A Syd-1 homologue regulates pre- and postsynaptic maturation in Drosophila

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Abstract

Active zones (AZs) are presynaptic membrane domains mediating synaptic vesicle fusion opposite postsynaptic densities (PSDs). At the Drosophila neuromuscular junction, the ELKS family member Bruchpilot (BRP) is essential for dense body formation and functional maturation of AZs. Using a proteomics approach, we identified Drosophila Syd-1 (DSyd-1) as a BRP binding partner. In vivo imaging shows that DSyd-1 arrives early at nascent AZs together with DLiprin-α, and both proteins localize to the AZ edge as the AZ matures. Mutants in dsyd-1 form smaller terminals with fewer release sites, and release less neurotransmitter. The remaining AZs are often large and misshapen, and ectopic, electron-dense accumulations of BRP form in boutons and axons. Furthermore, glutamate receptor content at PSDs increases because of excessive DGluRIIA accumulation. The AZ protein DSyd-1 is needed to properly localize DLiprin-α at AZs, and seems to control effective nucleation of newly forming AZs together with DLiprin-α. DSyd-1 also organizes trans-synaptic signaling to control maturation of PSD composition independently of DLiprin-α.

Introduction

Fast chemical synaptic transmission is mediated by precisely regulated neurotransmitter release from synaptic vesicles (SVs) at specialized presynaptic sites. This compartment, called the active zone (AZ), comprises a unique set of proteins (Schoch and Gundelfinger, 2006; Owald and Sigrist, 2009).

Genetic analyses of synapse assembly in Caenorhabditis elegans hermaphrodite-specific motor neuron synapses (HSNLs; Margeta et al., 2008) and in Drosophila neuromuscular junctions (NMJs; Collins and DiAntonio, 2007) have identified several presynaptic proteins important for AZ assembly (Owald and Sigrist, 2009). Syd-2/Liprin-α is needed for AZ formation at C. elegans HSNL synapses (Dai et al., 2006; Patel et al., 2006) and is important for proper AZ morphology in Drosophila (Kaufmann et al., 2002), and ELKS is essential downstream of Syd-2/Liprin-α (Dai et al., 2006). In Drosophila, the ELKS-related protein Bruchpilot (BRP) forms the electron-dense projection at AZs (T bar), and is crucial for AZ maturation (Kittel et al., 2006; Fouquet et al., 2009). Finally, Syd-1 (synapse defective 1), a multidomain RhoGAP-like protein, is required for C. elegans HSNL synapse assembly (Dai et al., 2006; Patel et al., 2006).

Here, a proteomics-based approach identified the Drosophila Syd-1 homologue (DSyd-1) as a BRP binding partner. Using stimulated emission depletion microscopy (STED; Kittel et al., 2006; Fouquet et al., 2009), we show that DSyd-1
Proteomics identify DSyd-1 as a physical interactor of BRP. [A] Monoclonal antibody Nc82 efficiently precipitates BRP (arrowhead), as seen in this SYPRO red-stained SDS-gel. Among other proteins, DSyd-1 was found to coprecipitate with BRP, as confirmed by MS/MS analysis. [B] Matrix showing yeast two-hybrid assay results confirming a direct physical interaction between BRP and DSyd-1. A C-terminal domain of BRP (aa 1,152–1,740) was positive for interaction with a C-terminal region of DSyd-1 (aa 1,301–1,844). Moreover, a bait N-terminal DSyd-1 (aa 1–400) fragment interacted with both the N-terminal fragment of BRP (aa 1–320) and a C-terminal BRP (aa 1,152–1,740) fragment. (C) Genomic fragment of BRP (aa 1–320) and a C-terminal fragment interacted with both the N-terminal region of DSyd-1 (aa 1,301–1,844). More specifically, BRP interacts with both a previously defined N-terminal region of DSyd-1 (aa 1,152–1,740) and a C-terminal domain of BRP (aa 1,741–1,150) physical interaction between BRP and DSyd-1. A C-terminal domain of BRP (aa 1,152–1,740) was found to coprecipitate with BRP, as confirmed by MS/MS analysis. [D] Walking ability (control: 15.69 ± 0.57 lines, n = 15; dsyd-1: 1.62 ± 0.69 lines, n = 8; dsyd-1 × rescue, 12.86 ± 0.99 lines, n = 10; dliprin-, 16.19 ± 0.65 lines, n = 7; control × dsyd-1: P = 0.0001; control × dsyd-1 × rescue, P = 0.02; control × dliprin-, P = 0.67; dsyd-1 × dsyd-1 × rescue, P < 0.0001). [E] Negative geotaxis (control: 8.32 ± 0.37 cm; dsyd-1: 2.92 ± 0.60 cm; dsyd-1 × rescue, 8.833 ± 0.17 cm; dliprin-, 8.67 ± 0.15 cm; all: n = 10; control × dsyd-1: P < 0.0001; control × dsyd-1 × rescue, P = 0.32; control × dliprin-, P = 0.91; dsyd-1 × dsyd-1 × rescue, P < 0.0001). Impaired locomotive behavior in dsyd-1 flies is rescued by pan-neural (elav-GAL4) reexpression of the dsyd-1 cDNA. Error bars indicate the SEM. *, P < 0.05; ***, P < 0.005; ns, P > 0.05. (F) A polyclonal antibody recognizes a band at the predicted molecular mass of 195 kD on immunoblots of w<sup>1118</sup> control fly head lysate (arrow). This band is missing in dsyd-1 head extracts. Statistics: Mann-Whitney test.

Results

The AZ protein BRP is an integral part of the electron-dense T bar and is needed for effective Ca<sup>2+</sup> channel clustering during synapse maturation (Fouquet et al., 2009). Thus, BRP may be a platform for protein–protein interactions and was well-suited as a starting point for an unbiased proteomics screen for novel Drosophila AZ proteins.

