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Synapsin Regulates Activity-Dependent Outgrowth of Synaptic Boutons at the Drosophila Neuromuscular Junction

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Patterned depolarization of Drosophila motor neurons can rapidly induce the outgrowth of new synaptic boutons at the larval neuromuscular junction (NMJ), providing a model system to investigate mechanisms underlying acute structural plasticity. Correlative light and electron microscopy analysis revealed that new boutons typically form near the edge of postsynaptic reticulums of presynaptic boutons. Unlike mature boutons, new varicosities have synaptic vesicles which are distributed uniformly throughout the bouton and undeveloped postsynaptic specializations. To characterize the presynaptic mechanisms mediating new synaptic growth induced by patterned activity, we investigated the formation of new boutons in NMJs lacking synapsin [Syn(−)], a synaptic protein important for vesicle clustering, neurodevelopment, and plasticity. We found that budding of new boutons at Syn(−) NMJs was significantly diminished, and that new boutons in Syn(−) preparations were smaller and had reduced synaptic vesicle density. Since synapsin is a target of protein kinase A (PKA), we assayed whether activity-dependent synaptic growth is regulated via a cAMP/PKA/synapsin pathway. We pretreated preparations with forskolin to raise cAMP levels and found this manipulation significantly enhanced activity-dependent synaptic growth in control but not Syn(−) preparations. To examine the trafficking of synapsin during synaptic growth, we generated transgenic animals expressing fluorescently tagged synapsin. Fluorescence recovery after photobleaching analysis revealed that patterned depolarization promoted synapsin movement between boutons. During new synaptic bouton formation, synapsin redistributed upon stimulation toward the sites of varicosity outgrowth. These findings support a model whereby synapsin accumulates at sites of synaptic growth and facilitates budding of new boutons via a cAMP/PKA-dependent pathway.

Key words: active zone; electron microscopy; forskolin; FRAP; synaptic vesicle; synaptotagmin

Introduction

Neuronal networks modify their activity in response to stimulation, and short-term changes in synaptic efficacy can lead to morphological changes in synaptic ultrastructure. Although postsynaptic structural modifications have been extensively studied (for review, see Holtmaat and Svoboda, 2009), the mechanisms of formation and differentiation of presynaptic boutons remain obscure. The Drosophila neuromuscular junction (NMJ) represents an excellent model system to study presynaptic restructurin...
brates to vertebrates (Kao et al., 1999). Synapsin phosphorylation by PKA promotes neurite outgrowth in *Xenopus laevis* embryos (Kao et al., 2002) and synapse formation in hippocampal cultured neurons (Perlini et al., 2011). Here, we examined the role of synapsin in activity-dependent synaptic growth at *Drosophila* NMJs, and identified an important function of synapsin in promoting vesicle clustering and transport into new synaptic varicosities.

**Materials and Methods**

*Drosophila* genetics. Flies were cultured on standard medium at 25°C. Flies of both sexes were used for all experiments. The *elav-Gal4* promoter (Bloomington Stock Center) was used to drive upstream activation sequence (UAS)-*CD8-GFP* (Bloomington Stock Center) expression throughout the nervous system. The synapsin-null mutant (cantonized Syn97) was a generous gift from Dr. Erich Buchner. The GFP-tagged and RFP-tagged synapsin lines (Syn-eGFP and Syn-mRFP) were produced as follows. The “run domain” isoform of synapsin (Klagges et al., 1996) was obtained from the Drosophila Genomics Resource Center (DGRC) gene collection (RE44971, stock no. 9229). The synapsin open reading frame was subcloned into pPGW and pPRW vectors downstream of a GAL4-bound UAS cassette by standard Gateway procedures to generate UAS-mRFP-synapsin and UAS-eGFP-synapsin constructs with N-terminal fusion tags. Microinjection of constructs was performed by Genetics Services. For fluorescence recovery after photobleaching (FRAP) and rescue experiments, the Syn-eGFP and Syn-mRFP lines were brought into the synapsin-null [Syn(–)] background to generate *elav-Gal4;UAS-CD8-GFP;Syn(–)*. The line *elav-Gal4;UAS-CD8-GFP;UAS-Syn-mRFP* was generated for simultaneously monitoring synaptic growth and synapsin movement. Canton-S (Bloomington Stock Center) was used as control. The GFP-tagged synaptotagmin line (Syt-eGFP; Zhang et al., 2002) and the UAS-GFP line were obtained from the Bloomington Stock Center.

