Dynamin photoinactivation blocks Clathrin and α-adaptin recruitment and induces bulk membrane retrieval

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Dynamin is a well-known regulator of synaptic endocytosis. Temperature-sensitive dynamin (shi61) mutations in Drosophila melanogaster or deletion of some of the mammalian Dynamins causes the accumulation of invaginated endocytic pits at synapses, sometimes also on bulk endosomes, indicating impaired membrane scission. However, complete loss of dynamin function has not been studied in neurons in vivo, and whether Dynamin acts in different aspects of synaptic vesicle formation remains enigmatic. We used acute photoinactivation and found that loss of Dynamin function blocked membrane recycling and caused the buildup of huge membrane-connected cisternae, in contrast to the invaginated pits that accumulate in shi61 mutants. Moreover, photoinactivation of Dynamin in shi61 animals converted these pits into bulk cisternae. Bulk membrane retrieval has also been seen upon Clathrin photoinactivation, and superresolution imaging indicated that acute Dynamin photoinactivation blocked Clathrin and α-adaptin relocalization to synaptic membranes upon nerve stimulation. Hence, our data indicate that Dynamin is critically involved in the stabilization of Clathrin- and AP2-dependent endocytic pits.

Introduction

During intense neuronal activity, synapses need to recycle synaptic vesicles to maintain neuronal communication. The best studied route for vesicle regeneration in neurons is Clathrin-mediated endocytosis (CME) but also bulk membrane uptake participates to recycle membrane (Holt et al., 2003; LoGiudice and Matthews, 2006; Watanabe et al., 2013; Winther et al., 2013). During CME, lipids, adaptors, and accessory proteins initiate vesicle formation (McMahon and Boucrot, 2011). In parallel, Clathrin, polymerized into cages, serves as a “mold” for new synaptic vesicles, preventing excessive membrane uptake and ensuring uniform vesicle size (Heerssen et al., 2008; Kasprowicz et al., 2008). Although numerous proteins have been implicated in CME, the exact molecular mechanisms by which these components coordinate the formation of new vesicles remains incompletely understood.

Dynamin is a large GTPase that is implicated in vesicle endocytosis in neuronal and nonneuronal cells (van der Bliek and Meyerowitz, 1991; Liu et al., 1998, 2011; Damke et al., 2001; Marks et al., 2001; Chen et al., 2002; Narayanan et al., 2005; Macia et al., 2006; Aguet et al., 2013). The protein binds to accessory endocytic proteins and lipids (David et al., 1996; Okamoto et al., 1999; Qualmann et al., 1999; Simpson et al., 1999; Bethoney et al., 2009; Sundborger et al., 2011), and in GTP-bound state, it assembles into rings at the base of deeply invaginated pits (Chen et al., 2002; Macia et al., 2006; Liu et al., 2011). GTP hydrolysis then induces conformational changes (Roux et al., 2006; Bashkirov et al., 2008; Chappie et al., 2010; Ford et al., 2011) that result in the separation of the vesicle and plasma membrane (McNiven et al., 2000); at the synapse, this process generates new vesicles that can participate in a new round of neurotransmitter release (Ferguson and De Camilli, 2012). Consistent with a role in membrane scission in vitro, temperature-sensitive dynamin fly mutants...
Figure 1. Photoinactivation of Dynamin blocks synaptic vesicle recycling. (A) Genomic dynamin construct tagged in the middle domain with a Flag-tetra-cysteine tag (shl-4C), PH, Pleckstrin homology; PRD, Proline-rich domain; GED, GTPase effector domain. (B–D) FlAsH fluorescence after incubation of yw controls [B] and shl12-12B; shl-4C [C] third instar fillets in FlAsH reagent shows labeling only in boutons of animals expressing Shi-4C [C]. (D) Anti-Dynamin

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(shi<sup>+/−</sup>) that were stimulated at restrictive temperature show a depletion of synaptic vesicles and numerous deeply invaginated pits that are stuck at the plasma membrane (Koenig and Ikeda, 1989; Chen et al., 1991, 2002; van der Bliek and Meyerowitz, 1991). Likewise, in mammalian neurons in which either one or two of the three Dynamin isoforms were deleted, a massive accumulation of invaginated pits is observed (Ferguson et al., 2007; Hayashi et al., 2008; Raimondi et al., 2011; Park et al., 2013). These studies in neurons indicate that a critical role for Dynamin in synaptic vesicle formation is to facilitate membrane fission. However, these genetic conditions in mice and flies may still reveal some aspects of Dynamin function, and it is currently unclear whether low levels of the second or third Dynamin isoform in mice or the presence of the temperature-sensitive Dynamin protein (Shi<sup>+/−</sup>) in flies at a restrictive temperature is concealing potential additional functions for the protein in synaptic vesicle formation.

In this work, we use acute and specific fluorescein-assisted light inactivation (FALI) of Dynamin to study endocytosis in the absence of Dynamin function. Surprisingly, loss of Dynamin function does not cause the accumulation of deeply invaginated pits, as seen in shi<sup>+/−</sup> mutants; instead, it results in the accumulation of giant bulk cisternae that can fill entire synaptic boutons and do not significantly participate in the synaptic vesicle cycle. Similarly, photoactivation of Dynamin in shi<sup>+/−</sup> mutants at restrictive temperature does not result in the accumulation of invaginated pits but in the formation of massive membrane-attached bulk cisternae, indicating that shi<sup>+/−</sup> retains the ability to prevent bulk endocytosis. Furthermore, these defects in membrane uptake are very reminiscent of those seen in clathrin mutant synapses with photoactivated Clathrin, and we therefore used superresolution imaging to localize Clathrin heavy chain (Chc) and the Clathrin adaptor subunit α-adaptin (α-Ada) at stimulated synaptic boutons. In contrast to controls, at neuromuscular junctions (NMJs) in which Dynamin was photoactivated, Chc and α-Ada both fail to relocate to and concentrate in the bouton periphery close to the presynaptic plasma membrane. Our data indicate that Dynamin is critical to coordinate Clathrin- and α-Ada–dependent steps during synaptic vesicle budding, and this function is retained in temperature-sensitive shi<sup>+/−</sup> mutants.