Proteomic identification of Drosophila Syd-1 as a BRP-linked protein

Using the monoclonal antibody Nc82, we immunoprecipitated BRP from adult fly head extracts. Although BRP was strongly enriched in Nc82 precipitates, it was not detected in control eluates as visualized by staining SDS-polyacrylamide gels specifically localizes to a discrete compartment at the AZ edge, coordinating the BRP-composed T bar at the center of the AZ. Flies lacking DSyd-1 show impaired locomotion and a reduced life span, which is rescued by nervous system expression of the protein. Fewer release sites form at their T bars, and that ectopic electron-dense accumulations also form distant from AZs. Thus, DSyd-1 inhibits inappropriate localization of BRP and its associated electron density. Both DSyd-1 and Dliprin-α accumulate early during the protracted AZ formation process. Notably, DSyd-1 was needed to properly localize Dliprin-α at AZs, but not vice versa. Thus, one function of the RhoGAP DSyd-1 seems to be to stably target Dliprin-α to maturing AZs, allowing Dliprin-α to execute its AZ assembly function. Independent of Dliprin-α, the presynaptic AZ-localized protein DSyd-1 is also involved in defining the amount and composition of glutamate receptors (GluRs) accumulating at maturing postsynaptic densities (PSDs).
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DSyd-1 is an AZ protein

In situ hybridization showed nervous system–specific expression of dsyd-1 (Fig. 2 A), with a similar onset of expression for brp, coincident with postmitotic differentiation (Wagh et al., 2009). Co-labeling revealed a strong overlap with BRPNc82 signals, which suggests that DSyd-1 is an AZ protein. To address this issue more explicitly, we first analyzed synapses within the mushroom body (MB) calyx. Here, postsynaptic specializations were labeled by expressing the GFP-labeled acetylcholine receptor subunit D2 (Fig. 2 D; Leiss et al., 2009; Raghu et al., 2009). DSyd-1–specific immuno-labeling was found to localize opposite to the D2 signal within the presynaptic terminals, which implies that DSyd-1 localizes to AZs (Fig. 2 D).

We then turned to the larval NMJ system (Fig. 3). Consistent with our observations in the MB calyx, the DSyd-1 antibody also specifically labeled AZs at NMJs (Fig. 3, A and B).

Both DSyd-1 and DLiprin-α localize at the AZ edge

Given that AZ assembly at HSNL synapses in C. elegans (Dai et al., 2006; Patel et al., 2006) involves a tight interplay between Syd-1 and Syd-2/Liprin-α, we reasoned that their homologues might operate together during synaptogenesis in flies. DLiprin-α is known to control proper segregation and shaping of AZs at the developing Drosophila NMJ (Kauffmann et al., 2002; Fouquet 2006). The DSyd-1 antibody gave a neuropil-specific staining in larval (not depicted) and adult brains (Fig. 2 B), which was completely absent in dsyd-1 mutant animals (Fig. 2 C) but restored upon pan-neuronal expression of UAS–dsyd-1pDNA (not depicted). Co-labeling revealed a strong overlap with BRPNc82 signals, which suggests that DSyd-1 is an AZ protein. To address this issue more explicitly, we first analyzed synapses within the mushroom body (MB) calyx. Here, postsynaptic specializations were labeled by expressing the GFP-labeled acetylcholine receptor subunit D2 within Kenyon cells (Fig. 2 D; Leiss et al., 2009; Raghu et al., 2009). DSyd-1–specific immuno-labeling was found to localize opposite to the D2 signal within the presynaptic terminals, which implies that DSyd-1 localizes to AZs (Fig. 2 D).

Figure 2. DSyd-1 localizes to central synapses. (A) In situ hybridizations show that dsyd-1 is expressed throughout the embryo’s CNS. st., stage. (B) Confocal z projection of adult Drosophila CNS. α-DSyd-1 staining co-localizes with BRPNc82 throughout the brain, but is absent in dsyd-1 animals (C). (D) DSyd-1 localizes opposite to postsynaptic acetylcholine receptors (Du75C) expressed in Kenyon cells at the adult MB calyx. Arrowheads in the inset panels (which show enlarged views) indicate pre-to postsynaptic alignment. Bars: (B and C) 50 µm; (D) 10 µm; (D, insets) 500 nm.
Reduction of evoked release at dsyd-1 mutant NMJs

To explore whether DSyd-1 was needed for proper synaptic neurotransmitter release at AZs, two electrode voltage clamp recordings of late third-instar larval NMJs were performed. Evoked excitatory junctional currents (eEJCs) were significantly reduced in dsyd-1 mutant larvae compared with controls (Fig. 4 A). These were significantly rescued by presynaptic expression of UAS–dsyd-1 cDNA using the motoneuronal driver ok6-GAL4 (Fig. 4 A). For comparison, recordings from mutants in the AZ organizing protein DLiprin-α were performed (compare Fig. 4 A with Kaufmann et al., 2002). Interestingly, eEJC amplitudes were decreased to a comparable level in dsyd-1 and DLiprin-α. Spontaneous miniature-current amplitudes, in turn, were on average not changed between dsyd-1 and controls (Fig. 4 B, but see “Presynaptic DSyd-1 controls the amount…”).

