arl8 (Figures S7C and S7D). While elegant previous work in C. elegans has revealed a regulatory interplay between Arl8 and the JNK pathway in the capture and dissociation of presynaptic proteins (Wu et al., 2013), our data imply that the function of Arl8 may not be restricted to the regulation of Unc104/KIF1A-based PLV packet processivity. Instead, our results suggest that Arl8 likely executes a more general function in linking presynaptically targeted PLV packages to the

Figure 7. AZ- and SV-Protein-Containing PLVs Accumulate in Neuronal Somata of arl8 Mutants

(A–I) Specific accumulation of presynaptic scaffold proteins and SV proteins in motoneuronal somata of *arl8* mutant *Drosophila* larvae. (A) Confocal images of ventral nerve cord (VNC) from wild-type (left) and *arl8* mutant (right) *Drosophila* larvae immunostained for BRP (green), showing a strong accumulation of BRP in the cortex (delineated by dotted lines) of the VNC. Scale bar, 100 μ m. (B) Zooms into the cortex of the VNC, showing accumulation and co-localization of BRP (green) and the SV protein Syt-1 (upper panel) as well as VGlut (lower panel). Scale bar, 3 μ m. (C) The early presynaptic scaffold protein Syd1 is not accumulating in the cortex of the VNC of *arl8* mutant larvae (upper panel), and their signals are not overlapping. Autophagy does not seem affected in the *arl8* mutant larvae as p62 levels are not increased (lower level) and BRP aggregates are not positive for p62. Scale bar, 3 μ m. (D–I) Quantifications of the representative images of (A)–(C) and Figure S6 (A, B, C, D, and F). (D) BRP mean intensity (normalized to 100): wild-type 100.0 ± 10.00 (n = 4), *arl8* mutant: 272.2 ± 9.391 (n = 4). (E) UNC1: wild-type 100 ± 12.46, *arl8* mutant 165.2 ± 5.87 (n = 4). (F) Syt1 mean intensity (normalized to 100): wild-type 100.0 ± 14.50 (n = 4), *arl8* mutant: 263.3 ± 13.70 (n = 4). (G) VGlut mean intensity: wild-type 100 ± 26.33 (n = 4), *arl8* mutant 255.3 ± 43.27 (n = 4). (H) Syd1 mean intensity: wild-type 100.0 ± 13.41 (n = 4), *arl8* mutant 121.1 ± 8.076 (n = 4) and (l) p62 mean intensity (normalized to 100): wild-type 100.0 ± 3.931 (n = 4), *arl8* mutant: 98.15 ± 6.612 (n = 4). Data are mean ± SEM. Student's t test, *p < 0.05, ***p < 0.001.

(J and K) Electron micrographs of neuronal somata from (J) wild-type and (K) *arl8* mutant larval brains. Blue, nucleus; brown, mitochondria; yellow, endoplasmic reticulum. Note the massive accumulation of PLVs with or without electron-dense cores in neuronal somata of *arl8* mutants (zoom, right). In somata from WT animals (zoom, left) single or very small groups of PLVs were detected. Scale bar, 100 nm, zoom (K) 20 nm.

(L) Mean diameter of PLVs and synaptic vesicle (SV) profiles: 66.19 nm ± 0.59 (n = 497 vesicles); SV, 30.74 nm ± 0.41 (n = 497 vesicles). PLVs are, thus, distinct from SVs.

(M) Quantification of mitochondrial volume fraction in the neuronal cytoplasm from the same preparation is not increased in *arl8* mutants compared to WT controls (WT 0.06803 \pm 0.006499, N [larvae brain] = 5, n [images of neuronal soma layer] = 49; *arl8* mutant 0.05848 \pm 0.005769, N = 5, n = 45), while (N) PLV volume fraction is strongly increased (WT 0.02149 \pm 0.003548, n = 5; *arl8* mutant 0.09272 \pm 0.01629, n = 5).

(O) 3D electron tomography analysis of PLV accumulations in neuronal somata. 3D tomogram segmentation from *arl8* mutant neuronal soma reveals a striking accumulation of spherical organelles (PLVs). PLVs show a specific morphology and are clearly distinct from other organelles. Blue, nuclear envelope; brown, mitochondrion; yellow, ER; green, PLVs. Scale bar, 150 nm.

(P) Cryo-ultrathin sections according to Tokuyasu from neuronal somata of WT and *arl8* mutant larval brains stained for BRP or synaptotagmin 1 (Syt-1) and decorated with 10 nm immunogold particles. Scale bar, 100 nm (left panels), 20 nm (zoomed panels on the right).

(Q) Multicolor gSTED microscopy of immunolabeled ultrathin cryosections (150 nm) from neuronal somata of WT and *arl*8 mutant larval brains stained for HRP (blue as a membrane marker), BRP (green), and synaptotagmin 1 (Syt-1, magenta). DAPI-stained nuclei are shown in white. Scale bar, 100 nm. BRP is directly apposed to Syt-1- containing PLVs.

See also Figures S6 and S7.

Unc104/KIF1A-based transport machinery in the neuronal soma (see Figures 7 and S6). It is further conceivable that Arl8 also contributes to the formation or maturation of PLVs, akin to the role of the HOPS complex in secretory organelle biogenesis in other cell types (Morlon-Guyot et al., 2015).