**Results**

**Synapses with photoactivated Dynamin do not maintain neurotransmitter release during intense stimulation**

Whereas mammals harbor three dynamin genes, *Drosophila melanogaster* harbors only one, *shi* (shibire; Cao et al., 1998). shi-null mutants (shi<sup>+/−;12-12B</sup>) die as embryos (Grant et al., 1998), precluding us from analyzing defects in synaptic function. To circumvent issues with early lethality or development, we resorted to acute FLAsH (4′,5′-bis[1,3,2-dithioarsolan-2-yl] fluorescein)-mediated FALI (FLAsH-FALI; Marek and Davis, 2002), a technology already used to inactivate Synaptotagmin I (Poskanzer et al., 2003), Chc (Kasprowicz et al., 2008), and Clathrin light chain (Cíc; Heerssen et al., 2008) at the *Drosophila* NMJ. Using recombineering in *Escherichia coli*, we retrieved the *shi* gene from BACR32K23 into P[acman] (Venken et al., 2006), and we then recombineered a 16–amino acid tetracysteine (4C) tag into the middle domain of Dynamin (Shi-4C; Fig. 1 A; Venken et al., 2008). We tagged the middle domain of Dynamin because FALI at this site would ensure Dynamin as well as Dynamin complex photoactivation. The middle domain is part of a stalk in the quaternary structure of Dynamin. This stalk is required to form dimers that are used as building units in the formation of Dynamin rings that assemble around the necks of newly forming vesicles (Chappie et al., 2010; Gao et al., 2010; Ford et al., 2011). We find that the *shi-4C* construct expressed under endogenous promoter control in shi<sup>+/−;12-12B-null mutants (shi<sup>12-12B-4C</sup>) localizes at boutons very similarly to endogenous Dynamin (Fig. 1, B–D). Assessing whether Shi-4C is functional, we find that the presence of Shi-4C fully rescues the lethality associated with *shi<sup>12-12B-4C</sup>–null mutants, and these rescued animals do not show obvious behavioral or developmental defects (Fig. S1, A and B). Moreover, *shi<sup>12-12B</sup>*, *shi-4C* animals show normal synaptic NMJ morphology (Fig. S1, C–E). In addition, excitatory junctional currents (EJCs) and excitatory junctional potentials (EJPs) recorded from *shi<sup>12-12B</sup>*, *shi-4C* animals in different external calcium concentrations (Fig. 1, E–H) elicited at low (1 Hz; Fig. 1, E and F) or high (10 Hz; Fig. 1 H, first 2 min) frequency nerve stimulation are comparable to controls. Finally, also, the amplitude distribution of spontaneous vesicle fusion events in *shi<sup>12-12B</sup>*, *shi-4C* is very similar to controls (Fig. 1 G). Hence, Shi-4C constitutes a functional protein that recapitulates normal Dynamin function.

Next, we tested whether photoactivation of the 4C-tagged Dynamin affects neurotransmitter release during low and high frequency stimulation. Shi-4C in *shi<sup>12-12B</sup>*, *shi-4C* animals was loaded with FLAsH, and EJCs were measured before and after 2 min of 508-nm light inactivation. As shown in Fig. 1 (E–G), the amplitude of EJCs recorded at low frequency stimulation in 0.5 or 2 mM calcium and miniature EJC amplitude recorded in 0.5 mM calcium before (Fig. 1, E and F, −FALI) and after (Fig. 1, E and F, +FALI) Dynamin photoinactivation are very similar. In contrast, during more intense (10 Hz) stimulation, Dynamin photoinactivation (2 min of 508-nm light) results in a fast drop of the EJP amplitude and most recordings reach 0 in <5 min after photoinactivation (Fig. 1 H), very similar to recordings made from...
at the restrictive temperature (Delgado et al., 2000; Verstreken et al., 2002). Under identical conditions, both yw controls or shi12-12B; shi-4C animals, in which Dynamin was loaded with FlAsH but not inactivated by light, maintain neurotransmitter release well (Fig. 1H, control). Hence, acute photoinactivation of Dynamin results in an inability to maintain neurotransmitter release during intense stimulation, consistent with a block in synaptic vesicle recycling.

**Photoinactivation of Dynamin results in bulk membrane uptake**

At a restrictive temperature, shi12-12B causes a block in synaptic membrane uptake during nerve stimulation (Koenig and Ikeda, 1989; Ramaswami et al., 1994; Delgado et al., 2000). To examine the effect of loss of Dynamin function on membrane uptake, we assessed FM 1-43 dye uptake during stimulation. FM 1-43 is a dye that binds to the membrane and becomes internalized into newly formed vesicles during nerve stimulation (Betz et al., 1996). We treated controls and shi12-12B; shi-4C animals with or without FlAsH and with or without illumination for 2 min with 508-nm light and finally stimulated them using KCl in the presence of FM 1-43 (Fig. 2, A–H). In boutons of controls treated with or without FlAsH and with or without illumination, internalized FM 1-43 organizes in a typical doughnut-like pattern, indicating that FlAsH incubation or 508-nm light illumination do not measurably affect synaptic endocytosis (Fig. 2, A–D). Similarly, FM 1-43 dye, internalized in shi12-12B; shi-4C larvae or in segments of such larvae in which Dynamin is not inactivated by FlAsH and light, distributes in a doughnut-like pattern (Fig. 2, E–G). Notably, in the segments in which Dynamin was photoinactivated by FALI, we observe clear membrane uptake (Fig. 2 H). The overall intensity of the internalized FM 1-43 in these areas is not different from controls (Fig. 2 I, black bars), but the dye distributes in large membranous structures (Fig. 2, H and I). This peculiar labeling pattern upon Dynamin inactivation is not the result of the KCl stimulation paradigm that was used, as we observe similar membrane internalization defects in Dynamin photoinactivated boutons that were electrically stimulated for 5 min at 10 Hz (Fig. 2, K and L). Hence, loss of Dynamin function results in uptake of bulk membrane, a phenotype that is very reminiscent of the defects observed upon photoinactivation of Chc or Clc (Heerssen et al., 2008; Kasprowicz et al., 2008) but at odds with the complete block in membrane uptake in shi12-12B at a restrictive temperature (Ramaswami et al., 1994; Delgado et al., 2000).
Next, to determine whether the defect in membrane uptake upon Dynamin photoactivation correlates with a deficit to recycle the internalized membrane and release it in a new round of fusion, we first stimulated animals to load FM 1-43 into synaptic boutons in which Dynamin was photoactivated and then, after a 10-min period of rest, stimulated the boutons a second time in the absence of dye, to assess unloading. In contrast to the efficient unloading of FM 1-43 observed in yw controls (Fig. 2, J, M, and N), in shi12-12B; shi-4C boutons with photoactivated Dynamin, the dye that was loaded in large membranous structures (Fig. 2, J and O) does not significantly unload (Fig. 2, J and P). Technical limitations preclude us from assessing very slow reformation of vesicles in the time frame of hours. Nonetheless, within the time frame of minutes, these data are in accordance with the acute inability to maintain neurotransmitter release during intense nerve stimulation and suggest that photoactivation of Dynamin largely blocks synaptic vesicle recycling.