Correlation analysis of DLiprin-α and DSyd-1 costainings (Fig. 3 G) indicated that both proteins closely colocalize (RDSyd-1:DLiprin-α = 0.81 ± 0.01; n = 12), significantly closer than BRP and DLiprin-α (RBRP:DLiprin-α = 0.66 ± 0.01; P < 0.0001, n = 12; Fig. 3 G). Moreover, the mean distances of individual DLiprin-α and DSyd-1 signals to neighboring spots or to the AZ center were comparable (Fig. 3 E). Thus, DSyd-1 and DLiprin-α together seem to define a common subcompartment surrounding the AZ core.
Neurotransmitter release deficits at dsyd-1 mutant NMJs might be explained by a drop in release probability of SVs, e.g., by a reduction of Ca\textsuperscript{2+} sensitivity of the SVs that are to be released. In this case, a change in short-term plasticity (paired pulse paradigm) or sensitivity to different extracellular Ca\textsuperscript{2+} concentrations should be observed. However, when we compared evoked release at two different Ca\textsuperscript{2+} concentrations, the ratio between dsyd-1 mutant and control was unchanged (Fig. 4 C).
which argues against a change in Ca\(^{2+}\) sensitivity. Moreover, no clear alteration in paired pulse behavior was observed (Fig. 4 D). Collectively, these data imply that the characteristics of SV release are (if anything) only moderately altered after loss of DSyd-1. Thus, the question arose as to the number of release sites (i.e., an individual PSD + adjacent AZ) forming at dsyd-1 mutant NMJs, and/or whether the number of releasable SVs was reduced.

Reduced numbers of synaptic release sites at dsyd-1 mutant NMJs

To account for SV numbers and distribution, we performed Drosophila vesicular glutamate transporter (DVGlut) immunostainings (Fig. S2 A; Daniels et al., 2004; Mahr and Aberle, 2006). Overall, both dsyd-1 and control NMJs showed comparable immunoreactivity (Fig. S2 B), which indicates that the absolute number of SVs per terminal was not substantially changed. However, the SV signal appeared somewhat uneven between individual boutons at dsyd-1 NMJs when compared with controls (Fig. S2 A). To evaluate whether this distribution would account for the observed release defect at low frequency stimulation (Fig. 4 A), SV distribution closely surrounding the electron-dense projection at AZs was evaluated in electron micrographs (Fig. S2, C and D). Here, the SV size (Fig. S2 E) as well as the number of SVs surrounding the AZs (Fig. S2, D and F) were comparable between control and dsyd-1 mutant animals. We also tested whether mitochondria were properly transported to the NMJ terminal in dsyd-1 mutants, using MitoGR (Fig. S2 G; Pilling et al., 2006). Here, the mean NMJ signal did not differ significantly between controls and mutants (Fig. S2 H).

To perform quantitative analysis of release sites, NMJs of third-instar larvae were stained (Fig. 4 E). The overall size of individual NMJs (as scored by HRP reactivity) was reduced in both dliprin-α and dsyd-1 mutant animals (Fig. 4, E and F). We scored numbers of release sites by counting (a) BRP spots (for AZs, Fig. 4 E) and (b) DGluRIID spots (for PSDs, not depicted; Qin et al., 2005). In dsyd-1 mutant larvae, a significant reduction of release sites was observed (Fig. 4, G and H). This reduction appeared identical when independently counting either BRP or DGluRIID spots, and was rescued by motoneuron-specific expression of UAS–dsyd-1\(^{cDNA}\) (Fig. 4, G and H).

Thus, presynaptic DSyd-1 is needed for developing NMJs to reach full morphological size and adopt a full complement of release sites. Consistent with previous studies, release site numbers were also reduced at dliprin-α mutant NMJs (Kaufmann et al., 2002); however, the phenotype is more pronounced than that observed in dsyd-1 NMJs (Fig. 4, E, G, and H).

Defective AZ assembly and ectopic BRP accumulations at dsyd-1 mutant terminals

Upon scoring BRP signals, we had the impression that atypically large spots formed at dsyd-1 NMJs. To resolve AZ morphology more accurately, we used STED microscopy for the further analysis.

Using this technique, we recently showed that BRP is a direct building block of T bars. The N terminus of BRP localizes close to Ca\(^{2+}\) channels at the AZ membrane, whereas its C-terminus (recognized by the BRP\(^{N\text{C2}}\)) defines the edge of the distal T bar platform, resulting in a typical donut-shaped appearance at wild-type NMJs (Fig. 5, A and A’, arrowheads; Fouquet et al., 2009).

At dsyd-1 mutant AZs, this donut-type distribution was compromised (Fig. 5, B and B’, arrowheads) but was partially restored by UAS–dsyd-1\(^{cDNA}\) reexpression (Fig. 5, C and C’). In dsyd-1, BRP organization at individual sites often appeared enlarged (Fig. 5, B and B’, arrowheads; and Fig. 5 D).
and misshapen. Thus, the STED analysis implied that T bar morphology was affected, with atypical formation of large assemblies (Fig. 5, B and B’).

Individual synaptic release sites (as defined by presynaptic BRP in conjunction with opposing PSDs) showed further abnormalities. Although the size of individual PSDs was enlarged in dsyd-1 mutants (see the following section), individual release sites (defined by the PSD) often comprised several BRP clusters (Fig. 5 E). Furthermore, spacing between individual AZs was irregular, and small BRP assemblies lacking adjacent GluR fields were observed (Fig. 5, B and B’, arrows). These might represent AZ assemblies, which do not progress to maturation properly due to a lack of nucleation assembly.

To address T bar morphology and the nature of increased BRP entities directly, we continued our studies using EM (Fig. 6 A) combined with 3D reconstruction of serial sections. In fact, at dsyd-1 mutant NMJs (Fig. 6, B and B’), T bars often appeared irregular in shape, with pedestals of very high diameter and multiple, atypically prominent filamentous projections in their distal parts (Fig. 6 B, arrowhead). Such misshapen T bars (as in Fig. 6 B) were never observed in controls (Fig. 6 A). Moreover, atypically small T bar–like assemblies were apparent (Fig. 6 B’). These might reflect immature release sites and correspond to the small BRP assemblies observed by STED (Fig. 5, B and B’, arrows).