**Live imaging.** Third instar larvae were dissected in low-Ca²⁺ hemolymph-like (HL) 3.1 saline (in mM: 70 NaCl, 5 KCl, 20 MgCl₂, 0.2 CaCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, 2.5 HEPES-HCl, 2.5 HEPES-NaOH, pH 7.2–7.4) at room temperature. Motor nerves were carefully cut below the ventral nerve cord, and the CNS was removed. The preparation was washed several times with the same low-Ca²⁺–HL 3.1 saline and allowed to rest for 5 min. Muscles 6/7 from abdominal segments 2–4 were imaged using a real-time laser-based confocal unit (PerkinElmer Life Sci) equipped with a CCD camera (Hamamatsu ORCA ER) using a 60×/1 numerical aperture water-immersion objective (Zeiss). Z stacks were taken at a 1 μm step to image the entire NMJ. The preparations were stimulated with high-K⁺ saline (in mM: 40 NaCl, 90 KCl, 20 MgCl₂, 1.5 CaCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, 2.5 HEPES-HCl, 2.5 HEPES-NaOH, pH 7.2–7.4). Alternatively, the axon was stimulated via a suction electrode using suprathreshold depolarization at a frequency of 30 Hz. N-(3-trimethylammoniumpropyl)-4-(6-[4-(diethylamino)phenyl]hexatrienyl pyridinium dibromide (FM 5-95; Invitrogen; 10 μM) was loaded during 5 min application of the high-K⁺

**Figure 1.** Synaptic outgrowth induced by patterned stimulation. **A.** Stimulation protocol: three spaced stimulations are applied to dissected third instar larva where axons have been severed from the motor neuron cell body. Either high-K⁺ application (90 mv KCl for 2 min) or electrical stimulation of the nerve (30 Hz for 5 min) was used. **B.** New boutons (arrows) formed upon stimulation. Stacks of confocal images (extended view) of CD8-GFP at NMJ arbors before and after stimulation. **C.** Both stimulation paradigms (high K⁺ or 30 Hz frequency) produce a significant growth, although it is more prominent with high-K⁺ stimulation. A very modest growth (−1 bouton per segment, white bar) was also observed at resting conditions (30 min). p < 0.0001 per one-way ANOVA; rest: n = 28; 30 Hz: n = 28; high K⁺: n = 42. **D.** New boutons are typically formed either at the edge or outside of the SSR. Images of CD8-GFP before and after the stimulation (top and middle) and double immunolabeling of the same preparation for HRP and NC82 (bottom). **E.** Double labeling of stimulated preparations for HRP and NC82 shows NC82 labeling within a new bouton (arrow), indicating that BRP is present. **F.** Only a minority of new boutons contain BRP (NC82 labeling), and <1 new bouton per segment shows FM 5-95 loading. The dotted line indicates the overall number of new boutons with this stimulation paradigm. Data collected from eight larvae. **G.** FM 5-95 loading of stimulated preparations shows that new boutons do not normally recycle vesicles (arrowheads, no FM 5-95 labeling), but occasionally FM 5-95 labeling of new boutons was observed (arrow). Subsequent stimulation in the absence of FM 5-95 (right) produces destaining of the entire preparation, including the new bouton (arrow).
solution. The dye was washed for 5 min in Ca^{2+}-free solution (in mM: 70 NaCl, 5 KCl, 20 MgCl$_2$, 0 CaCl$_2$, 10 NaHCO$_3$, 5 trehalose, 115 sucrose, 2.5 HEPES-HCl, 2.5 HEPES-NaOH, pH 7.2–7.4). Destaining was performed during 7 min high-K$^+$ solution application with no dye added. All images were analyzed using Velocity software (Improvision). For quantitative fluorescence measurements, we calculated the fluorescence above background, as described by Akbergenova and Bykhovskaia (2007). The same area and background values were used for the preparations before and after the stimulation, and all images were contrasted with identical settings.