PLVs likely represent a specialized form of axonally targeted neuronal secretory lysosomal vesicles (Luzio et al., 2014; Marks et al., 2013) similar to those found in other cell types such as hematopoietic cells and melanocytes, where they mediate the secretion of chemical signals or enzymes or promote lipid turnover and facilitate membrane growth and repair (Blott and Griffiths, 2002; Luzio et al., 2007, 2014; Marks et al., 2013; Reddy et al., 2001; Setty et al., 2008). Interestingly, a secretory lysosome-related organelle-based mechanism has recently been shown to mediate lumen formation during epithelial tube anastomosis in Drosophila (Caviglia et al., 2016) and in secretory organelle formation in parasitic eukaryotes (Morlon-Guyot et al., 2015), suggesting that the function of secretory lysosome-related organelles in the biogenesis of specialized membrane compartments may not be restricted to the nervous system. How the PLV-based presynaptic biogenesis pathway is segregated from the conventional lysosomal pathway for protein and lipid degradation is an open question. One possibility is that PLVs are formed by a somatic biogenesis route shared with other lysosome-related organelles (e.g., via BLOC/AP-3 and/or Vps41; Asensio et al., 2013; Li et al., 2016; Newell-Litwa et al., 2009) but then divert from the canonical route toward the formation of conventional degradative lysosomes by Arl8- and Unc104/ KIF1A-mediated transport of these vesicles into the axon. Another, non-exclusive possibility is that PLVs following presynaptic delivery of AZ and SV proteins further mature within the periphery (e.g., by progressive acidification and acquisition of degradative enzymes) into retrogradely targeted lysosomes (Gowrishankar et al., 2017) that degrade malfunctional proteins in the neuronal soma. Future studies will be needed to address these possibilities.

Interestingly, we observe that AZ and SV proteins are partially co-transported along axons in live Drosophila larvae and mouse hippocampal neurons, presumably as an aggregate of PLVs (Tao-Cheng, 2007) held together by AZ proteins (Figures 7P and 7Q). Our data thus support the view that the presynapse is made, in part, as a pre-assembled functional unit with its main components, e.g., AZ and SV proteins and possibly others (for example, endocytic proteins), being transported on a common organelle (e.g., PLV) that delivers its content to the presynapse (Ahmari et al., 2000). It is important to note that not all BRP or Bsn puncta are co-transported with SV proteins in PLVs (see Figures 1 and 6) and that early-acting AZ scaffold assembly proteins, such as Syd-1 and Liprin-a, do not significantly accumulate in neuronal somata of arl8 mutant motoneurons. Axons, in addition to small clear SVs, also contain dense core granules, the components of which may conceivably be co-transported with a fraction of AZ proteins and may correspond to the previously described post-Golgi-derived (Maas et al., 2012) piccolobassoon transport vesicles for AZ proteins (Shapira et al., 2003; Zhai et al., 2001). How exactly PLVs relate to these piccolo-bassoon transport vesicles remains to be determined. Based on their size (about 70 nm), it is conceivable that PLVs are closely related to piccolo-bassoon transport vesicles, although PLVs do not always show a distinctive electron-dense core characteristic of piccolo-bassoon transport vesicles in mammalian neurons (see Figure 7J). Hence, it is possible that PLVs and piccolo-bassoon transport vesicles are distinct organelles that cooperatively mediate presynaptic biogenesis. Another non-exclusive possibility is that neurons may be able to regulate PLV cargo content depending on the developmental stage, the type of synapse, or their physiological status (Hallermann et al., 2010), resulting in transport vesicles of similar size but variable AZ and SV cargo protein content and thus of varying appearance. This would allow neurons to adapt presynaptic biogenesis to their needs for delivery of AZ, SV, or endocytic protein components.

While our data support an important role for the Arl8-Unc104/ KIF1A-dependent pathway of presynaptic biogenesis, we note that remnant presynaptic compartments are formed even in the absence of *arl8*. Recent data have indeed revealed additional kinesin adaptors, such as the Rab7-FYCO1 complex, that may partially overlap with the function of Arl8 in the transport of lysosome-related organelles (Raiborg et al., 2015). Although no functional role of FYCO1 in the nervous system has been described yet, this may be a fruitful area for future studies.

Finally, the identification of PLV packets as precursor organelles for presynaptic biogenesis also sheds new light on the intimate relationship between lysosomal alterations and neuronal dysfunction in human diseases (Luzio et al., 2014; Marks et al., 2013). For example, homozygous mutations in TBC1D23, a putative GTPase-activating protein regulated by Arl8, have been identified as a genetic cause for a non-degenerative form of pontocerebellar hypoplasia characterized by microcephaly, motor delay, and ataxia (Marin-Valencia et al., 2017). Whether and how these and other disorders are caused by defects in PLV-mediated presynaptic biogenesis will need to be investigated in future studies.

STAR*METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one video and can be found with this article online at https://doi.org/10.1016/j.neuron.2018.08.004.