At control NMJs, electron-dense material is restricted to the T bar assembly at the center of the AZ (as defined by planar apposition between pre- and postsynaptic membrane). However, ectopic electron-dense material was easily observed at dsyd-1 mutant NMJ terminals. Such material frequently appeared at the edge of AZs (Fig. 6 C), and was only loosely (Fig. 6 D, arrowhead), if at all (Fig. 6 E), associated with the presynaptic plasma membrane. Floating electron-dense material, highly decorated with SVs, was observed in the bouton interior (Fig. 6 E).

As BRP seems to be a principal component of the electron-dense T bar (Fouquet et al., 2009), these ectopic electron-dense assemblies in dsyd-1 mutants should contain BRP. Ectopic BRP reactivity at the bouton center and throughout the axon was also consistently detected by light microscopy (Fig. 6, E and F). In agreement with our EM data showing electron-dense material in association with SV-like material, ectopic axonal BRP accumulations colocalized with the SV marker DVGlut (Fig. S3; Daniels et al., 2004; Mahr and Aberle, 2006).

Collectively, fewer full-sized AZs formed in dsyd-1 mutants, most likely because of the failure of some AZs to progress to maturation. However, excessive amounts of BRP were observed at the remaining AZs and within the neighboring plasma membrane and the presynaptic cytoplasm. Thus, DSyd-1 appears to be necessary to distribute AZ material adequately among a sufficient number of forming and maturing AZs. That the NMJ comprises a reduced number of immature AZs in dsyd-1 mutants might contribute to the deposit of excess AZ material at remaining sites, effectively overgrowing them.

**Presynaptic DSyd-1 controls the amount and composition of postsynaptic GluRs**

At the Drosophila NMJ, ionotropic receptors (assembling as heteromeric tetramers by selecting four from five subunits) mediate the postsynaptic response to glutamate. Three subunits—DGluRIIC, IID, and IIE—are essential for receptor formation and function and are seemingly contained within all GluR complexes (Petersen et al., 1997; Marrus et al., 2004; Qin et al., 2005; Schmid et al., 2008). To assess PSDs in dsyd-1 mutants, we looked into the distribution and signal intensity for different GluR subunits (Fig. 7, A–F).

When we stained dsyd-1 mutants for DGluRIID, we recognized that individual GluR fields (reflecting individual PSDs) were dramatically enlarged at dsyd-1 mutant NMJs.
Dsyd-1 controls postsynaptic GluR field size and composition. (A–C) Co-labeling of DGluRIID and BRPNc82 for control [A], dsyd-1 mutant [B], and presynaptically rescued [C] NMJs. Individual PSDs are indicated by arrowheads. (D–F) Co-labeling of DGluRIIA and DGluRIIB for control [D], dsyd-1 mutant [E], and presynaptically rescued [F] NMJs. (G) Integrated DGluRIIA signal (control: 32.25 ± 0.67 au, n = 344; control × dsyd-1: 1.24 ± 0.08, P = 0.008, Mann-Whitney test; compare with the traces in Fig. 4 A). (H) Integrated DGluRIIB signal (control: 33.88 ± 0.66 au, n = 1,064; dsyd-1: 66.85 ± 2.09 au, n = 667; dsyd-1 × dsyd-1 rescue post: 36.31 ± 0.87 au, n = 830; control × dsyd-1: P = 0.001; control × dsyd-1 × dsyd-1 rescue: P > 0.05; dsyd-1 × dsyd-1 rescue: P < 0.001). (I) Integrated DGluRIB signal (control: 46.40 ± 0.99 au, n = 934; dsyd-1: 23.85 ± 0.60 au, n = 783; dsyd-1 × dsyd-1 rescue: 35.46 ± 0.89 au, n = 770; control × dsyd-1: P = 0.001; control × dsyd-1 × dsyd-1 rescue: P < 0.001; dsyd-1 × dsyd-1 rescue: P < 0.001) size in dsyd-1 mutants. (J) GluR field composition (control: 0.89 ± 0.06, n = 7; dsyd-1: 1.99 ± 0.19, n = 8; dsyd-1 × dsyd-1 rescue: 1.24 ± 0.08, n = 6; control × dsyd-1: P < 0.001; control × dsyd-1 × dsyd-1 rescue: P > 0.05; dsyd-1 × dsyd-1 rescue: P < 0.01). Statistics: one-way ANOVA. Error bars indicate the SEM. **, P < 0.01; ***, P < 0.005; ns, P > 0.05. Bars: (A–C) 1 μm; (D–F) 2 μm.
We further analyzed embryonic synapse morphology using BRP as a presynaptic marker and DGluRIID as a postsynaptic marker in single and double mutant combinations (Fig. 8, B and C). Compared with controls, BRP reactivity was clearly elevated at dsyd-1, but only very mildly at dliprin-α mutant NMJs (Fig. 8 B). Notably, BRP levels at dliprin-α; dsyd-1 double mutant NMJs were comparable to those at dliprin-α, rather than to those at dsyd-1 mutant NMJs (Fig. 8 B). Thus, loss of DSyd-1 leads to an increase in recruitment of BRP to AZs, which is dependent on the presence of DLiprin-α. Increased levels of BRP were also observed at dsyd-1 mutant larval NMJs (Fig. 5).

Levels of DGluRIID were drastically increased at dsyd-1 and equally at dsyd-1; dliprin-α double mutant NMJs, but only mildly elevated in dliprin-α-deficient synapses (Fig. 8, B and C). Thus, DSyd-1 is involved in regulating GluR field size, independently of DLiprin-α.