**Immunohistochemistry.** Larvae were fixed for 45 min in HL 3.1 saline containing 4% formaldehyde. Following washing in PBST (0.1% Triton X-100 containing 1 × PBS solution), larvae were preincubated in the blocking solution containing 2% normal goat serum, 2% bovine serum albumin, and 0.05% sodium azide for 1 h. Primary antibody was applied overnight at 4°C. The secondary antibody was applied for 4–6 h at room temperature. Antibodies were diluted as follows: mouse NC82 [anti-bruchpilot (anti-Brp), 1:100; Developmental Studies Hybridoma Bank (DSHB)]; mouse DLG (Discs large; anti-DLG, 1:100; DSHB); horseradish peroxidase (HRP) conjugated to Alexa488 and Texas red (anti-HRP, 1:200; Jackson Immuno Research); Texas red-conjugated goat anti-mouse (1:200; Santa Cruz Biotechnology). Confocal imaging of fixed tissue was performed using an oil immersion 50×/0.9 objective (Olympus).

**EM.** Preparations were fixed in the 2.5% glutaraldehyde, 4% paraformaldehyde in 90 mM sodium cacodylate buffer with 0.02 mM CaCl$_2$ added and pH adjusted to 7.2–7.4. The preparations were kept in the fixative in the microwave at 40°C for 2 min and then at room temperature for 15 min (protocol adapted from Akbergenova and Bykhovskaia, 2010). After brief washing with 90 mM sodium cacodylate buffer, preparations were postfixed in 1% osmium tetroxide OsO$_4$ with 1.5% KFeCN for 30 min, washed in distilled water, incubated in 1% OsO$_4$ for 30 min, and then incubated in a 2% aqueous solution of uranyl acetate UO$_2$(CH$_3$COO)$_2$ 2H$_2$O for 1 h and washed. The preparations were then dehydrated through a graded series of acetone and embedded in a 1:1 mixture of EMBed-812 and SPURR (Electron Microscopy Sciences). Ultrathin sections (30–60 nm) were cut with a Leica Ultracut ultramicrotome mounted on copper slot Formvar-coated grids and examined with a JEM 100C transmission electron microscope (JEOL).

**Results**

**Structural analysis of newly formed synaptic boutons reveals early stages of synapse development**

Using a modified synaptic growth assay that employs a spaced stimulation protocol (Fig. 1A; modified from Ataman et al., 2008), we followed synaptic growth (Fig. 1B) using transgenic lines expressing a fluorescently tagged neuronal membrane protein (CD8-GFP). The stimulation was performed either by high-K$^+$ application (90 mM for 2 min) or by high-frequency electrical stimulation of the nerve (30 Hz for 5 min). Both protocols robustly induced budding of new presynaptic boutons within 30 min, providing an easily quantifiable assay for rapid activity-induced synaptic remodeling (Fig. 1C). Importantly, all the experiments were performed using NMJs where the axon was severed from the cell body, demonstrating that the mechanisms underlying budding and outgrowth are local to the nerve terminal. Double immunolabeling of stimulated preparations with presynaptic (anti-HRP) and postsynaptic (anti-DLG) markers demonstrated that new boutons are typically formed at the edge of postsynaptic specializations, and are not usually surrounded by postsynaptic DLG (Fig. 1D). These newly formed boutons have been previously termed “ghost boutons” due to their lack of postsynaptic maturation at this stage of development.