The specificity of Dynamin FLAsH-FALI
Photoactivation of FLAsH results in the production of singlet oxygen radicals that are less reactive than other radicals and specifically target methionines in a radius of 7–40 Å. This technology thus permits more selective inactivation of tagged proteins than some other forms of FALI (Yan et al., 2006). Nonetheless, the possibility remains that Dynamin-binding partners are (partially) inactivated in our study as well, thereby confounding the interpretation of the results. We therefore performed several tests as to assess the specificity of the Dynamin loss-of-function phenotype. First, we panneuronally expressed RNAi to shi in the nervous system (dicer-2/+; shi RNAi105971/+; nSybGal4/+). This tool results in a significant decrease in Dynamin immunofluorescence at synaptic boutons (Fig. 3, A–C). Expression of shi RNAi causes internalization of FM 1-43 in large membranous structures (Fig. 3, D–H), qualitatively reminiscent of the phenotype seen upon Dynamin photoactivation. Second, we generated flies that express, at endogenous levels, tetracysteine-tagged EndoA (EndophilinA; Endo-4C; see Materials and methods; Fig. 3 I). EndoA is a Dynamin-binding partner, and the presence of Endo-4C rescues the lethality associated with endoA-null mutants (endoA). Although controls internalize FM 1-43 dye efficiently (Fig. 3, J, K, and M), photoactivation of EndoA-4C using FLAsH-FALI does not result in the appearance of large FM 1-43–labeled membrane structures, but it causes a significant reduction in dye uptake, similar to observations made in endoA-null mutants (Fig. 3, L and M; Verstreken et al., 2002). These results indicate that the defect in FM 1-43 dye distribution upon Dynamin photoactivation is associated with loss of Dynamin function and that it is not a nonspecific effect of FLAsH-FALI.

Loss of Dynamin function induces bulk membrane internalization at the ultrastructural level
To further characterize the membrane inclusions formed upon stimulation of boutons during which Dynamin was photoinactivated, we performed transmission EM (TEM). We photoactivated Dynamin in synaptic boutons of shi12-12B; shi-4C using FALI, stimulated the samples, and imaged synaptic boutons. As a control, we also imaged boutons in which Dynamin was not photoactivated. Similar to stimulated yw controls or to stimulated shi12-12B; shi-4C without FALI or to shi null mutants at permissive temperature, synaptic boutons in FLAsH-treated shi12-12B; shi-4C in which Dynamin is not photoactivated are filled with regular-sized synaptic vesicles (Fig. 4, A, B, I, M, and O). In contrast, stimulated synaptic boutons of shi12-12B; shi-4C animals after photoactivation of Dynamin show an accumulation of large membranous profiles (Fig. 4, C–H and K). In some images, we observe these profiles to be connected to the presynaptic membrane (Fig. 4, G and H). Additionally, synaptic terminals in which Dynamin was photoactivated almost completely lack normal-sized synaptic vesicles (Fig. 4, I–K), and in sharp contrast to stimulated shi null mutants kept at the restrictive temperature (Fig. 4, J–N [asterisks], P, and Q; Estes et al., 1996), they do not display the typical invaginated pits at their presynaptic membrane (Fig. 4, E, F, and L). Although the membrane internalization defects upon photoactivation of Dynamin are substantial, other synaptic features, including mitochondrial number and active zone density, are not affected. Finally, we also observe similar membrane internalization defects in electron micrographs of boutons from stimulated shi RNAi–expressing animals, indicating that the defect is specific (Fig. S2, A–D). Hence, loss of Dynamin function results in qualitatively very different membrane uptake defects compared with the defects seen in shi null mutants, and collectively, the data indicate that Dynamin prevents the uptake of large membranous cisternae upon stimulation.

Photoactivation of Dynamin blocks Clathrin and α-Ada recruitment upon stimulation
Loss of Dynamin function results in the internalization of large membranous structures that appear very similar to those seen upon loss of Chc or Clc function (Heerssen et al., 2008; Kasprowicz et al., 2008). We therefore hypothesized that in neurons, in addition to membrane fission, Dynamin would also be required to coordinate Clathrin-dependent steps in the formation of synaptic vesicles. To address this question, we used superresolution microscopy and assessed Chc and α-Ada localization before and after stimulation of synaptic boutons. Immunohistochemistry using anti-Chc antibodies is difficult in Drosophila larvae, and overexpression of a Clc-GFP fusion causes strong morphological defects at the larval NMJ. We therefore used yeast recombination and phiC-31–mediated genomic integration to express HA-Chc at endogenous levels. The presence of this construct rescues the lethality associated with chc null mutants (Bazinet et al., 1993; Kasprowicz et al., 2008). Additionally, anti-HA labeling of larval fillets that express HA-Chc shows clear presynaptic boutonic labeling that is absent in yw controls (presynaptic boutonic anti-HA intensity in yw is 2.8 ± 0.3 arbitrary units [a.u.] and in yw; HA-Chc/+ is 63.2 ± 4.2 a.u.; t test: P < 0.0001), indicating that the tagged protein is expressed and functional.
Figure 3. **Dynamin FIAsh-FALI is specific.** (A–C) Labeling of controls (dicer-2/+; nSybGal4/+; A) and larvae expressing shi RNAi (dicer-2/+; shi RNAi/+; nSybGal4/+; B) third instar larval boutons with anti-Dynamin (Dyn) and anti-HRP and quantification of boutonic anti-Dynamin labeling intensity. Error bars show SEMs; t test: **, P < 0.001 (n = 10 NMJs from five larvae per genotype). (D–F) FM 1-43 dye uptake measured after 5 min of stimulation with KCl in controls (dicer-2/+; nSybGal4/+; D), larvae expressing RNAi to shi (dicer-2/+; shi RNAi/+; nSybGal4/+; E) and in heterozygous mutant shi larvae that express RNAi to shi (shi12-12B/dicer-2; shi RNAi/+; nSybGal4/+; F). (G and H) Quantification of the number of FM 1-43–labeled accumulations (Accum.) per boutonic area (G) and relative FM 1-43 labeling intensity (int.; H) in controls (dicer-2/+; nSybGal4/+), in larvae expressing RNAi to shi (dicer-2/+; shi RNAi/+; nSybGal4/+), and in heterozygous mutant shi larvae that express RNAi to shi (shi12-12B/dicer-2; shi RNAi/+; nSybGal4/+). Error bars show SEMs; ANOVA (post hoc Tukey’s test): **, P < 0.001. In G, n = 24, 36, and 60 boutons from three, seven, and five animals. In H, n = 24, 36, and 20 boutons from three, five, and four animals. (I) Strategy used to generate a genomic Endo-4C construct. The 4C is inserted between the BAR and SH3 domain. ATG is the start codon. (J–L) FM 1-43 labeling in w and w; endo-4C; endo1 animals treated (+) or not treated (−) with FIAsh for 10 min and/or illumination for 5 min (+). All preparations were stimulated with KCl in the presence of FM 1-43 for 1 min, washed, and imaged. (M) Quantification of FM 1-43 labeling intensity after loading of w and w; endo-4C; endo1 before and after FALI normalized to the w control. Error bars show SEMs; ANOVA (post hoc Tukey’s test): ***, P < 0.0001. n = 24, 60, and 64 boutons from six, four, and seven animals. Bars, 5 µm.
Figure 4. Photoinactivation of Dynamin results in massive bulk membrane uptake, whereas shi\textsuperscript{ts1} mutant boutons at restrictive temperature show an accumulation of invaginated pits. (A and B) Electron micrographs of yw (A) and shi\textsuperscript{12-12B}; shi-4C (B) control boutons stimulated for 5 min with KCl without FALI. Arrows, T bar; m, mitochondria. (C–H) Electron micrographs of shi\textsuperscript{12-12B}; shi-4C boutons in which Dynamin was photoinactivated using FALI and subsequently stimulated for 5 min with KCl. High magnifications of the membrane with an active zone decorated with a T bar (E) and of inner membrane inclusions (F–H). Arrowheads, submembrane; arrow, T bar; m, mitochondria. (I) Cumulative probability distributions of vesicular profile diameter size in yw (n = 1,291 vesicles from three larvae), shi\textsuperscript{12-12B}; shi-4C controls not treated with FALI (n = 1,291 vesicles from three larvae), and shi\textsuperscript{12-12B}; shi-4C treated with FALI (n = 2,824 vesicles from three larvae). (J–L) Quantification of different boutonic features: the number of synaptic vesicles with a diameter <80 nm per area (J), the number of synaptic vesicles with a diameter >80 nm per area (K), and the number of invaginated pits per area (L) in yw controls (n = 33 bouton profiles from three larvae), shi\textsuperscript{12-12B}; shi-4C after FALI (n = 27 bouton profiles from three larvae), and shi\textsuperscript{ts1} at a restrictive temperature (33°C; n = 16 profiles from three larvae). Error bars show SEMs; ANOVA (post hoc Tukey’s test): ***, P < 0.0001. (M and N) Electron micrographs of shi\textsuperscript{ts1} boutons stimulated for 5 min with KCl at permissive (25°C; M) and restrictive (33°C; N) temperature. Asterisks, invaginated pits; arrow, T bar; m, mitochondria. (O–Q) Higher magnification of the active zones in shi\textsuperscript{ts1} boutons stimulated for 5 min with KCl at permissive (O) and restrictive temperature (P) and of invaginated pits in shi\textsuperscript{ts1} boutons at restrictive temperature (Q). Note the lack of synaptic vesicles around the active zone in shi\textsuperscript{ts1} at restrictive temperature. Bars: (A–H, M, and N) 0.5 µm; (O–Q) 0.1 µm.
Figure 5. *Stimulus-dependent Chc and α-Ada recruitments are blocked upon Dynamin photoinactivation.* (A–H) Superresolution imaging of HA-Chc fusion proteins with anti-HA antibodies, using structured illumination microscopy in not stimulated (−KCl) and stimulated (+KCl; 90 mM for 5 min) preparations. (A and B) Labelling of *yw*; HA-chc controls (yw) and *shi12-12B/Y*; HA-chc/shi-4C without FALI at rest (−KCl). Note the presence of Chc in the bouton center.
Next, we assessed boutonic HA-Chc localization before and after neuronal stimulation in controls and in boutons in which Dynamin was photoactivated. In nonstimulated control boutons or boutons in which Dynamin is not photoactivated, HA-Chc is widely distributed (Fig. 5, A, B, and F; and Fig. S3, A and B; see also Materials and methods). However, after stimulation, the protein relocates and concentrates at the bouton periphery at the expense of its localization in the bouton center (Fig. 5, C, D, and G; and Fig. S3, C and D). In contrast, photoactivation of Dynamin blocks this activity-dependent relocalization of HA-Chc, and despite the neuronal stimulation, the protein remains distributed throughout the bouton (Fig. 5, E and H; and Fig. S3 E). Attesting to the specificity of this phenotype, boutons in which Dynamin was knocked down using RNAi display qualitatively very similar phenotypes (Fig. S4, A–C). Hence, the data suggest that Dynamin is needed to coordinate Clathrin-dependent steps during the formation of synaptic vesicles.