**DSyd-1 is needed for proper AZ localization of DLiprin-α, but not vice versa**

Using extended in vivo imaging of identified release sites, we recently showed that accumulation of DLiprin-α precedes accumulation of DGluRIIA as well as—at hours—the arrival of BRP throughout AZ assembly (Rasse et al., 2005; Schmid et al., 2008; Fouquet et al., 2009).

To place DSyd-1 into the temporal context of AZ assembly, we coexpressed GFPDSyd-1 (which, when pan-neuronally expressed, rescues the sluggish behavior of dsyd-1 mutant adults) and BRP-shortmStraw in motoneurons. As expected from immunostainings (Figs. 2 and 3), presynaptic expression of DSyd-1 labeled AZs (Fig. 9, A and B). Individual NMJs were reimaged after 12 h, and substantial growth of the NMJ along with the addition of new AZs (Figs. 2 and 3), presynaptic expression of DSyd-1 labeled AZs (Fig. 5). We went on to co-image DLiprin-αGFP and mStrawDSyd-1, suggesting that both proteins arrived at synaptic sites in very close temporal proximity (Fig. 9 B, arrows and arrowheads). Thus, newly forming AZs are characterized by DLiprin-α and DSyd-1–positive clusters from early on.

Genetic analysis in *C. elegans* has placed the putative RhoGAP DSyd-1 upstream of Syd-2/Liprin-α in the assembly hierarchy (Dai et al., 2006; Patel et al., 2006). We questioned whether both factors would reciprocally influence their distribution and AZ localization (Fig. 10, A–E). As expected

![Figure 8. Embryonic dsyd-1 phenotypes.](image)
We identified the Drosophila Syd-1 homologue (DSyd-1) as a binding partner of BRP. We found (Fig. 9; Fouquet et al., 2009) that DLiprin-α and DSyd-1 mark presynaptic sites where, subsequently, AZs (and adjunct PSDs) originate and mature, where BRP and Ca\(^{2+}\) channels accumulate at later time points than DLiprin-α and DSyd-1. DLiprin-α previously has been shown to be important for proper AZ formation (Kaufmann et al., 2002). Thus, consistent with reduced numbers of AZs forming at NMJs of dsyd-1 and dliprin-α mutants (Fig. 4 G; and Kaufmann et al., 2002) and with both proteins being localized to AZs, the accumulation of DLiprin-α and DSyd-1 at nascent AZs may be instrumental for transforming selected sites into AZs, a process we refer to as “AZ nucleation activity.” However, as the morphological size of dsyd-1 NMJs is reduced, as is the AZ number (Fig. 4 F, G), in principle, other growth processes might also become rate-limiting at dsyd-1 mutant NMJs.

In other words, reduced AZ numbers could also be a consequence of a reduction in morphological NMJ growth. Studying the coupling between morphological growth and AZ formation will be important for determining the relevance of morphological size to total AZ number.

Work on en passant synapses of the C. elegans HSNL motor neuron implies that, in genetic terms, Syd-1 operates upstream of Syd-2/Liprin-α. This is based on the fact that a Syd-2/Liprin-α dominant allele can bypass the requirement of syd-1 (Dai et al., 2006), which indicates that the protein’s essential role in AZ assembly at HSNL synapses is mediated via Syd-2/Liprin-α.

(Fouquet et al., 2009), at control NMJs (Fig. 10 A), DLiprin-α and BRP colabeled individual AZs in a regular pattern (Fig. 10 A, arrowheads). Notably, DLiprin-α showed a highly irregular distribution at dsyd-1 mutant terminals (Fig. 10 B), with many AZs (identified via BRP) lacking adequate DLiprin-α labeling (Fig. 10 B, arrowheads). Large DLiprin-α spots distant from BRP spots were often observed, which indicates the presence of ectopic accumulations of DLiprin-α (Fig. 10 B, arrows). After coexpression of DSyd-1 together with DLiprin-α at dsyd-1 NMJs, however, most BRP-positive AZs showed DLiprin-α labeling (Fig. 10 C, arrowheads). In contrast, DSyd-1 targeted normally to AZs in dliprin-α mutants (compare Fig. 10 D with Fig. 10 E). Thus, presynaptic DSyd-1 is needed to properly localize DLiprin-α to AZs, but DLiprin-α is apparently not needed to target DSyd-1.

We also asked whether DSyd-1 would localize to brp mutant terminals. BRP arrives late during synapse assembly and is needed for proper maturation of release sites, as shown for the distribution of calcium channels (Fouquet et al., 2009). Although DSyd-1 targeted to AZs (Fig. 10 F), the distribution of the protein appeared somewhat “smeared,” suggesting that BRP is needed for the proper spacing of DSyd-1 at mature AZs.

Discussion

Mechanisms which regulate assembly and maturation of presynaptic AZs are not well understood (Jin and Garner, 2008). We identified the Drosophila Syd-1 homologue (DSyd-1) as a binding partner of BRP. We found (Fig. 9; Fouquet et al., 2009) that DLiprin-α and DSyd-1 mark presynaptic sites where, subsequently, AZs (and adjunct PSDs) originate and mature, whereas BRP and Ca\(^{2+}\) channels accumulate at later time points than DLiprin-α and DSyd-1. DLiprin-α previously has been shown to be important for proper AZ formation (Kaufmann et al., 2002). Thus, consistent with reduced numbers of AZs forming at NMJs of dsyd-1 and dliprin-α mutants (Fig. 4 G; and Kaufmann et al., 2002) and with both proteins being localized to AZs, the accumulation of DLiprin-α and DSyd-1 at nascent AZs may be instrumental for transforming selected sites into AZs, a process we refer to as “AZ nucleation activity.” However, as the morphological size of dsyd-1 NMJs is reduced, as is the AZ number (Fig. 4 F, G), in principle, other growth processes might also become rate-limiting at dsyd-1 mutant NMJs. In other words, reduced AZ numbers could also be a consequence of a reduction in morphological NMJ growth. Studying the coupling between morphological growth and AZ formation will be important for determining the relevance of morphological size to total AZ number.