Labeling for the active zone (AZ) marker BRP (Wagh et al., 2006) with the NC82 monoclonal antibody (which specifically labels synaptic AZs in Drosophila) demonstrated that the majority of new boutons do not possess AZs, although some NC82 labeling was detected in ~30% of new boutons (Fig. 1E,F). This finding raised the possibility that a small subset of newly formed boutons may be functional and recycle synaptic vesicles. To address this question, we labeled stimulated preparations with the endocytic marker FM 5-95 (the dye was loaded during 5 min high-K$^+$ application after the outgrowth was induced with the
New boutons did not uptake the dye (Fig. 1A). We found that the vast majority of new boutons did not uptake the dye (Fig. 1G), including most boutons that displayed NC82 staining (Fig. 1F). Approximately 5% of all new boutons showed uptake of FM 5-95 (Fig. 1F). Importantly, however, the boutons that did uptake dye were also destained by a subsequent high-K⁺ application, suggesting that the observed FM 5-95 staining in these new boutons is due to local vesicle recycling rather than trafficking of stained vesicles from adjacent boutons.

To further analyze presynaptic development during the early growth of new synaptic varicosities, we performed ultrastructural analysis of stimulated NMJs and identified newly formed boutons using EM. Serial sectioning was performed in parallel to the analysis of stimulated NMJs and identified newly formed boutons in Syn(-) preparations. We found that in both cases, ghost boutons were significantly reduced in the absence of synapsin (Fig. 4A,B). This defect was rescued by reintroducing synapsin into the Syn(-)mutant background. However, synapsin overexpression did not promote further outgrowth. Next, we generated CD8-GFP transgenics in the Syn(-) background and assayed synaptic growth directly, using the high-K⁺-stimulation protocol shown in Figure 1A. We found that activity-induced synaptic growth in Syn(-) larvae was reduced by ~60% (Fig. 4C,D).

Since Syn(-) NMJs had reduced numbers of ghost boutons at rest, we assayed whether loss of synapsin altered NMJ structure. In agreement with Godenschwege et al. (2004), we found that the loss of synapsin did not affect the number of synaptic boutons per NMJ in third instar larvae at rest [71.81 ± 3.08, n = 16 in Syn(-)] vs 71.56 ± 4.28, n = 16 in Syn(+) at muscles 6/7; 24.96 ± 1.86, n = 27 in Syn(-) vs 22.73 ± 1.79, n = 22 in Syn(+) at muscle 4]. These findings indicate that synapsin function is primarily required for newly generated boutons induced by strong activity, suggesting an important role for the protein in translating neuronal activity to the formation of new vesicle clusters and the budding of new varicosities.

If this is the case, we might expect that synapsin(−)mutant would not only bud fewer boutons in response to strong activity, but that the new boutons that do form would contain fewer synaptic vesicles. To test this prediction, we investigated newly formed boutons in Syn(-) NMJs. To assay vesicle content in clustered at the periphery as observed in mature boutons (Fig. 3B). In a small subset of newly formed varicosities, we occasionally observed T-bars surrounded by vesicles (Fig. 3C). Strikingly, although vesicles were docked in the vicinity of T-bars, they did not oppose any detectable postsynaptic specializations in the muscle, although presynaptic and postsynaptic membranes were clearly detectable and separated by ~20 nm (Jahromi and Atwood, 1974), suggesting that a synaptic connection may be forming. Thus, differentiating presynaptic boutons, which possessed vesicles and occasionally AZs in the absence of any detectable postsynaptic structures, suggest a subset of new boutons can begin to mature during the stimulation paradigm. These results indicate that formation of presynaptic specializations precedes the formation of postsynaptic specializations during activity-induced synaptic growth at the Drosophila NMJ.

**Synapsin promotes budding and outgrowth of new varicosities via cAMP-dependent pathway**

To investigate presynaptic mechanisms controlling budding and outgrowth, we focused on the phosphoprotein synapsin, which has been demonstrated to play a role in both synaptic plasticity and neurodevelopment. First, we assayed synaptic growth in genetically modified Syn(-) larvae. Initially, we counted the number of ghost boutons lacking postsynaptic specializations in resting and high-K⁺-stimulated Syn(-) preparations. We found that in both cases, ghost boutons were significantly reduced in the absence of synapsin (Fig. 4A,B). This defect was rescued by reintroducing synapsin into the synapsin(−)mutant background. However, synapsin overexpression did not promote further outgrowth. Next, we generated CD8-GFP transgenics in the Syn(-) background and assayed synaptic growth directly, using the high-K⁺-stimulation protocol shown in Figure 1A. We found that activity-induced synaptic growth in Syn(-) larvae was reduced by ~60% (Fig. 4C,D).