The AP2 complex, which connects the Clathrin lattice to the membrane, acts as a hub during vesicle formation in non-neuronal cells (Puthenveedu and von Zastrow, 2006; Loerke et al., 2009; Mettlen et al., 2009; Auge et al., 2013). We therefore also examined the localization of α-Ada before and after stimulation in controls and at synapses in which Dynamin was photoactivated. Similar to HA-Chc localization, we found that α-Ada becomes more concentrated in the bouton periphery upon neuronal stimulation of controls, and this effect is lost at NMJs in which Dynamin was photoactivated (Fig. 5, I–P; and Fig. S3, F–J). Similarly, we observe a comparable phenotype upon RNAi-mediated knockdown of Dynamin (Fig. S4, D–F). Hence, the data suggest that Dynamin coordinates vesicle budding by stabilizing or recruiting α-Ada and Chc at the membrane.

**Stimulus-dependent Clathrin and α-Ada recruitment in shi12B/Y mutants**

Given that photoactivation of Dynamin causes massive membrane internalization, whereas shifting shi12B/Y to a restrictive temperature blocks membrane uptake, we wondered whether HA-Chc and α-Ada still relocalize to the bouton periphery in shi12B/Y mutants at a restrictive temperature upon stimulation. We therefore expressed HA-Chc in shi12B/Y and stimulated the larval fillets at permissive (22°C) or at restrictive temperature (33°C). Anti-HA labeling and anti–α-Ada labeling in stimulated shi12B/Y at 33°C show HA-Chc and α-Ada labeling concentrating in the bouton periphery (Fig. 6, A and B). This labeling of HA-Chc and α-Ada in shi12B/Y at 33°C is different from the block in HA-Chc and α-Ada relocalization upon photoactivation of Dynamin (Fig. 5), but it is less pronounced than the relocalization seen in control animals or in shi12B/Y animals at permissive temperature (Fig. 5 and Fig. 6, A and B). This difference may be caused by the formation of the deeply invaginated pits in shi12B/Y that may result in a “broader rim” of Clathrin and α-Ada at the membrane. Nonetheless, our data indicate that shi12B/Y at restrictive temperature retains, at least in part, the ability to facilitate Clathrin and α-Ada relocalization upon stimulation, a function that is lost upon photoactivation of Dynamin.

Our data are consistent with a model in which, in shi12B/Y at restrictive temperature, Dynamin is trapped in a metastable, aggregated state, at the neck of nascent vesicles (Chen et al., 2002). In this capacity, Dynamin would still support Clathrin-dependent endocytic functions that prevent bulk membrane uptake while having lost its activity to mediate GTP-dependent membrane fission. To test this model, we photoactivated Dynamin in shi12B/Y mutants and assessed membrane internalization. We therefore generated shi12B/Y flies that also express Shi-4C (shi12B/Y; shi-4C/+). Given that Dynamin forms multimeric complexes and based on quantification of the intensity of boutonic labeling using anti-Dynamin antibodies in shi12B/Y (258.7 ± 4.7 a.u.) and in shi12B/Y; shi-4C/+ larvae (401.8 ± 46.3 a.u.; t test: P < 0.05), we estimate that for every two Shi12B/Y molecules, about one Shi-4C is expressed in shi12B/Y; shi-4C/+ animals (Fig. 6 C). Hence, assuming similar incorporation of these two types of Dynamin in Dynamin multimers, virtually every Dynamin multimeric complex harbors both types of Dynamin (for multimers without Shi-4C, P = 3.01 × 10⁻⁵; for multimers without Shi12B/Y, P = 3.03 × 10⁻³; Fig. 6 C). Given that both Shi12B/Y and Shi-4C act dominantly (Fig. S5, A–C; Kitamoto, 2001), such that even when wild-type Dynamin is present they result in strong membrane uptake defects (see following paragraph), Shi12B/Y can first be trapped in a metastable state at restrictive temperature. In a second phase, using FALL, photoactivation of Shi-4C can then be used to also acutely photoinactivate Shi12B/Y–Shi-4C complexes.