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AUTHOR CONTRIBUTIONS

A.V., U.R., A.G.P., and S.R. performed all *Drosophila* imaging experiments and analyzed data. E.R. conducted all electrophysiological experiments. C.O. and D.V. analyzed hippocampal neurons and contributed biochemical experiments. C.Q., S.H., and D.P. performed electron microscopy and tomography. M.L. and D.P. contributed STED and correlative light electron microscopy. S.J.S. and V.H. designed the study aided by R.L., analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bruchpilot (Nc82), mouse, used 1:250	Developmental Studies Hybridoma Bank	Nc82
Cysteine String Protein (6D6), mouse, used 1:100	Developmental Studies Hybridoma Bank	6D6
Syntaxin (8C3), mouse, used 1:100	Developmental Studies Hybridoma Bank	8C3
Synapsin (3C11), mouse, used 1:100	Developmental Studies Hybridoma Bank	3C11
Stoned B, rabbit, used 1:500	V. Haucke lab	N/A
Dap160, rabbit, used 1:500	O. Shupliakov lab	N/A
Glutamate Receptor IID, rabbit, used 1:500	S.J. Sigrist lab	N/A
VGlut, rabbit, 1:500	H. Aberle lab	N/A
Synaptotagmin, rabbit, 1:1,000	BD Bioscience	N/A
Lamp1, rabbit, 1:100	Abcam	ab30687
Spinster, guinea pig, 1:500	P. Sweeney lab	N/A
CathepsinL, mouse, 1:200	R&D Systems	MAB22591
Futsch, mouse, 1:100	Developmental Studies	22C10
Disc large	Hybridoma Bank Developmental Studies	4F3
	Hybridoma Bank	
p62, rabbit, 1:250	G. Juhasz lab	N/A
UNC13A, guinea pig, 1:500	S.J. Sigrist lab	N/A
Synapse defective-1 (Syd-1), rabbit, 1:250	S.J. Sigrist lab	N/A
Liprin-α, rabbit, 1:500	S.J. Sigrist lab	N/A
Atg8a, rat, 1:250	G. Juhasz lab	N/A
ATPSynthase	Abcam	14748
GFP (mouse, rabbit, chicken) 1:500	Invitrogen/Life Technologies	N/A
HRP-Alexa647 (conjugated antibody), 1:500	Jackson ImmunoResearch	123-605-021
Atto647 (anti-mouse) 1:500 used for STED	Atto-Tec	N/A
Bassoon (mouse, monoclonal, used at 1:400 in IF)	Abcam	Ab82958
RFP (rabbit, polyclonal, used at 1:400 in IF)	MBL	PM005
GFP (chicken, polyclonal, used at 1:2000 in IF)	Abcam	ab13970
Ankyrin G made in mouse (used at 1:200 in IF)	Thermo Scientific	33-8800
Arl8A&B (mouse, monoclonal, used at 1:50 in IF)	Santa Cruz	398635 (H-8)
Lamp1 (rat, monoclonal, used at 1:400 in IF)	BD Biosciences	Clone1D4B
Myc (polyclonal, mouse, used at 1:200 in IF)	V. Haucke lab	N/A
Alexa488 goat anti-chicken (used at 1:800 in IF)	Abcam	ab150169
Alexa 647 goat anti-mouse (used at 1:800 in IF)	Invitrogen/Life Technologies	A21235
Alexa 568 donkey anti-mouse (used at 1:800 in IF)	Invitrogen/Life Technologies	A10037
Alexa 647 goat anti-rat (used at 1:800 in IF)	Invitrogen/Life Technologies	A21247
Rabbit anti-myc (used at 1:1,000 in WB)	Abcam	ab9106
Mouse anti-GFP (used at 1:2,500 in WB)	Clontech	632381
Goat anti-rabbit IRD800 (used at 1:10,000 in WB).	LI-COR Biosciences	925-32210
Goat anti-mouse IRDye800 (used at 1:10,000 in WB).	LI-COR Biosciences	925-32211
Alexa 488 goat anti-mouse (used at 1:500 in IF)	Invitrogen	A11029
Alexa 568 goat anti-rabbit (used at 1:500 in IF)	Invitrogen	A11011
Chemicals, Peptides, and Recombinant Proteins		
siRNA smart pool mouse arl8a	Dharmacon	68724

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CellPress

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
HEK 293T cells	ATCC	CRL-3216
Experimental Models: Organisms/Strains		
w1118 as wild type	Bloomington	N/A
OK6-GAL4, UAS-BRP-D3-Straw (UAS-BRP is a truncated BRP from aa 473-1227)	Sigrist lab, see Fouquet et al., 2009	N/A
UAS-Lamp1-GFP	Krämer lab (UT Southwestern, Dallas, TX, USA)	N/A
UAS-BRP-D3-GFP/OK6-GAL4;	Ok6-Gal4: Bloomington	#64199
UAS-Spinster-RFP/+;	Sweeney lab (Univ. of York, UK)	N/A
OK6-GAL4,UAS-BRP-D3-Straw/UAS-Arl8-GFP	UAS-Arl8-GFP: Sigrist lab	N/A
OK6-GAL4/UAS-Lamp1-GFP	as above	N/A
PBac(RB)Gie ^{e00336} /PBac(RB)Gie ^{e00336}	Bloomington	#17846
OK6-GAL4/UAS-D3-Straw, PBac(RB)Gie ^{e00336}	as above	N/A
OK6-GAL4/UAS-Arl8; PBac(RB)Gie ^{e00336} /PBac(RB)Gie ^{e00336}	as above	N/A
OK6-GAL4/UAS-Arl8	as above	N/A
UAS-BRP-D3-Straw/OK6-GAL4,UAS-Rab7-GFP	UAS-Rab7: Bloomington	#42705
UAS-BRP-D3-Straw/OK6-GAL4,UAS-Rab7-GFP	as above	N/A
OK6-GAL4/UAS-Arl8-GFP	as above	N/A
UAS Spinster/+, OK6-GAL4-UASDicer2/+	UAS-Dicer2: VDRC	N/A
UAS Spinster/+, OK6-GAL4-Dicer2/+	as above	N/A
OK6-GAL4-Dicer2/UAS-Arl8-RNAi	UAS-Arl8-RNAi: VDRC	#26085
OK6-GAL4-UAS-Dicer2/UAS-VPS39-RNAi	UAS-VPS39 RNAi: VDRC	#40427
OK6-GAL4/+; PBac(RB)Gie ^{e00336} /PBac(RB)Gie ^{e0033}	as above	N/A
Oligonucleotides		
Fwd (Kpnl): 5'-GACTGGTACCGAATGTTGGCCCTCATCA ACAGGATCCTC-3'	MWG	N/A
Rev (Notl): 5'-AGTGCGGCCGCCAACGACTTTGGCTTTTC GAATGTTGAATTAAC-3'	MWG	N/A
Recombinant DNA		
Gateway entry vector pENTR4 (Invitrogen)	Addgene	#17424
pUASt-destination vector (pTW, pTWG)	DGRC	1129, 1076
cDNA encoding full length Drosophila Arl8	DGRC	LD29185
GFP-Basson 95-3938	Eckardt D. Gundelfinger (LIN, Magdeburg)	Dresbach et al., 2006
Arl8b-HA pcDNA3	This study	N/A
Arl8b-mCherry pcDNA3	This study	N/A
vGlut1-mCherry	Franck Polleux (Columbia university, New York)	Kwon et al., 2016
Lamp1-mCherry	Addgene	#79998; Shcherbakova et al., 2016
vGlut1-VENUS	Etienne Herzog (FBN, Bordeaux)	Herzog et al., 2011
Lamp1- miRFP703	Addgene	#79998
Rab7-EGFP	Peter van der Sluijs (UMC, Utrecht)	N/A
EGFP-Synapsin1a	Hung-Teh Kao (Brown University, Rhode Island)	N/A
dArl8-GFP	Sean Munro, (MRC LMB Cambridge, UK)	N/A
dUnc104/imac-myc	Thomas Schwarz (Children's Hospital, Boston)	clone 6-4-1