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newly formed boutons, we performed immunolabeling for the vesicle-associated proteins Synaptotagmin (Syt) and Cysteine String Protein (CSP) in Syn(+) and Syn(−) boutons. We found that both Syt and CSP fluorescence was significantly reduced in newly formed Syn(−) boutons (Fig. 5A–H). Furthermore, Syn(−) NMJs had a significant proportion of new boutons that did not show any Syt or CSP labeling (Fig. 5G). In contrast, such boutons were rare in Syn(+) NMJs. In addition, HRP labeling revealed that new boutons in Syn(−) preparations are significantly smaller than those in Syn(+) preparations (Fig. 5F). Since it was shown earlier that Syn(−) terminals have significantly reduced vesicle content (Li et al., 1995; Rosahl et al., 1995; Gitler et al., 2004; Samigullin et al., 2004), and that this phenomenon is conserved between vertebrates and invertebrates (Hilliker et al., 1999; Humeau et al., 2011), we also used immunolabeling to assess vesicle content in mature Syn(minus) boutons. We found that although CSP and Syt levels are reduced in mature Syn(−) boutons (Fig. 5H), as might be expected from EM analysis (Akbargenova and Bykhovskaia, 2010), Syt depletion is not as severe as in newly formed Syn(−) boutons (Fig. 5E). Thus, synaptic vesicle depletion in Syn(−) synapses may be the result of their impaired growth and development.

Since synapsin is a PKA target in vertebrates and invertebrates, and since the cAMP/PKA pathway has been shown to contribute to neuronal development in many organisms, including Drosophila (Kim and Wu, 1996; Ueda and Wu, 2012), we tested whether raising cAMP levels would promote activity-dependent synaptic outgrowth. NMJ preparations were pretreated with forskolin (10 μM) for 1 h, and then a high-K+ patterned stimulation (Fig. 1A) was used. We found that forskolin pretreatment significantly promoted activity-dependent synaptic growth (Fig. 6), and that this effect was completely abolished in Syn(−) preparations. These data suggest that raising cAMP levels may promote phosphorylation of synapsin, which in turn enhances activity-dependent synaptic growth.

Upon stimulation, synapsin redistributes toward the sites of bouton outgrowth

Since synapsin was shown to dissociate from vesicles and disperse from synaptic boutons during activity in hippocampal cultures (Chi et al., 2001), we hypothesized that synapsin redistribution followed by vesicle reclustering may contribute to activity-induced budding of new boutons. To examine these mechanisms, we investigated the dynamics of synapsin trafficking during the growth process. To assay synapsin localization dynamically during synapse stimulation, we generated transgenic animals expressing eGFP-tagged synapsin (Syn-eGFP) and used FRAP to investigate synapsin trafficking between boutons at rest and during stimulation. We found that fluorescence recovery was significantly enhanced when preparations were stimulated after photobleaching (Fig. 7A). Interestingly, this was not the case for terminally positioned boutons (Fig. 7B), which showed no recovery either in the absence or presence of stimulation. This result suggests that synapsin may be redistributed locally between adjacent boutons, and that synapsin movement to terminal points of this trafficking pathway is compromised. To test whether synapsin is redistributed from adjacent boutons or trafficked over the entire NMJ, we repeated the above experiment while bleaching a bouton of terminal bouton position. Data collected from 42 Syn(+) and 28 Syn(−) segments with high-K+ stimulation, and 54 Syn(+) and 22 Syn(−) segments with electrical stimulation (≥6 larvae per line per condition).