We stimulated shi12B/Y; shi-4C/+ animals at the restrictive temperature (33°C) without photoactivating Dynamin and tested for endocytosis defects using FM 1-43. This condition results in a block in membrane uptake (Fig. 6, E and H) as compared with control (shi12B/Y; shi-4C/+ at 22°C (Fig. 6 D), indicating Shi12B/Y can indeed inhibit membrane uptake in a dominant fashion. Next, we tested for membrane uptake in stimulated shi12B/Y; shi-4C/+ animals in which we photoactivated Dynamin using FALL at the permissive temperature. Here, we find that FM 1-43 concentrates in large inclusions (Fig. 6 G), indicating that also

and at the bouton periphery as quantified in F [n = 10 boutons from three larvae]; see Materials and methods and also Fig. S3. (K–M) Labeling of yw;Ha-chc(w) and shi12B/Y;Ha-chc/shi-4C, stimulated with KCl without Dynamin inactivation [blue; C and D] and with Dynamin inactivation using FALL (E). Note that in the stimulated controls [C and D], Chc becomes more concentrated in the bouton periphery than in animals in which Dynamin was inactivated [E] as quantified in G and H [n = 11–12 boutons from four to five larvae]; see Materials and methods and also Fig. S3. (I–P) Superresolution imaging of α-Ada using structured illumination microscopy in not stimulated (−KCl) and stimulated (+KCl; 90 mM for 5 min) preparations. (I and J) Labeling of yw controls (yw) and shi12B/Y; shi-4C/+ without FALL at rest (−KCl). Note the presence of α-Ada in the bouton center and at the bouton periphery as quantified in N [n = 9–10 boutons from three to four larvae]; see Materials and methods and also Fig. S3. (K–M) Labeling of yw (yw) and shi12B/Y; shi-4C/+; stimulated with KCl without Dynamin inactivation [blue; K and L] and with Dynamin inactivation using FALL (M). Note that in the stimulated controls [K and L], α-Ada becomes more concentrated in the bouton periphery than in animals in which Dynamin was inactivated [M] as quantified in O and P [n = 9–10 boutons from four to five larvae]; see Materials and methods and also Fig. S3. SEM is shown in the lighter shade. Arrows, plasma membrane. Bars, 2 μm.
controls (Fig. 4 A). Conversely, boutons of stimulated shi12-12B; shi-4C/+/+ animals at restrictive temperature show numerous deeply invaginated pits, and they are almost devoid of synaptic vesicles (Fig. 7, B, G, and I). Interestingly, when Dynamin is photoinactivated in the same shi12-12B; shi-4C/+/+ animals kept at restrictive temperature, the boutons do not display an accumulation of invaginated pits upon stimulation (Fig. 7, C, D, and I), instead these boutons harbor massive membrane cisternae that are sometimes intertwined and double invaginated, appearing as double membranes (Fig. 7, C, D, G, and H), similar to shi12-12B; shi-4C with photoinactivated Dynamin (see previous section; Fig. 4, C–H). To further assess whether the presynaptic membrane surface or the membrane surface of these cisternal structures contain invaginated pits, we also performed 3D electron tomography on thick 300-nm sections (Fig. 7, E and F; and Video 1). Inspection of the plasma membrane surface does not reveal invaginated pits (Fig. 7 E), and reconstructions of individual cisternal structures (Fig. 7 F) show

Figure 6. Photoinactivation of Dynamin converts invaginated pits in shi1/1 mutants into bulk cisternae. (A and B) Quantification of the HA-Chc [A, n = 11 boutons from seven larvae each] and α-Ada [B, n = 7–10 boutons from six to seven larvae] labeling intensity in a line over the largest bouton diameter (and normalized to the length of the bouton diameter; see Materials and methods) in KCl-stimulated shi1/1; HA-chc/+ at permissive (RT) temperature and shi1/1; HA-chc/+ at restrictive temperature (33°C); see Materials and methods. Averages are in black lines, and SEMs are in gray shades. rel., relative. (C) Model of the distribution of different Dynamin molecules (Shi-4C and Shi1ts) in endocytic pits (based on anti-Dynamin labeling intensity—see Stimulus-dependent Clathrin and α-Ada recruitment in shi1 mutants in the Results section) and calculation of the probability that Dynamin rings consist only of Shi1ts or only of Shi-4C in shi1/1; shi-4C/+ larvae. (D–I) FM 1-43 labeling in shi1/1; shi-4C/+ larvae treated (+; F–I) or not treated (−; D and E) with FlAsH for 10 min and illuminated for 2 min (+; G and I) at permissive (D, F, and G) or restrictive (E, H, and I) temperature. All preparations were stimulated for 5 min with KCl in the presence of FM 1-43. Note that shi1/1; shi-4C/+ animals at restrictive temperature without FLAI phenocopy shi1/1 animals and do not internalize FM 1-43, whereas shi1/1; shi-4C/+ animals at restrictive temperature after FLAI also internalize FM 1-43 in cisternal inclusions, similar to shi12-12B; shi-4C animals in which Dynamin was photoinactivated. Bar, 5 μm.

Shi-4C acts dominantly (Fig. S5). Finally, we kept FlAsH-treated shi1/1; shi-4C/+ animals at 33°C to trap Shi1ts in a metastable state followed by photoinactivation of Shi-4C and then assessed FM 1-43 dye uptake upon stimulation. Despite the fact that Shi1ts is kept at a restrictive temperature, illustrated by the undetectable FM 1-43 internalization outside of the illuminated area (Fig. 6 H), boutons within the illuminated area, in which Shi-4C was photoinactivated, now show FM 1-43–labeled membrane inclusions (Fig. 6 I). Hence, our data suggest that Dynamin in shi1/1 retains the ability to prevent bulk membrane retrieval, but when inactivated, bulk cisternae formation ensues.