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
ImageJ	Open Source Software	https://imagej.net/Welcome
ImageJ Coloc 2	Open Source Software	https://imagej.net/Welcome
ImageJ Plugin KymoAnalyzer v.1.01	Open Source Software	https://imagej.net/Welcome

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Volker Haucke (haucke@fmp-berlin.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster

Fly strains were reared under standard laboratory conditions and raised at 25°C and 70% humidity on semi-defined medium (Bloomington recipe). For RNAi and Arl8 overexpression experiments flies were kept at 29°C. For electrophysiological recordings, only male larvae were used, for all other experiments both male and female animals were used. See Key Resources Table for genotypes and strains used.

Mice

All animal experiments involving mice (C57/BL6) were reviewed and approved by the ethics committee of the "Landesamt für Gesundheit und Soziales" (LAGeSo) Berlin and were conducted accordingly to the committee's guidelines.

- Health/immune status: The animals have a normal health and immune status. The animal facility where the mice are kept is regularly checked for standard pathogens. The health reports can be provided upon request.
- Mice used for all experiments were naive. No drug tests were done.
- Sample size estimation: No estimation of simple size was done as sample sizes were not chosen based on pre-specified effect size. Instead, multiple independent experiments were carried out using several biological replicates specified in the legends to figures.
- How subjects/samples were allocated to experimental groups: Hippocampal neurons from several newborn mice of identical genotype from the same litter were pooled and analyzed. For neurons from wild-type mice neuronal cultures were randomly allocated to different treatments (drugs, transfection with plasmids etc.).
- Gender of subjects or animals: Mice from both genders were used.
- Neuronal cultures were prepared by surgically removing the hippocampi from postnatal mice (male or female) at p1-5, followed by trypsin digestion to dissociate individual neurons. Cultures were grown in MEM medium (Thermo Fisher) supplemented with 5% FCS and 2% B-27. 2 μM AraC was added to the culture medium at 2 days *in vitro* (DIV) to limit glial proliferation. Cells were transfected at DIV 3 or 6 using a Calcium Phosphate transfection kit (Promega).

HEK293T Cells

HEK293T cells were obtained from ATCC (#CRL-3216). Cells were cultured in DMEM with 4.5g/L glucose (Lonza) containing 10% heat-inactivated FBS (GIBCO) and 100 U/ml penicillin, 100 μ g/ml streptomycin (GIBCO) during experimental procedures. HEK293T cells have a complex karyotype but due to the presence of multiple X chromosomes and the lack of any trace of Y chromosome derived sequence likely are of female origin.

METHOD DETAILS

Molecular Cloning of Constructs for Production of Transgenic Flies

cDNA encoding full-length *Drosophila* Arl8 (LD29185) was obtained from *Drosophila* Genomics Resource Center (DGRC). Primers used for amplification and insertion into the Gateway entry vector pENTR4 (Invitrogen) are listed in the Key Resources Table.

For production of transgenic flies, the gene cassette containing Arl8 cDNA was then transferred to Gateway destination vector pTW (no tag or GFP tag) following manufacturer's instructions (Invitrogen). In brief, entry clone, destination vector and TE buffer were mixed at room temperature and incubated for 20 min. The Clonase enzyme mix was then added and the whole reaction was incubated for 1 hr at 25°C. The reaction was terminated by addition of Proteinase K for 10 min at 37°C. Chemically competent *E.coli* Top10 cells were then transformed and grown at 37°C. Miniprep DNA preparations of positive clones were sent for injection into w1118 fly embryos to BestGene (USA).