Figure 4. Neuronal outgrowth is inhibited in the absence of synapsin. A, HRP/DLG immunolabeling of stimulated preparations shows ghost boutons (arrows) with no DLG labeling. B, Stimulation promotes the growth of ghost boutons (p < 0.0001 per 2-way ANOVA), and the number of ghost boutons is significantly reduced in Syn(−) preparations (p < 0.05). Data collected from 36, 33, 20, and 29 segments (wild type [Syn(+)], mutant [Syn(−)], rescue [Rescue], and over-expression [OE], respectively) in unstimulated preparations and 43, 35, 28, and 32 segments in stimulated preparations (>6 larvae per line per condition). C, Assessing activity-dependent formation of new boutons in Syn(−) preparations with GFP-tagged neuronal membranes using live confocal imaging. D, Activity-dependent formation of new boutons is reduced in Syn(−) preparations. Synapsin gene deletion produces a significant (p = 0.01 per 2-way ANOVA) reduction in the number of new boutons formed either upon high-K+ patterned application (high K+) or upon electrical stimulation of the nerve (30 Hz). Data collected from 42 Syn(+) and 28 Syn(−) segments with high-K+ stimulation, and 54 Syn(+) and 22 Syn(−) segments with electrical stimulation (≥6 larvae per line per condition).
found that Syt-GFP fluorescence did not recover in the absence of stimulation, and the recovery observed upon stimulation was significantly weaker than synapsin recovery (Fig. 8A, B). These results suggest that activity stimulates movement of synapsin in a vesicle-dissociated form. Finally, FRAP analysis of a line expressing cytosolic GFP alone demonstrated prominent trafficking of the cytosolic marker between boutons, with the recovery of GFP fluorescence being independent of stimulation and significantly exceeding the recovery of synapsin. Interestingly, the movement of either vesicle or cytosolic marker at terminally positioned boutons did not appear different from the redistribution of the marker at more centrally positioned parts of the NMJ, although synapsin movement shows a prominent distinction at terminal boutons, where its movement is compromised (Fig. 7A, B). This pattern points to a possibility of directed synapsin transport, as opposed to passive diffusion. Together, these experiments demonstrate that stimulation promotes redistribution of synapsin between boutons, and that synapsin largely redistributes in a vesicle-dissociated form.

These results suggest a model whereby synapsin may redistribute toward the sites of outgrowth during stimulation, where it may help promote the formation of new boutons. To test this hypothesis, we generated transgenic lines coexpressing CD8-GFP and Syn-mRFP to investigate how both markers are altered upon pretreatment. We found that, upon stimulation, synapsin fluorescence tends to accumulate in the vicinity of sites where new boutons are formed (Fig. 9A; note the increase in Syn-mRFP fluorescence at the branch where three new boutons were formed). Furthermore, new boutons typically contain prominent synapsin fluorescence (Fig. 9A, arrows), suggesting trafficking from existing boutons to newly formed varicosities. To quantify Syn-mRFP redistribution, we measured the fluorescence intensity before and after the stimulation at sites of outgrowth versus control sites with no outgrowth (Fig. 9B). The sites with single new boutons were analyzed separately from sites where multiple new boutons formed. The area where fluorescence was measured and the background threshold were kept identical. To quantify Syn-mRFP redistribution, we measured the fluorescence intensity before and after the stimulation at sites of outgrowth versus control sites with no outgrowth (Fig. 9B). The sites with single new boutons were analyzed separately from sites where multiple new boutons formed. The area where fluorescence was measured and the background threshold were kept identical.
Discussion