Stimulation of shi1/1 mutant synapses causes the formation of deeply invaginated pits (Fig. 4, L, N, and Q). We therefore investigated the membrane inclusions formed upon photoinactivation of Dynamin in shi1/1 boutons using TEM on stimulated shi1/1; shi-4C/+ boutons in different conditions. Boutons of shi1/1; shi-4C/+ animals stimulated at 22°C are abundantly packed with synaptic vesicles (Fig. 7, A and G), similar to yw controls (Fig. 4 A). Conversely, boutons of stimulated shi1/1; shi-4C/+ animals at restrictive temperature show numerous deeply invaginated pits, and they are almost devoid of synaptic vesicles (Fig. 7, B, G, and I). Interestingly, when Dynamin is photoinactivated in the same shi1/1; shi-4C/+ animals kept at restrictive temperature, the boutons do not display an accumulation of invaginated pits upon stimulation (Fig. 7, C, D, and I), instead these boutons harbor massive membrane cisternae that are sometimes intertwined and double invaginated, appearing as double membranes (Fig. 7, C, D, G, and H), similar to shi12-12B; shi-4C with photoinactivated Dynamin (see previous section; Fig. 4, C–H). To further assess whether the presynaptic membrane surface or the membrane surface of these cisternal structures contain invaginated pits, we also performed 3D electron tomography on thick 300-nm sections (Fig. 7, E and F; and Video 1). Inspection of the plasma membrane surface does not reveal invaginated pits (Fig. 7 E), and reconstructions of individual cisternal structures (Fig. 7 F) show...
Figure 7. Invaginated pits in shits1 mutants are converted into bulk cisternae upon Dynamin photoinactivation at the ultrastructural level. (A–D) Electron micrographs of shits1/Y; shi-4C larvae treated with FlAsH for 10 min (+; B–D) or not treated (−; A) and illuminated for 2 min (+; B and D) or not illuminated (−; A and B) at permissive (RT; A) or restrictive (33°C) temperature (B–D) stimulated for 5 min with KCl. Bars: (A–C) 0.5 µm; (D) 0.25 µm. Arrowheads, submembrane inclusions; arrows, T bar; m, mitochondria; asterisks, invaginated pits. (E and F) Model of a bouton after surface rendering of a tomogram of shits1/Y; shi-4C larvae after FALI (+/+; E and F) at restrictive temperature 33°C stimulated for 5 min with KCl (see also Video 1). (E) Note that some of the membrane inclusions are so massive that they are intertwined and folded inside each other. (F) Individual tomography models of different membrane inclusions. Gray, plasma membrane; blue, red, yellow, purple, and green, membrane inclusions. (G–I) Quantification of the number of synaptic vesicles <80 nm per area (G), the number of synaptic cisternae >80 nm per area (H), and the number of invaginated pits per area (I) in shits1/Y; shi-4C larvae at 22°C (RT) not treated with FALI (−/−), at 33°C not treated with FALI (−/−), and at 33°C after FALI (+/+). Error bars show SEMs; ANOVA (post hoc Tukey’s test): *, P < 0.01; ***, P < 0.0001. n = 13, 10, and 14 bouton profiles from three larvae each.
that they are connected to the plasma membrane but also do not contain a significant number of invaginated pits (Fig. 7, F and I). Collectively, these data suggest that Shi\textsuperscript{11} retains specific aspects of Dynamin function involved in coordinating Clathrin-dependent steps that prevent bulk membrane retrieval while blocking membrane fission.

Discussion

Dynamin is one of the best-studied endocytic proteins, but the effect of acute, complete, or very strong Dynamin inactivation in the absence of developmental effects in neurons has not been reported (Koenig and Ikeda, 1989; Merrifield et al., 2002; Macia et al., 2006; Ferguson et al., 2007; Raimondi et al., 2011). The available data based on biochemistry, genetics, and pharmacology indicate an essential function for Dynamin in the fission of vesicles from the plasma membrane. Here, we provide evidence that during synaptic vesicle formation, Dynamin is critically needed to also coordinate the recruitment of Clathrin and AP2, factors needed for vesicle budding. Previous data already indicated that Clathrin prevents bulk endosome-like structure formation (Heerssen et al., 2008; Kasprowicz et al., 2008), and a role for Clathrin adaptors in preventing bulk cisternae retrieval is also consistent with the observation that mutations in such adaptors increase the incidence of detecting cisternal-like structures in different species (González-Gaitán and Jäckle, 1997; Zhang et al., 1998; Nonet et al., 1999; Gu et al., 2013). Hence, our data suggest that by stabilizing Clathrin- and AP2-dependent events, Dynamin takes on a critical role during synaptic vesicle biogenesis. Recent work in nonneuronal systems links a role for Dynamin in controlling actin polymerization (Ferguson et al., 2009), but it is currently not known whether this function relates to the role for Dynamin in facilitating Clathrin recruitment at synapses. Dynamin (and Clathrin) also appears important for the formation of synaptic vesicles from the membranes that were retrieved by bulk endocytosis. Without excluding slow Dynamin- or Clathrin-independent vesicle formation mechanisms that operate at time frames much longer than the 10 min we tested, we find that the photoinactivation of Dynamin at the NMJ very severely blocks synaptic vesicle recycling as gauged by a block in FM 1-43 unloading, and it also results in an inability to maintain neurotransmitter release during intense stimulation. Hence, efficient synaptic vesicle recycling requires Dynamin.

Our work also leads us to conclude that Dynamin is dispensable for bulk membrane retrieval in neurons. This conclusion contradicts earlier studies that indicated a phosphorylation-dependent role of Dynamin in bulk membrane uptake (Evans and Cousin, 2007; Clayton et al., 2009, 2010; Wu et al., 2009; Smillie and Cousin, 2012). In this model, Calcineurin-dependent dephosphorylation of Dynamin controls the interaction with Syndap1, which was shown to facilitate bulk cisternae formation (Anggono et al., 2006; Clayton et al., 2009). These inconsistencies may be the result of the difference in preparations used. We used a live preparation and different genetic methodologies that were thoroughly tested for specificity, to inactivate Dynamin function, all yielding consistent results. Likewise, also in cultured dynamin mutant nonneuronal mouse cells, the formation of bulk cisternae is apparent (Park et al., 2013). Hence, the data indicate that bulk cisternae form more readily in the absence rather than the presence of Dynamin. Consistent with this conclusion, in stimulated fly daps mutants, in which Dynamin mislocalizes, larger vesicles and cisternae are also apparent (Marie et al., 2004; Verstreken et al., 2005; Winther et al., 2013).

A role for Dynamin in preventing bulk membrane retrieval at synapses seems to be at odds with the accumulation of invaginated pits in Shi\textsuperscript{11} mutants or in neurons in which Dynamin was inhibited by pharmacological means (Macia et al., 2006; Newton et al., 2006). However, in all these conditions, some Dynamin function almost certainly remained or Dynamin may have been trapped in a metastable state. First, triple dynamin mouse knockout neurons have not yet been generated, and dynamin-null mutant fruit flies die early during development. In nonneuronal triple knockout fibroblasts, the loss of dynamin does cause bulk membrane retrieval (Park et al., 2013), but it remains to be seen whether the molecular mechanisms of stimulus-dependent bulk membrane uptake in neurons compares with bulk retrieval in fibroblasts. Second, drugs used to inhibit Dynamin function have been reported to be not entirely specific (Park et al., 2013). Off-target effects or incomplete inhibition of all the functions of Dynamin may complicate the interpretation of the data. Finally, the temperature-sensitive Shi\textsuperscript{11} protein that in a dominant fashion causes the buildup of invaginated pits at restrictive temperature appears to be locked in a metastable state visible by TEM. This mutant Dynamin protein may still harbor some function. Consistent with this idea, we show that when we photoinactivate Dynamin and Dynamin complexes that consist of both Shi-4C and Shi\textsuperscript{11} at restrictive temperature, invaginated pits are lost, and massive bulk cisternae formation ensues. This phenotype is very reminiscent of that seen upon photoinactivation of Dynamin itself, suggesting that despite blocking membrane recycling, the Shi\textsuperscript{11} protein retained some functionality in that it prevents bulk membrane retrieval. Hence, strong inhibition of Dynamin function reveals a critical role for the protein in preventing the formation of bulk cisternae. We believe this mechanism may turn out to be evolutionarily conserved because heavily stimulated mouse dynamin1 knockout neurons also show bulk endosome-like structures; however, here, the formation of invaginated pits was not entirely restricted (Hayashi et al., 2008), a feature potentially caused by the presence of the other Dynamin isoforms.