RT-PCR from Drosophila Larvae Tissue

cDNA was extracted from whole larvae of 5 animals/genotype (wild-type and arl8 mutant). After smashing the larvae, cells were solubilized in 1 mL TRIzol reagent (Life Technologies). After 5min incubation at RT, 200 μ L chloroform was added to the mixture and the tube was shaken vigorously for 15 s, let stand for 3 min and spun at 4°C for 15min at 12,000xg. The upper, aqueous phase was then transferred to a fresh plastic tube. 500 μ L isopropanol was then added and the tube was flipped several times prior to 10 min incubation at RT and centrifugation at 4°C for 10min at 12,000xg. The supernatant was discarded and the resulting pellet was first washed with 1ml 75% ethanol and then spun at 4°C for 5min at 12,000xg. The supernatant was discarded again and the pellet was allowed to air-dry. It was then solubilized in 50 μ L Millipore water and placed in a heat-block at 60°C for 10min. The amount of thus extracted RNA was measured by UV spectroscopy. To obtain cDNA from mRNA, samples were immediately used for reverse transcription PCR (RT-PCR), whereby a complementary DNA strand is synthetized on an RNA template (first-strand synthesis) which can then be used as coding sequence for PCR amplification. 11 μ L of the extracted RNA was mixed with 1 μ L dNTPs (Superscript III RT kit) and 1 μ L polyTprimers (GIBCO). The RNA was denatured by incubating the mix at 65°C for 5min followed by cooling for 1min on ice to allow primer annealing. The reverse transcription was performed using the Superscript III RT kit (Life Technologies) by adding 4 μ L of 5 × RT buffer and 1 μ L each of 0.1 M DTT, RNAsin and Superscript III RT to the mix and incubating it for 5 min at 25°C and then 45 min at 50°C. The obtained cDNA was stored at -20°C until use. cDNA was used for PCR, primers are listed in the Key Resources Table.

Co-immunoprecipitation of Proteins from HEK293T Cells

HEK293T cells were obtained from ATCC (#CRL-3216). Cells were cultured in DMEM with 4.5g/L glucose (Lonza) containing 10% heat-inactivated FBS (GIBCO) and 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO) during experimental procedures. Cells were routinely tested for mycoplasma contamination. *Drosophila* dArl8-GFP (a kind gift from Dr. Sean Munro, MRC LMB Cambridge, United Kingdom) and dUnc104/imac-myc (imac/unc-104 (clone 6-4-1, kindly provided by Dr. Thomas Schwarz Children's Hospital Boston, USA) were transfected into HEK293T cells using calcium phosphate. 48h post-transfection cells were washed twice with ice-cold PBS, lyzed in ice cold lysis buffer (0.1% saponin, 20 mM HEPES pH7.4, 130 mM NaCl, 2 mM MgCl₂, 10 mNaF, protease inhibitor cocktail from Sigma) and protein concentrations were measured by Bradford assay. As input for each immunoprecipitation (IP) 3 mg total protein was used. For IP of dUnc104/imac-myc 10 μL myc-trap magnetic bead slurry (Chromo Tek, myc-TRAP_MA) was used. Protein lysates and myc-trap beads were incubated on a rotating wheel for 2h at 4°C. Afterward, beads were washed five times in lysis buffer, boiled for 5 min in Laemmli buffer and lysates were loaded on SDS-PAGE (8%) for analysis. Immunoblotting was done on nitrocellulose membranes. Nitrocellulose membranes were incubated with the primary antibodies (rabbit anti-myc, Abcam and mouse anti-GFP, Clontech) at 4°C overnight. On the next day, primary antibodies were detected by goat anti-rabbit IRD800 and goat anti-mouse IRDye800 (LI-COR Biosciences, 925-32210 and 925-32211, 1:10000). Immunoblots were scanned on an LI-COR Odyssey fluorescent reader.

Immunostaining of Drosophila Larvae

Larval filets were dissected and stained as previously described (Owald et al., 2010). For NMJ stainings, filets were fixed in 4% PFA for 10 min, except for aUNC13A, which is fixed for 10 min in MeOH. For brains/axon, filets were fixed with Bouin for 10 min for better antibody penetration. Larvae were mounted in ProLong Gold (Thermofischer) or MOWIOL (SigmaAldrich). The HRP signal was used as template for a mask, restricting the quantified area to the shape of the NMJ, quantification following (Andlauer and Sigrist, 2012). For brains the mean fluorescence intensity of cortex area only was quantified.

For STED, larval filets were fixed with ice-cold methanol for 5 or 10 min (following the protocol above). Larvae were mounted in ProLong Gold (Thermofischer) or MOWIOL (SigmaAldrich).

NMJ Outgrowth Analysis

Immunostainings of larval filets (L3) were performed for HRP and p62 (to visualize the muscle). Confocal images were taken of abdominal segments A2-A5, always 4 segments/animal and 8 animals/genotype (wild-type, arl8 mutant). Innervating NMJs (one NMJ/muscle) on muscle 6/7, 4, 2 and 1 were counted and represented in [%].

Confocal Microscopy Analyses of Drosophila

Confocal microscopy was performed with a Leica SP8 microscope (Leica Microsystems, Germany). Images of fixed and live samples were acquired at room temperature. Confocal imaging of NMJs and VNC was done using a z-step of 0.25 µm. The following objective was used: 63 × 1.4 NA oil immersion for confocal imaging, 100 × 1.4 NA for STED. All images were acquired using the LAS X software (Leica Microsystems, Germany). Images from fixed samples were taken from 3rd instar larval NMJs (segments A2-A5) or VNCs. Images for figures were processed with ImageJ software to enhance brightness using the brightness/contrast function. If necessary, images were smoothened (0.5 pixel Sigma radius) using the Gaussian blur function. Confocal stacks were processed with Fiji (http://fiji.sc) (Schindelin et al., 2012). Quantifications of AZs (scored via BRP) were performed following an adjusted manual (Andlauer and Sigrist, 2012), briefly as follows. The signal of a HRP-Cy5 antibody was used as template for a mask, restricting the quantified area to the shape of the NMJ. The original confocal stacks were converted to maximal projections, and after background subtraction, a mask of the synaptic area was created by applying a certain threshold to remove the irrelevant lower intensity pixels. The segmentation of