In the present study, we took advantage of the Drosophila NMJ to investigate stages and presynaptic mechanisms of activity-induced synapse formation. In Drosophila larvae, both glutamatergic (Ataman et al., 2008) and octopominergic (Koon et al., 2011) terminals show outgrowth in response to patterned depolarization. Previous work has suggested that some of these new structures undergo degradation via glial and muscle-mediated mechanisms, while others are likely to stabilize and develop into mature synaptic boutons (Fuentes-Medel et al., 2012). To identify stages in synaptic development, we performed EM analysis of newly formed synaptic structures following patterned stimulation with high K⁺. Our EM analysis revealed highly differentiated presynaptic compartments, which included synaptic vesicles and occasionally AZs in the absence of postsynaptic specializations, suggesting that development of presynaptic boutons precedes formation of postsynaptic specializations during this stimulation paradigm. To elucidate potential presynaptic mechanisms of new varicosity outgrowth, we examined the role of synapsin, which plays critical roles in synaptic vesicle clustering (for review, see Shupliakov et al., 2011), synaptic plasticity (Bykhovskaia, 2011; for review, see Fassio et al., 2011), and neurodevelopment (for review, see Valtorta et al., 2011). We found that new bouton outgrowth is severely compromised in the absence of synapsin, with fewer boutons being formed. Furthermore, new boutons that do form in the absence of synapsin are smaller and contain fewer vesicles. Finally, raising cAMP levels by forskolin pretreatment significantly promotes synaptic growth in control but not in synapsin (−) preparations. Using live confocal imaging of larvae with synapsin tagged with mRFP or eGFP, we also detected movement of synapsin in response to patterned depolarization, preferentially directed to sites of outgrowth. These observations suggest a model whereby synapsin may dissociate from vesicles in response to depolarization, move toward sites of outgrowth, form new vesicle clusters, and possibly participate in actin bundling and budding of new presynaptic boutons.

Stages of synapse formation and differentiation

Growth and maturation of new synapses ultimately involves both presynaptic and postsynaptic restructuring. Although postsynaptic growth and formation of dendritic spines has been extensively studied (for review, see Holtmaat and Svoboda, 2009), the mechanisms of formation and activation of new presynaptic boutons remain obscure, even though it has been shown that structural plasticity of presynaptic terminals is associated with learning (Li et al., 2011; Ruediger et al., 2011). Although tremendous progress has been achieved recently in monitoring axonal dynamics (Allegra Mascaro et al., 2013; Grillo et al., 2013), such studies in the CNS do not allow for detailed investigation of the ultrastructure of growing synaptic boutons. In contrast, the Drosophila larval NMJ provides an excellent model system for such studies, since each motor neuron is easily identifiable, and presynaptic boutons can be visualized by using genetically encoded markers (for review, see Collins and DiAntonio, 2007). We took advantage of this preparation and used correlative light and EM to investigate the ultrastructure of newly formed boutons during activity-induced synapse formation.

This analysis enabled us to identify several ultrastructural characteristics of newly formed boutons and to make implications regarding the stages of their development. It has been previously shown (Ataman et al., 2006) that Drosophila mutants with alterations in Wingless signaling have abnormal synaptic structures with boutons lacking postsynaptic specializations and AZs: ghost boutons. A subsequent study (Ataman et al., 2008) showed that new boutons form in response to patterned depolarization at the preparations with intact axons, and that these new boutons lack postsynaptic markers (DLG), as well as the markers of AZs (BRP). The latter study also indicated that the formation of the new boutons involves the Wnt/Wg pathway and depends on
transcription and translation. We used a shorter depolarization protocol at the larval NMJ preparation with innervating axons severed from the motor neuron cell body. We found that even though the formation of new boutons is less prominent, new boutons can still be formed within 30 min with the axon cut, indicating a component of synapse formation that is local to nerve terminals. Furthermore, a small subset of newly formed boutons possesses the AZ marker BRP and can recycle synaptic vesicles, indicating this protocol is likely to induce a program where some boutons begin the process of maturation to functional connections.

Our EM analysis revealed that although new boutons typically lack AZs, occasionally they included T-bars surrounded by vesicles. New boutons were filled with vesicles, but the vesicles usually lacked the organization typical for mature boutons. Membranous structures in the vicinity of the extrasynaptic space indicated the possibility that SSR formation is beginning, although a mature SSR structure was never observed around new boutons. Thus, we found that the new presynaptic specializations, including compartments with vesicles, AZs, and vesicle recycling capabilities, can be formed very rapidly and in the absence of the protein synthesis. We next examined the presynaptic mechanisms that can mediate this rapid formation of new presynaptic specializations.