Although Dynamin is essential for membrane fission, our work now suggests that the protein also serves an essential role in stabilizing synaptic endocytic complexes that involve Clathrin and AP2. This notion is consistent with the emerging role for Dynamin in nonneuronal cells during the early steps of Clathrin-coated pit formation (Conner and Schmid, 2003; Loerke et al., 2009; Mettlen et al., 2009). Studies of Dynamin in cell culture, in which GTPase domain mutants were overexpressed, suggested a role for the protein in Clathrin-coated pit formation (Damke et al., 2001). Moreover, in vitro imaging experiments revealed that the level of Dynamin2 at new Clathrin-coated pits negatively correlates with the lifetime of a pit, further suggesting that the maturation of a nascent bud depends on Dynamin2 in these cells (Aguet et al., 2013). In neurons, Dynamin directly interacts with several synaptic endocytic factors that, at least in part, may act during...
earlier phases of endocytosis as well, including Dap160/Intersectin, Syndaplin, and Amphiphrisyn (David et al., 1996; Okamoto et al., 1997; Qualmann et al., 1999; Simpson et al., 1999; Koh et al., 2004; Ferguson et al., 2009; Winther et al., 2013). Also before fission, another Dynamin-binding partner, EndoA, has been shown to localize to the neck of deeply invaginated pits in a Dynamin-dependent manner, suggesting it aids in the Dynamin-catalyzed fission reaction (Sundborger et al., 2011). Hence, it is tempting to speculate that Dynamin’s involvement at synapses is needed for proper endocytic complex assembly at different stages of vesicle formation. We now find that during the first steps of synaptic vesicle formation, Dynamin would stabilize AP2, culminating in Clathrin polymerization and cage formation, a process that prevents the retrieval of bulk endosomes and facilitates the formation of uniformly sized synaptic vesicles.

Materials and methods

Genetics and behavior tests
Fly stocks were maintained on standard maize meal and molasses medium. Mutant and RNAi stocks were obtained from the Bloomington Drosophila Stock Center and from the Vienna Drosophila RNAi Center or were gifts. shi:12A26 is a shi mutant and was generated by C. Poodry (University of California, Santa Cruz, Santa Cruz, CA) by γ-rays, resulting in genomic rearrangements (Grant et al., 1998). shi:12A26 flies were given to us by M. Ramaswami (Trinity College, Dublin, Ireland). shi3 harbors a G273D mutation (Chen et al., 2002). The upstream activation sequence–shRNAi is v10597I obtained from the Vienna Drosophila RNAi Center, endo1 is an endoA null allele that corresponds to P[EP]729 outcrossed to w1118 for several generations (Verstreken et al., 2002), and chc1 is a chcn null mutant (Bazinet et al., 1993). Transgenes are described in the Molecular biology section of the Materials and methods. To collect third instar larvae for Flash-FAL, embryos were raised on black currant plates with fresh yeast paste; for RNAi expression, flies were crossed on standard medium, at RT for 3 d, and then shifted to 25°C. Third instar larvae were directly collected from the vials.

The flight and negative geotaxis assays were performed using batches of five flies. For the flight assay, flies were placed in an empty vial and tapped down. Flies able to fly were scored as 1, whereas those that did not were scored as 0. Negative geotaxis tests were also performed using batches of five flies. Flies were tapped down in an empty vial, and the number of flies that crossed a 4 cm mark within 5 s was counted.

Molecular biology
The shi:4C construct was generated by retrieving the genomic region of the shi gene (CG18102) and the nearby 5′-located gene (CG15916) using E. coli recombination from BACR32K23 with primers listed in Table S1 (Venken et al., 2006, 2008). A FLAG-4C tag (a peptide fusion of FLAG and an optimized Flash-binding tetrasacysteine tag; Martin et al., 2005) was added at the beginning of the middle domain of the shi gene by recombinase-mediated tagging (Venken et al., 2006, 2008). The endo-4C construct was generated using recombination in Saccharomyces cerevisiae. Two overlapping PCR fragments that encompass endoA and incorporate a 4C tag (without FLAG) in between the BAR (Bin1/Amphiphysin/Arp167) and SH3 domain and that harbor homology arms to pFl44sw+AttB (Khuong et al., 2010) were prepared using the primers listed in Table S1. pFl44sw+AttB was cut with BamHI, and the primers and plasmid were combined using yeast recombination (Merhi et al., 2011). To generate HA-chc, the genomic region of the chc gene (CG9012) and the nearby 5′-located gene CG32582 were first retrieved by E. coli recombination from BACR25C18 into the attB/attP[acman]-AMP-R vector (Venken et al., 2006), adopted for S. cerevisiae, using two homology arms to pFl44sw+AttB (Khuong et al., 2013) were prepared using the primers listed in Table S1. pFl44sw+AttB was cut with BamHI, and the primers and plasmid were combined using yeast recombination of overlapping PCR fragments that contain the HA tag. The primers used to generate the homology arms and the overlapping PCR fragments with the HA tag are listed in Table S1. Recombined constructs were sequenced, and transgenic animals were generated using pHc31-mediated integration in BACpalyw[2-3P]VK0033 for shi-4C and y+ w71; p[Cy2]Parp40 for Endo-4C and for HA-chc.

Photoinactivation of Shi-4C and Endo-4C and visualization of Flash fluorescence
To load the 4C tag with Flash (Invitrogen), third instar larvae were dissected in HL3 [110 mM NaCl, 5 mM KCl, 10 mM Hepes, 30 mM sucrose, 5 mM trehalose, and 10 mM MgCl₂, pH 7.2; Stewart et al., 1994] and incubated for 10 min in 1 μM Flash in the dark. Subsequently, preparations were washed in Bal (Invitrogen) to remove unbound Flash and three times with HL3. Photoinactivation of Dynamin or EndoA was performed on synapses in segments A3 or A4 by illuminating the NMJs in a hemisegement with epifluorescent 500 ± 12 nm (Intensilight CHGFI; Nikon) band pass–filtered light (excitation filter, 500/24 nm; dichroic mirror, 520 nm) for 2 min (Dynamin) or 5 min (EndoA) using a 40×, 0.8 NA water immersion objective on a microscope (Eclipse F1 or A1R; Nikon) at RT. For experiments at high temperature (the restrictive temperature for shi 20°C), we preincubated dissected larvae in warm (33°C) HL3 for 2 min after incubation with Flash and washing in Bal and subsequently kept them on a heating plate during the experiment to maintain 33°C. Photoinactivated synapses were analyzed on muscles 12 and 13.