single spots was done semi-automatically via the command "Find Maxima" embedded in the Fiji and by hand with the pencil tool and a line thickness of 1 pixel. To remove high-frequency noise a Gaussian blur filter (0.5 pixel Sigma radius) was applied. The processed picture was then transformed into a binary mask using the same lower threshold value as in the first step. This binary mask was then projected onto the original unmodified image using the "min" operation from the ImageJ image calculator. For sum / total intensities all intensities of the corresponding channel of one NMJ were added up, n represents the number of NMJs, For STED, deconvolution was performed with Huygens Software using the deconvolution wizard setting background value and signal to noise ratio manually.

In Vivo Live Imaging and Analysis of Drosophila Larvae

In vivo imaging of intact *Drosophila* larvae was performed as previously described (Füger et al., 2007). Briefly, third instar larvae were put into a drop of Voltalef H10S oil (Arkema, France) within an airtight imaging chamber. The larvae were anaesthetized before imaging with 10 short pulses of a desflurane (Baxter,IL, UAS) air mixture until the heartbeat completely stopped. Axons immediately after exiting the ventral nerve cord were imaged using confocal microscopy. Kymographs were plotted using a custom-written Fiji script.

Transfection of Hippocampal Neurons in Culture

Neuronal cultures were prepared by surgically removing the hippocampi from postnatal mice at postnatal day 1-2, followed by trypsin digestion to dissociate individual neurons. Cultures were grown in MEM medium (Thermo Fisher) supplemented with 5% FCS and 2% B-27 and L-Glutamine. 2μ M AraC was added to the culture medium at 2 days *in vitro* (DIV) to limit glial proliferation. Cells were transfected at DIV 3 using a Calcium Phosphate transfection kit (Promega), for knockdown of Arl8A, overexpression of Arl8B-HA or Arl8B-mCherry and the expression of AZ and SV proteins together with Lamp1, Arl8B or Rab7 for live imaging (see below). For transfection 1 μ g plasmid DNA, 250 mM CaCl₂ and water (for each well of a 12-well plate or 2μ g plasmid DNA for each well of a 6-well plate for live cell imaging) were mixed with equal volume of 2x HEPES buffered saline (50 μ L per) and incubated for 20 min allowing for precipitate formation, while neurons were starved in NBA medium for the same time at 37° C, 5% CO₂. For knock down experiments 500nM siRNA (Arl8A smart pools from Dharmacon) was additionally included and precipitated together with the plasmid DNA. Precipitates were added to neurons and incubated at 37° C, 5% CO₂ for 30 min. Finally, neurons were washed twice with HBSS medium and transferred back into their conditioned medium.

Immunostaining of Hippocampal Neurons in Culture

Cultured hippocampal neurons were fixed at DIV 7.5 or DIV8 using 4% (w/v) paraformaldehyde (PFA) and 4% sucrose in PBS for 15 min at RT. Fixed neurons were blocked and permeabilized with PBS containing 10% (v/v) normal goat serum and 0.1% Triton X-100 for 30 min, followed by incubation with primary antibodies (anti-GFP, Abcam and anti-AnkyrinG, Thermo Scientific or anti-Bassoon, Abcam and anti-RFP, MBL) in 5% (v/v) normal goat serum and 0.1% Triton X-100 in PBS overnight at 4°C. Unbound primary antibody was removed with PBS while bound was detected with corresponding secondary antibodies (coupled to Alexa Fluor 488, 568 and 647, Life technologies) for 45 min in 5% (v/v) normal goat serum in PBS plus 0.1% Triton X-100. Neuronal nuclei were visualized with DAPI (0.02 μ g/mL in H₂O). Coverslips were mounted with Immumount (Thermo-Fisher).

Confocal Imaging of Hippocampal Neurons

Imaging of DIV7.5 and DIV8 hippocampal neurons was performed with a Zeiss Axiovert 200M equipped with the Perkin-Elmer Ultra View ERS system and a Hamamatsu C9100 EM-CCD camera under the control of Volocity software (Perkin-Elmer). Fluorescent intensities were quantified using ImageJ by the use of custom-written macros. Proximal axons were identified by Ankyrin G staining.

Live Imaging of Hippocampal Neurons in Culture

Cultured hippocampal neurons were transfected at DIV3 as described above. The following plasmids were co-transfected at DIV3: (1) eGFP-Bsn (95-3938) together with vGlut1-mCherry and Lamp1-miRFP703 or (2) eGFP-Bsn (95-3938) and Lamp1-mCherry or (3) eGFP-Bsn (95-3938) and vGlut1-mCherry or (4) vGlut1-VENUS and Lamp1-mCherry or (5) EGFP-Rab7 and Arl8B-mCherry. Axonal co-trafficking was measured at DIV4. Neurons were imaged in basic buffer (170mM NaCl, 3.5mM KCl, 0.4mM KH₂PO₄, 20mM N-Tris [hydroxyl-methyl]-methyl-2-aminoethane-sulphonic acid (TES), 5mM NaHCO₃, 5mM glucose, 1.2mM Na₂SO₄, 1.2mM MgCl₂, 1.3mM CaCl₂) using a spinning disc confocal microscope (CSU-X1, Nikon), equipped with an incubation chamber (37°C), a 60x objective (water, Nikon), a EMCCD camera (AU-888, Nikon). Images were acquired with 16-bit sampling a frame rate of 2 s for a total time of 300 s (laser lines: 488 nm, 561 nm; 638nm; exposure times: 200 ms each). Analysis and kymographs were done with ImageJ using the KymoAnalyzer v1.01 plugin.