The role of synapsin in budding of new boutons

In addressing this question, we focused on synapsin, which has been shown to regulate synaptic development and plasticity (for review, see Valtorta et al., 2011; Bykhovskaya, 2011). Synapsin is the most abundant presynaptic phosphoprotein that reversibly associates with synaptic vesicles, and it is a target for multiple protein kinases, including PKA (Czernik et al., 1987). Synapsins have been shown to cluster synaptic vesicles (Li et al., 1995; Hilfiker et al., 1999; Siksou et al., 2007), possibly by forming connectors via dimerization and cross-linking vesicles into a mesh-work-like organization (for review, see Shupliakov et al., 2011). Binding of dephosphosynapsin to actin promotes the formation of actin bundles (Bähler and Greengard, 1987), although actin filaments were not found inside vesicle clusters, but instead were located over the periphery of vesicle clusters (Bloom et al., 2003; Sankaranarayanan et al., 2003). Synapsin disperses in response to activity (Chi et al., 2001), and experiments at *Aplysia* suggest that synapsin dispersion may depend on cAMP/PKA pathway (Angers et al., 2002). Synapsin expression in neuroblastoma cells induced formation of presynaptic-like structures (Han et al., 1991), and synapsin expression in non-neuronal cells gave rise to reorganization of actin filaments (Han and Greengard, 1994). Increased levels of synapsin were also shown to promote neuronal development, as well as synaptic maturation and differentiation (for review, see Valtorta et al., 2011), while synapsin deficiency slows neuronal differentiation (Ferreira et al., 1994, 1998) and synapse formation (Ferreira et al., 1995). Expression of synapsin mutants mimicking phosphorylation at the PKA site promote neurite outgrowth (Kao et al., 2002) and synapse formation (Perlini et al., 2011). However, it is still obscure how synapsin function promotes the formation of new synapses.

We took advantage of imaging capabilities at the *Drosophila* larval NMJ to investigate how new synaptic boutons are formed in
the absence of synapsin and also to investigate activity-dependent synapsin movement. In *Drosophila*, synapsin is encoded by a single gene (Klagges et al., 1996), and synapsin knock-out flies are viable, although they show impaired behavior (Godenschwege et al., 2004) and altered vesicle cycling (Akbergenova and Bykhovskaya, 2007, 2010). We investigated the activity-dependent formation of synaptic boutons in Syn(−) larvae and found that it is compromised in several ways. First, the number of new boutons formed in response to stimulation in Syn(−) larvae was significantly reduced. Second, newly formed boutons that did emerge were smaller in Syn(−) larvae. Third, in the absence of synapsin, the content of vesicle proteins CSP and Syt was reduced in newly formed boutons. Finally, raising cAMP levels with forskolin pretreatment failed to promote activity-dependent outgrowth in Syn(−) larvae, although it significantly promoted synaptic growth in controls. These results suggest that synapsin is critical for activity-dependent formation of new boutons, that its role in synapse formation is likely to be mediated via cAMP-dependent phosphorylation, and that it may involve clustering of synaptic vesicles and their delivery into newly formed synaptic compartments.

In addition to these defects in activity-dependent synaptic growth in the absence of synapsin, we found that activity promotes synapsin movement toward the sites of synaptic outgrowth in control animals. Earlier studies (Chi et al., 2001) have shown that activity promotes dissociation of synapsin from vesicles and dispersion from boutons toward the axons. Similarly, our FRAP experiments show that activity stimulates synapsin movement, and that it is likely to occur in a vesicle-dissociated form. In addition, live confocal imaging experiments using CD8-GFP and Syn-mRFP double labeling demonstrate that synapsin movement is directed toward the sites of outgrowth. Together, these findings suggest that synapsin moves toward the sites of synaptic outgrowth during stimulation and promotes formation of new boutons. Although it remains to be elucidated how synapsin drives new bouton formation, we hypothesize that the protein participates in forming new synaptic vesicle clusters at budding sites, and may enhance actin reorganization to stimulate budding and recruitment of new synaptic vesicle clusters into newly forming presynaptic varicosities.

References