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release sites formed (DiAntonio, 2006). Individual PSDs form distinctly from preexisting ones, and mature over hours, switching from DGluRIIA to IIB incorporation throughout maturation in a manner dependent on presynaptic signaling (Rasse et al., 2005; Schmid et al., 2008). DSyd-1 might mediate such a maturation signal, as *dsyd-1* mutants show excessive amounts of DGluRIIA incorporation at PSDs. This regulation is likely not (or only partially) due to compensation for reduced presynaptic glutamate release, as *dliprin-α* mutants (with similarly reduced transmission levels) do not show this dramatic increase in GluR levels.

Despite enlarged receptor fields and specifically elevated DGluRIIA levels, average miniature event amplitudes were comparable between *dsyd-1* animals and controls, which we currently cannot account for. A possible explanation might comprise regulatory processes rendering populations of receptors non-/partially functional. Nonetheless, EJC decay time constants of *dsyd-1* mutants resemble those found at *dgluRIIB*-deficient (and thus GluRIIA dominated) NMJs (Schmid et al., 2008).

Which processes are downstream of the DSyd-1–mediated DLiprin-α activity at nascent AZs? Liprin family proteins steer transport in axons and dendrites (e.g., of AMPA receptors) to support synaptic specializations (Wyszynski et al., 2002; Shin et al., 2003; Wagner et al., 2009). Notably, in *dsyd-1* mutants, although many AZs lacked proper amounts of DLiprin-α, large ectopic accumulations of DLiprin-α were observed. At the same time, ectopic accumulations of BRP/electron density were observed in the absence of DSyd-1. It is tempting to speculate that the presynaptic DSyd-1 protein helps the AZ localization of an adhesion protein, which via trans-synaptic interaction might steer the incorporation of postsynaptic GluRs (for a model, see Fig. 10 G). A potential role of the Neurexin–Neuroligin axis should be evaluated in this context (Li et al., 2007; Südhof, 2008).

*Drosophila* NMJs express two functionally distinct GluR complexes, DGluRIIA and IIB, which influence the number of release sites formed (DiAntonio, 2006). Individual PSDs form distinctly from preexisting ones, and mature over hours, switching from DGluRIIA to IIB incorporation throughout maturation in a manner dependent on presynaptic signaling (Rasse et al., 2005; Schmid et al., 2008). DSyd-1 might mediate such a maturation signal, as *dsyd-1* mutants show excessive amounts of DGluRIIA incorporation at PSDs. This regulation is likely not (or only partially) due to compensation for reduced presynaptic glutamate release, as *dliprin-α* mutants (with similarly reduced transmission levels) do not show this dramatic increase in GluR levels.

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speculate that these ectopic pools of DLiprin-α provoke the aberrant accumulation of electron densities in dysd-1 mutants, which is consistent with the transport function of DLiprin-α (Miller et al., 2005) and the direct interaction of DLiprin-α/Syd-2 and ELKS/BRP (Patel and Shen, 2009). Consistently, large BRP accumulations observed in dysd-1 embryos were no longer present in dysd-1; dliprin-α double mutants, which indicates that the presence of DLiprin-α is needed to provoke these overaccumulations of BRP when DSyd-1 is missing.

In the absence of DSyd-1, BRP was inappropriately localized, even within the cytoplasm, forming ectopic electron-dense material (which is consistent with its role as building block for the electron-dense T bars). Such “precipitates” also occurred at and close to non-AZ membranes. Moreover, at dysd-1 AZs, large malformed T bars formed. Thus, it appears plausible that DSyd-1 keeps BRP “in solution” to organize its proper consumption at AZs. An alternate and not mutually exclusive explanation may be that axonal BRP precipitates also reflect defects in axonal transport due to the absence of DSyd-1. The presence of several binding interfaces between BRP and DSyd-1 may be considered as a basis for regulating their interplay.

BRP accumulation in the center of the AZ is also in the center of the functional and structural AZ assembly process (Kittel et al., 2006; Wagh et al., 2006; Fouquet et al., 2009). It appears likely that BRP assembly is regulated on multiple levels. Notably, although BRP accumulation is severely compromised in mutants for the kinesin imac (Pack-Chung et al., 2007), it is not fully eliminated. Moreover, the serine/arginine protein kinase SRPK79D was recently shown to associate with BRP and to repress premature “precipitation” of BRP in the axons (Johnson et al., 2009; Nieratscher et al., 2009). Furthermore, mutants for the serine/threonine kinase unc51I have recently been shown to suffer from BRP targeting defects (Waikar et al., 2009). Phosphorylation of DSyd-1 (e.g., within serine-rich stretches toward the C terminus) might be involved in regulating proper long-range transport (“blocking precipitation on the way”) as well as proper delivery of BRP at nascent AZ sites.

Recently, the Rab3 GTase has been shown to be crucial for effective nucleation of BRP at AZs (Graf et al., 2009). In an interesting parallel to dysd-1 defects, rab3 mutant NMJs showed fewer BRP-positive AZs; however, if present, BRP levels were increased. Nonetheless, instead of overgrown T bars, as observed in dysd-1 mutants, rab3 mutants rather showed multiple T bar AZs (Graf et al., 2009). It will be interesting to investigate whether these pathways act in parallel or converge, along with their relationships to other synaptogenic signals (Giautrozgiou et al., 2009; Oswald and Sigrist, 2009).