Cultured hippocampal neurons were transfected with GFP-Bsn (95-3938) and Arl8B-mCherry at DIV6 and used for live cell imaging at DIV9.

Here, a Nikon Eclipse Ti microscope was used, equipped with an incubation chamber (37°C), a 60x TIRF objective (oil-immersion, Nikon), a sCMOS camera (Neo, Andor), a 200 W mercury lamp (Lumen 200, Prior), and a dual-color TIRF setup in epifluorescent mode (laser lines: 488 nm, 568 nm; exposure times: 200 ms each), all operated by open-source ImageJ-based micromanager software. Images were acquired with a frame rate of 2 s for a total time of 60 s.

For fluorescence recovery after photobleaching (FRAP) the following plasmids were co-transfected as described above: (1) Lamp1-mCherry and eGFP-Bsn(95-3938), (2) Arl8b-mCherry and synapsin1a-eGFP and (3) Lamp1-mCherry and synapsin1a-eGFP. Axon terminals and synaptic boutons were imaged at DIV6 or 8 respectively. The same basic buffer as above was used for imaging. Images were acquired with 16-bit sampling using a Zeiss LSM 710 confocal laser scanning microscope equipped with an incubation chamber (37°C). For FRAP, the 488 nm line of the argon laser and the 561 nm line of the DSSP laser was used in combination with a Plan-Apochromat × 63/1.40 oil DIC objective. After 10 s of baseline recording, both channels were bleached and a total time of 300 frames was acquired with 1 s interval. ImageJ was used for image processing.

Pearson's Correlation Coefficient in Hippocampal Neurons and Fly VNC

Cultured hippocampal neurons were transfected at DIV3 as described above with Arl8b-mCherry and Lamp1-miRFP703. Wild-type hippocampal neurons and transfected neurons were fixed on DIV4 using 4% (w/v) PFA in PHEM (pH = 6.9) buffer for 10 min at RT. Fixed wild-type neurons were blocked and permeabilized with PBS containing 0.5% (w/v) BSA and 0.05% Tween-20 for 5 min, followed by blocking with PBS containing 0.5% (w/v) BSA for 1h at RT. Afterward, coverslips were incubated with primary antibodies (Arl8A&B, Santa Cruz and Lamp1, BD Biosciences) in 0.5% (w/v) BSA and 0.05% Tween-20 in PBS overnight at 4°C. Unbound primary antibody was removed with PBS while bound was detected with corresponding secondary antibodies (coupled to Alexa Fluor 568 and 647, Life technologies) for 1h at RT in in 0.5% (w/v) BSA and 0.05% Tween-20 in PBS. Neuronal nuclei were visualized with DAPI (0.02 μ g/mL in PBS). Coverslips were mounted with Immumount (Thermo-Fisher). A spinning disc confocal microscope (CSU-X1, Nikon), with a 63x objective (oil, Nikon) and an EMCCD camera (AU-888, Nikon) was used for image acquisition of neuronal somata. Confocal stacks of whole somata were acquired for analysis. Pearon's correlation coefficients for overexpressed and endogenous proteins were determined with Coloc 2 in ImageJ. As control one channel was flipped horizontally to rule out random co-localization.

For fly VNCs staining was performed as described above. A z stack was acquired and one focal plane choosen for analysis after ROI selection of a single cell. ImageJ Coloc_2 was used to determine the Pearson'R above threshold. As control one channel was flipped horizontally to show random co-localization of the image. Analyzed where overall 9-15 cells of 2-3 VNCs.

Staining of HeLa Cells

Anti-myc made in mouse (self-made, 1:200); Alexa568 goat anti mouse (Invitrogen, A11031, 1:400).

3D-Time-Gated STED Imaging

STED imaging with time-gated detection was performed on a Leica SP8 TCS STED microscope (Leica Microsystems) equipped with a pulsed white light excitation laser (WLL; ?80 ps pulse width, 80 MHz repetition rate; NKT Photonics) and two STED lasers for depletion at 592 nm and 775 nm. The pulsed 775 nm STED laser was triggered by the WLL. Three-channel STED imaging was performed by sequentially exciting Alexa 488, Cy3 and ATTO647N at 488 nm, 568 nm, and 646 nm, respectively. Emission from Alexa 488 was depleted with 592 nm, whereas the 775 nm STED laser was used to deplete both Cy3 and ATTO647N. Time-gated detection was set from 0.3–6 ns for all dyes. Fluorescence signals were detected sequentially by hybrid detectors at appropriate spectral regions separated from the STED laser. Single optical slices were acquired with an HC PL APO CS2 100 × /1.40-N.A. oil objective (Leica Microsystems), a scanning format of 1,024 × 1,024 pixel, 8 bit sampling, and 6 fold zoom, yielding a voxel dimension of 18.9x18.9 nm. To minimize thermal drift, the microscope was housed in a heatable incubation chamber (LIS Life Imaging Services). BRP rings were measured from planar oriented Active Zones using a custom written Fiji script.