To visualize Flash, preparations were treated as in the previous paragraph but without the photoinactivation step. Imaging of Flash fluorescense was performed on a confocal microscope (LSM 510 Meta; Carl Zeiss) using a 63×, 1.0 NA water immersion lens (zoom 4) at RT.

Electrophysiology
Two electrode voltage clamp experiments (holding potential at ~70 mV) to record EJC s were performed in HL3 with CaCl₂ as indicated (Verstreken et al., 2009; Khuong et al., 2010), and miniature were recorded in the presence of 0.5 mM CaCl₂ and 0.5 mM MgCl₂. The holding potential was ~70 mV, and the input resistance of the cells was ≥4 MΩ. Current clamp experiments to record EP s were performed in HL3 with 2 mM CaCl₂ from muscle 6, segment A2 or A3. Motor neurons were stimulated using a suction electrode at ~2x threshold. EJC and EP measurements were amplified with an amplifier (Axoclamp 900A; Axon instruments), digitized with a Digidata 1440A (Axon instruments), and stored and processed using Clampex 10.2 (Axon instruments). Data were Bessel filtered at 1 kHz.

Fluorescence imaging and quantification
FM 1-43 labeling was performed by incubating dissected larvae either in HL3 with 4 μM FM 1-43 (Invitrogen), 1.5 mM CaCl₂, and 90 mM KCl for 5 min (1 min for EndoA) or in HL3 with 1.5 mM CaCl₂ and electrically stimulating motor nerves at 10 Hz for 5 min. Subsequently, noninternalized dye was removed by washing with HL-3. For unloading, nerve terminals were stimulated for a second time in HL3 with 90 mM KCl for 10 min and washed with HL-3 before imaging. For experiments at high temperature (restrictive temperature for shi 20°C), dissected fillets were kept on a heating plate during the experiment to maintain 33°C. Quantifications of the FM 1-43 intensity in unloading experiments were corrected for bleaching by multiplying the intensity value (calculated by the bleaching coefficient) by the ratio of fluorescence when imaging twice the same NMJ of the same genotype ( IMAGE J; NIH). Images of FM 1-43 were captured with a confocal microscope (LSM 510 Meta) and 63x, 1.0 NA water immersion lens (zoom 4) or with a confocal microscope (A1R) and a 60x, 1.0 NA water immersion lens (zoom 4) at RT and stored using LSM 510 or NIS elements AR 4.1 software packages (Nikon), respectively. For quantification of intensities, the mean fluorescence levels in individual boutons minus the background fluorescence in muscles was calculated and averaged. To score accumulations of FM 1-43 inside boutons, samples were blindly scored, and such structures were manually counted and normalized to the bouton surface area (measured in Fiji; National Institutes of Health). Boutons on muscles 12 and 13 were imaged and analyzed.

For immunohistochemistry, larvae were dissected in HL-3 and fixed in 4% formaldehyde for 20 min. Samples were permeabilized in 0.4% Triton X-100 and incubated overnight at 4°C with the following antibodies: mouse anti–disc large (DLG) at 1:50 raised against the second PDZ domain of DLG (Parnas et al., 2001), rabbit anti-HRP at 1:2,000 (Jackson ImmunoResearch Laboratories Inc.,), mouse anti-HA at 1:500 (Eurogentec), mouse anti–Dynamin at 1:500 (BD), and rabbit anti–α-Ada (1:500) raised against the 360 C-terminal amino acid sequence of Drosophila α-Ada (González-Gaitán and Jäckle, 1997). After washes with PBS, Alexa Fluor 488– and 555–conjugated secondary antibodies (Invitrogen) were added at 1:500 for 2 h at RT, washed, and mounted in Vectashield (Vector Laboratories). Images were captured either on a confocal microscope (LSM 510 META) with a 63x, NA 1.4 oil lens or on a confocal microscope (A1R) with a 60x, 1.4 NA oil lens (zoom 1 and 4) and stored using LSM 510 or NIS elements AR 4.1 software packages, respectively. All images were taken.
at RT. Labeling intensity in single section confocal images was quantified as the mean gray value of boutonic fluorescence corrected for background in the muscle. Furthermore, NMJ length and bouton number were quantified as to obtain a line scan view of the intensity profile through the center of a bouton (line width and plot profile tools in Fiji). Finally, to obtain the mean labeling distribution across boutons, we calculated the mean intensity for each point along the 500 pixels.

For electron tomography tilt series, micrographs at 200 kV and 0.25°/frame were recorded from 200–300-nm-thick sections collected on Formvar carbon-coated grids covered withcollodial gold particles (15 nm) using an electron microscope (JEM-2100; JEOL). Micrographs were recorded from −60 to 60° at 2° intervals using Recorder software (JEOL) and a 1,024 × 1,024-pixel bottom-mounted charge-coupled device camera (MultiScan; Gatan). Tomograms were generated with the etomo module in IMOD, using the gold particles for alignment. 3D models were built by semi-automated surface rendering and computed using 3dmod in IMOD.

Image processing
Individual color channels were merged using Fiji, and images were adjusted for brightness and contrast using Photoshop 7.

Statistical analysis
Statistical analysis was performed using the appropriate statistical test (t test or analysis of variance [ANOVA] with Tukey’s test) for comparisons between groups, as described in the figure legends. The statistical significance of differences was defined with α < 0.05.

Online supplemental material
Fig. S1 shows that dynamin null mutants that express the tetracysteinetagged Dynamin do not show overt behavioral defects and normal third instar larval morphology. Fig. S2 shows TEM micrographs of boutons from animals that express RNAi to downregulate Dynamin expression in the nervous system. Fig. S3 shows a series of superresolution z stacks of α-Ada labeling in boutons in which Dynamin was or was not down-regulated by RNAi and that were stimulated or not stimulated using KCl. Fig. S4 shows a series of superresolution z stacks of α-Chc and α-Ada labeling in boutons in which Dynamin was or was not down-regulated by RNAi and that were stimulated or not stimulated using KCl. We thank the Bloomington Drosophila Stock Center, the Vienna Drosophila RNAi center, the Drosophila Genomics Resource Center, and the Developmental Studies Hybridoma Bank, B. André, H. Bellen, M. Cousin, M. González-Gaitán, B. Hassan, E. Lauevers, V. Morais, M. Ramaswami, J. Slabbaert, and D. Schmucker, and members of the Verstreken laboratory for reagents, help, or comments. We thank S. Murck and N. Corfout from the VIB Bio Imaging Core, Light Microscopy and Imaging Network facility, and KU Leuven Cell Imaging Core and P. Boekee from the KU Leuven EM core facility and VIB Center for the Biology of Disease.

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