Materials and methods

Proteomics

Protein extraction protocols were modified from Luo et al. (1997). Wild-type adult fly heads were mechanically homogenized in deoxycholate buffer (500 mM Tris, pH 9.0, and 1% sodium-deoxycholate containing protease inhibitor cocktail [Roche]) followed by incubation at 36°C for 30 min. 0.1% Triton X-100 was added thereafter, and the lysate was incubated at 4°C for 30 min. After centrifugation for 15 min at 16,000 g, the supernatant was used in immunoprecipitations with the monoclonal antibody BRPm82 (provided by E. Buchner, Universität Würzburg, Würzburg, Germany) or mouse IgG heavy chain for control (Dianova) cross-linked to protein A–Sepharose (Bio-Rad Laboratories). After incubation at 4°C for 2 h, beads were washed in deoxycholate/Triton X-100 buffer. In a first approach, proteins were removed en masse from the BRPdsyd-1; Protein A beads with 100 mM glycine, pH 2.0, reduced with diithiothreitol, carboxymethylated using iodoacetamide, and digested with trypsin (Betschinger et al., 2003). Peptides were extracted with formic acid (FA) and separated by reverse-phase liquid chromatography (RP-LC) on a PepMap C18 reversed-phase column. Eluting peptides were transferred online to an ion trap mass spectrometer (LTQ; Thermo-Fisher Scientific).

In a second approach, proteins were eluted from the MAB Nc82; Protein A beads with SDS sample buffer. The samples were separated by one-dimensional SDS-PAGE (NuPAGE 4–12% gradient gel; Invitrogen), and proteins were visualized using SYPRO red (Invitrogen). The elution and control lanes contained proteins with matching mouse IgGs, which were each cut in 2-mm-thick stripes so that the regions of both lanes aligned with each other. Each individual stripe was digested in gel with trypsin (sequencing grade; Roche), and peptides were extracted according to Shevchenko et al. (1996). Dried samples from in-gel digests were dissolved in 10% (vol/vol) acetonitrile (CH3CN; LiChrosolv grade; Merck & Co., Inc.) and 0.1% (vol/vol) FA. The gradient applied was 90% (vol/vol) buffer A to 55% (vol/vol) buffer A in 60 min, 55% (vol/vol) buffer A to 10% (vol/vol) buffer A in 5 min, and 5 min with 10% (vol/vol) buffer A. Before separation of the peptides by nano-LC, samples were desalted with online coupled precolumns (3 mm) consisting of the same chromatography material. The electrospray was generated with fused-silica 10-µm PicoTip needles (New Objective, Inc.) and was operated at ~1.8–2.3 kV. Fragment spectra of sequenced peptides were searched against all entries of the nonredundant Database from the National Center for Biotechnology Information using the software search algorithms MASCOT (Matrix Science Ltd.). For the database search, no constraints on molecular weight or biological species were applied.

Both approaches identified DSyd-1 in BRPdsyd-1 immunoprecipitates as physical interactors of BRP; however, DSyd-1 was not detected in control immunoprecipitations.

Yeast two-hybrid

dysd-1 constructs were obtained by PCR on pUAS/dysd-1 (see the Molecular cloning paragraph) and cloned into pGADT7 and pGBK7T7 (both from Takara Bio Inc.). BRP constructs have been described previously (Fouquet et al., 2009). In principle, all experiments were conducted as described previously (Fouquet et al., 2009). All cotransformation experiments were conducted according to the yeast two-hybrid protocols of Takara Bio Inc., using the strain AH109. In brief: to ensure the presence of both cotransformed plasmids, the yeast was plated on minimal synthetic defined (SD)/−His/−Leu/−Trp medium plates. After growing for 2–3 d, at least 10 clones each were analyzed on SD/−Adr/−His/−Leu/−Trp/X-gal medium plates to select for positive interaction. If >90% of the clones grew (and turned blue in color), this was regarded as positive interaction. As a positive control, pGBK7T7-p53 was cotransformed with pGADT7 containing the SV40 large T antigen. Negative controls consisted either of laminin as bait together with the prey to be tested or the corresponding bait together with the empty prey vector (Fouquet et al., 2009).

Genetics

Fly strains were reared under standard laboratory conditions (Sigrist et al., 2003). Either w or w1118 strains were used as background for generation of transgenes (BestGene, Inc.). dysd-1 mutants (dysd-1,J2d4, eliminating the complete dysd-1 and partially deleting the 3′ hept locus; and dysd-1,J2d3, eliminating the complete dysd-1 and partially deleting the 5′ ferrochelatase locus) were constructed and validated by genomic PCR according to Parks et al. (2004). For diliprin, diliprinA mutant strain diliprinA,J2d15 (Kaufmann et al. 2002) was used. diliprinA,J2d15 and diliprinA,J2d12; dysd-1,J2d3 were kept using the T2; CyO;GFP:TM3GDF compound balancer (Eisenberg et al., 2005).

Genotypes used for in vivo imaging were (all from a w background): (a) ok-X-GAL4, UAS-BRPdsherry+; UAS–DSDyd-1/++; (b) ok-X-GAL4, UAS–GFPDiliprin-α/+; UAS–nmcDSDyd-1/++; (c) UAS-MitoGFP/ok-X-GAL4
G.GGTGGGCGACGAAATCCT-3
A2: (A1) 5'-GAACTGATCTTCCATTTTCCGC-
A3: (A1) 5'-GAGC-
the “analyze particles” tool was applied within predefined region of interest surrounding single PSDs. The sum of the area of all BRP-labeled particles was measured for each PSD, and the number of particles was subsequently counted. No Gaussian blur was applied here.