FM1-43 Dye Uptake in Drosophila Larvae

The FM dye uptake experiment was performed by Stefanie Nunes Rosa following the protocol published previously (Verstreken et al., 2008). In short, 3rd instar larvae were dissected as described above and NMJs on muscle 4, segments A2 and A3 were analyzed. The larval filets were first stimulated with high K+ saline containing 4μ M FM1-43 dye for 1 min and washed (3x, 5min) with Ca²⁺-free saline. Ca²⁺ was added to extracellular haemolymph-like saline HL-33 (70 mM NaCl, 5 mM KCl, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, 5 mM Hepes, and 1 mM CaCl₂, pH 7.2) to a final concentration of 1.5mM.

For image acquisition, a 40x water immersion lens on an upright epifluorescence Olympus BX51WI microscope (equipped with a Hamamtsu Orca ER cooled CCD digital camera and a Visitron lambda DG-4 ilumination system) was used. Single optical slices of boutons were acquired and their fluorescence intensity was measured by drawing a region of interest (ROI) around boutons and subtracting the background intensity. For each NMJ a mean value was established and subsequently normalized to wild-type levels. ImageJ was used for image processing and GraphPad Prism5 for statistical analysis.

Electron Microscopy

Drosophila larval brains were dissected, fixed with glutaradehyde, postfixed with osmium tetroxide, dehydrated in methanol and embedded into Epoxy resin. Following polymerization, \sim 60 nm sections were collected and contrasted for transmission electron microscopy morphometric analysis. Long vesicle diameter (Figure 7L) was measured with ImageJ, mitochondrial and PLV volume fraction in the cytoplasm was analyzed by superimposing a grid over the neuronal somata according to stereology routine. For tomography 3D reconstructions \approx 200 nm sections were collected on coated slotted grids and imaged with a Tecnai F20 TEM.

10 nm gold particles were used as fiducials. Tomograms were built with a help of IMOD and ETomo software. 3D reconstructions were rendered with the help of Microscopy image browser and Imaris.

For immunoelectron microscopy brains were fixed in 4% formaldehyde for 20 min, stained by methylene blue cryoprotected in 2.3M sucrose and plunge-frozen on pins for Tokuyasu sectioning (Tokuyasu, 1973).

For immunogold labeling, ultrathin sections were collected on coated grids, blocked, and stained by BRP last 200 primary and 10 nm gold coupled secondary antibodies. After washing, sections were contrasted and covered by polyvinyl alcohol and tungstosilicic acid hydrate. Immunolabelling for gSTED imaging was performed on 150nm cryosections that were collected on acid cleaned and silanized high precision coverslips. Coverslips were blocked in PBS containing 1% BSA and 0.1M Glycine. Staining was performed with anti-BRP (last 200, 1:500) and Rabbit α Synaptotagmin-1 (1:500) followed by secondary antibodies α Guinea pig Alexa488 (Life Technologies), α Rabbit Atto647N (Active Motif), Cy3 α HRP antibody (Jackson ImmunoResearch 1:250) and Hoechst 33258 (Life Technologies, 5ug/ML). Samples were mounted in Prolong Gold and cured for 24h at room temperature.

Live Imaging of Vesicle pH Quenching in Drosophila Larvae

Larvae were dissected in Ca²⁺-free HL3 and mounted in a Sylgard block (184, Dow Corning, Midland, MI, USA) to be transferred to a recording chamber containing 200 μ L HL3 for imaging on an upright microscope (Olympus BX51WI). Imaging was performed using a water-immersion 40x objective (UAPO 40x 1.15). Muscle 4 of abdominal segment 3 was imaged during 60 s (50 ms, acquisition rate 10 Hz). After 15 s of recording 1,5M NH₄Cl was added to the chamber.

Electrophysiological Analysis of Drosophila Larvae

Two-electrode voltage clamp (TEVC) recordings were performed essentially as previously described (Qin et al., 2005). All experiments were performed on male, third-instar larval NMJs (muscle 6 of abdominal segments A2/A3), raised on semi-defined medium (Bloomington recipe) at 25°C. Recordings were made from cells with an initial V_m between -50 and -70mV and input resistances of $\ge 4 M\Omega$, using intracellular electrodes with resistances of 10-25 M Ω , filled with 3M KCI. eEJCs were recorded at a voltage clamp of -60 mV and mEJCs were recorded at a voltage clamp of -80 mV. The eEJCs were low-pass filtered at 5kHz and sampled at 10kHz. Larvae were dissected in Ca²⁺-free haemolymph-like solution (HL3; Stewart et al., 1994; Composition (in mM): NaCl 70, KCl 5, MgCl₂ 20, NaHCO₃ 10, trehalose 5, sucrose 115, HEPES 5, pH adjusted to 7.2). The bath solution was HL3 containing 1.5 mM CaCl₂. The exemplary traces are averaged traces unless otherwise noted. The stimulation artifact of eEJCs was removed for clarity. Data were analyzed using Clampfit version 10.7.0.3. (Molecular Devices, LLC, Sunnyvale, CA, USA, 2016). Data are presented as mean \pm SEM, n indicates the number of cells examined.

Experimental Design

A strategy for randomization, stratification or blind selection of samples has not been carried out. Sample sizes were not chosen based on pre-specified effect size. Instead, multiple independent experiments were carried out using several sample replicates as detailed in the figure legends.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantified data were analyzed using the GraphPad Prism6 software: Mann-Whitney test and Student's t test were used for experiments with two genotypes and one-way analysis of variance (ANOVA) for experiments with more than two. Data are reported as mean \pm SEM unless stated otherwise and n represents the number of samples analyzed. Significance is denoted using asterisks *p < 0.05, **p < 0.01, ***p < 0.001 and p > 0.05 is not significant (ns).