To define the DVGUT and Mito-SP signal intensity of NMs, a region of interest was applied by surrounding the 1b innervations (based on the HRP signal), and the mean pixel intensity was measured. To compare several experiments, the mean signal was subsequently normalized to the corresponding HRP signal.

To compare different time points in live imaging experiments, all images were normalized by adjusting the brightest pixel composing the NMJ to 255 arbitrary units (au).

Two-electrode voltage clamp recordings

Two-electrode voltage clamp recordings were essentially performed as described by Fouquet et al. (2009). In brief: for dsy1-1 (ok6-GAL4/+; dsy1-1), dsy1-1 rescue (ok6-GAL4/); dsy1-1, UAS–dsy1-1mRNA/dsy1-1), and controls (w[1118]; ok6-GAL4/+), as well as dpprinα and controls (w[1118]), recordings were made from late third-instar larvae (muscle 6, segments A2 and A3; experimental groups consisted of either males or females only). For all experiments, the recording solution consisted of HL3: 70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM tretahole, 115 mM sucrose, 5 mM Hepes, and 1 or 0.5 mM CaCl2, pH adjusted to 7.2. The cells from which recordings were made had an input resistance ≥ 4 MΩ. Intracellular electrodes were filled with 3 M KCl, and resistances ranged from 10 to 25 MΩ. Stimulation artifacts of eEJCs were removed for clarity. Paired pulse stimulation protocols and analyses were essentially performed as in Kittel et al. (2006). Paired pulse intervals were either 10 ms or 30 ms, and experiments were performed in 0.5 mM extracellular calcium. For determination of the base line of the second pulse at the 10-ms interpulse interval, the decay of the first pulse was extrapolated.

Transmission EM

For high-pressure freezing, ~2–10 (22–24 h) staged Drosophila embryos were placed in aluminum specimen carrier 200 µm deep (type A; Leica), filled with yeast paste, and covered with a lid (specimen carrier type). The samples were frozen immediately in a high-pressure freezing machine (HPM010; Bal-Tec) and rapidly transferred to liquid nitrogen for storage.

Subsequently, 55–65-nm (gray-silver) sections were cut using an ultramicrotome (EM Ultracut 6; Leica). Sections were collected on formvar-coated 100-nm mesh grids. Sections were dried and post-stained with uranyl acetate and lead citrate as described previously (Schmid et al., 2006). Micrographs were taken with a 1024 × 1024 charge-coupled device (CCD) detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems GmbH) in a transmission EM (EM 902A; Carl Zeiss, Inc.) operated in bright field mode.

Reconstructions

For 3D-reconstructions of larval T bar walls (w118; dsy1-1), 3–5 serial 60-nm sections were reconstructed with the free software Reconstruct (Fiala, 2005).

Behavioral analysis

Female animals were tested within 48 h after eclosion and at least one night at 18°C. Before testing, flies were anesthetized on ice and wings were clipped. Experiments were performed under a red light, and animals were allowed to adapt to darkness for at least 1 h before testing. To test walking ability, flies were placed on a flat surface with a 2 × 2-cm grid and allowed to walk freely for 10 s. The number of lines crossed was counted. Negative geotaxis was measured with flies placed on the bottom of an empty, scaled food vial, and the maximum height (max = 9 cm) reached within 30 s was recorded.

Statistics

Data were analyzed with Prism (GraphPad Software). Asterisks are used to denote significance (*, P < 0.05; **, P < 0.01; ***, P < 0.005; ns, P > 0.05).

Online supplemental material

Fig. S1 shows the amino acid sequence of Dsy1-1 with peptides identified via MS highlighted in red. Fig. S2 deals with the distribution of SVs and mitochondria in dsy1-1 mutants. Fig. S3 shows that axonal BRP and DVGUT colocalize. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908055/DC1.

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References


Downloaded from on June 19, 2017
**Figure S1. MS/MS identification of DSyd-1.** (A) Amino acid sequence of DSyd-1, with the peptides identified in BRP coimmunoprecipitations via MS/MS highlighted in red. LD28013 of *dsyd-1* is indicated in gray. The peptide comprising the DSyd-1 antibody epitope is highlighted in blue. (B) Proteins found to coprecipitate with BRP in two independent approaches.

<table>
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Figure S2. **SV distribution and size is mildly affected in dsyd-1.** (A) HRP and DVGlut costaining of control and dsyd-1 NMJs. Mean DVGlut intensity is comparable between mutant and control. Bar, 5 µm. (B) Control: 0.51 ± 0.05, n = 7; dsyd-1: 0.50 ± 0.05, n = 10, P = 0.88 according to a Mann-Whitney test. DVGlut distribution seems to be inhomogeneous in dsyd-1 mutants (A; blue arrowhead indicates bouton with strong reactivity, white arrowhead indicates bouton with weak reactivity). (C–F) An ultrastructural analysis (bouton overview shown in C). The relative cumulative frequency of vesicle diameter in dsyd-1 mutants and controls is comparable (E), as is the SV distribution surrounding the T bar at AZs (D and F). (G) Co-labeling for MitoGFP and DGluRIID. (H) The mean intensity of MitoGFP is not altered significantly (control: 0.89 ± 0.14, n = 10; dsyd-1: 0.80 ± 0.08, n = 10, P = 0.97 according to a Mann-Whitney test). Error bars indicate the SEM. ns, P > 0.05. Bars: (A) 5 µm; (C) 700 nm; (D) 150 nm; (G) 1 μm; (G, insets) 500 nm.

Figure S3. **Ectopic BRP and DVGlut colocalizing in dsyd-1 axon.** Some axonal BRP-positive spots are also positive for the SV marker DVGlut in dsyd-1. Panels on the right show enlarged views of the boxed regions. Bars: (left) 3 µm; (right) 500 nm.