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

David Owald,1,2 Wernher Fouquet,1,2 Manuela Schmidt,3 Carolin Wichmann,1 Sara Mertel,1,2 Harald Depner,1,2 Frauke Christiansen,1,2 Christina Zube,1 Christine Quentin,1,2 Jörg Körner,2 Henning Urlaub,4 Karl Mechtler,5 and Stephan J. Sigrist1,2

1Department of Genetics, Institute for Biology, Freie Universität Berlin, 14195 Berlin, Germany
2Bioimaging Center, Rudolf Virchow Center, 97078 Würzburg, Germany
3European Neuroscience Institute, 37077 Göttingen, Germany
4Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany
5Research Institute of Molecular Pathology, 1030 Vienna, Austria

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...
Figure 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 co precipitate 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 an 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 location of dsysd-1 on chromosome arm 3R at 100D2–100D3. dsysd-1-deficient animals were constructed using Drosophila lines carrying transposon-mediated flippase recognition target sites (Parks et al., 2004) that neighbor the dsysd-1 locus (black, dsysd-1ex1.2; gray, dsysd-1ex3.4 in gray). We obtained two deficiencies that were confirmed with genomic PCR. In both cases, the entire dsysd-1 locus (red) was excised, whereas in one case (dsyd-1ex1.2, black line), the 5′ ferrochelatase was affected; and in the other case, the 3′ heph (dsyd-1ex3.4, gray line) locus was affected. Taking these deficiencies in trans eliminates both copies of dsysd-1; however, this leaves one intact copy of each heph and ferrochelatase. (D and E) Behavioral tests demonstrate a requirement for DLPinxx in the adult CNS. (D) Walking ability (control: 15.69 ± 0.57 lines, n = 15; dsysd-1: 1.62 ± 0.69 lines, n = 8; dsysd-1ex3.4: 12.86 ± 0.99 lines, n = 10; dlpinx+: 16.19 ± 0.65 lines, n = 7; control × dsysd-1: P = 0.0001; control × dsysd-1ex3.4, P = 0.02; control × dlpinx+: P = 0.67; dsysd-1 × dlpinx+: P < 0.0001). (E) Negative geotaxis (control: 8.32 ± 0.37 cm; dsysd-1: 2.92 ± 0.60 cm; dsysd-1ex3.4: 8.83 ± 0.17 cm; dlpinx+: 8.67 ± 0.13 cm; all: n = 10; control × dsysd-1: P < 0.0001; control × dsysd-1ex3.4, P = 0.32; control × dlpinx+: P = 0.91; dsysd-1 × dlpinx+: P < 0.0001). Impaired locomotive behavior in dsysd-1 flies is rescued by pan-neural (elav-GAL4) reexpression of the dsysd-1 cDNA. Error bars indicate the SEM. *, P < 0.05; ***, P < 0.005; ns, P > 0.05. (F) A polyclonal antibody of DSyd-1 antibody recognizes a band at the predicted molecular mass of 195 kDa on immunoblots of control fly head lysate (arrow). This band is missing in dsysd-1 head extracts. Statistics: Mann-Whitney test.

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 synaptic 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).

Results

The AZ protein BRP is an integral part of the electron-dense T bar and is needed for effective Ca2+ 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.
DSyd-1-deficient flies suffer from impaired locomotion and decreased lifespan

We generated dsyd-1–deficient animals using Flippase-mediated trans-deletion of flippase recognition target site–containing transposon lines (Parks et al., 2004) flanking the dsyd-1 locus (Fig. 1 C). Two dsyd-1–deficient lines (dsyd-1 ex1.2 and dsyd-1 ex3.4) were isolated, and deletions were confirmed by genomic PCR (Parks et al., 2004). A combination of both chromosomes results in flies specifically deficient for dsyd-1 (Fig. 1 C). Although dsyd-1 adults appeared morphologically normal and, under optimal culturing conditions, eclosed close to the Mendelian ratio, they rarely survived longer than a week (>80% died within one week, n = 36). In contrast, >80% of control flies (n = 159) lived for at least two weeks. Moreover, the early lethality was completely overcome by elav-GAL4–driven pan-neuronal expression of the composite full-length cDNA (upstream activator sequence [UAS]–dsyd-1 cDNA) in the mutant background (n = 42). Notably, dsyd-1 animals showed severely impaired locomotion, as revealed by two independent experimental settings, which was rescued by pan-neuronal expression of UAS–dsyd-1 cDNA as well (Fig. 1, D and E).

We raised a polyclonal antibody against a C-terminal peptide of DSyd-1 (DSyd-1 antibody) that identified a band of predicted size (195 kD) in adult head extracts and that was missing in extracts of dsyd-1 mutants (Fig. 1 F).

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 (Du77F) 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.

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 (Kaufmann et al., 2002; Fouquet et al., 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-1 cDNA (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 Dα7 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 Dα7 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).
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…”).

dliprin-α together seem to define a common subcompartment surrounding the AZ core.

Correlation analysis of DLiprin-α and DSyd-1 costainings (Fig. 3 G) indicated that both proteins closely colocalize (R_{DSyd-1:DLiprin-α} = 0.81 ± 0.01; n = 12), significantly closer than BRP and DLiprin-α (R_{BRP: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 core were comparable (Fig. 3 E). Thus, DSyd-1 and DLiprin-α together seem to define a common subcompartment surrounding the AZ core.

et al., 2009). However, we noted that adults lacking DLiprin-α did not display the severe locomotor deficits seen in dsyd-1 mutants, which suggests that both proteins may also have differentiated functions (Fig. 1, D and E).

Using STED microscopy, we recently showed that DLiprin-α forms discrete clusters surrounding the BRP-defined center of AZs (Fig. 3 C; Fouquet et al., 2009). We found DSyd-1 in clusters of similar size and distribution (Fig. 3, D and E) to DLiprin-α clusters. The number of DLiprin-α clusters varied according to the size of AZs (as judged by BRP immunoreactivity) ranging from one cluster at small AZs to four or five clusters at mature-sized AZs (Fig. 3 F).

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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 Ca²⁺ 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 Ca²⁺ concentrations should be observed. However, when we compared evoked release at two different Ca²⁺ concentrations, the ratio between dsyd-1 mutant and control was unchanged (Fig. 4C).
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 + adjunct 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 MitoGFP (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-1RNAi (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 BRPNE2) 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-1RNAi 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.
Figure 7. **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 = 1,314; dsyd-1: 74.86 ± 2.98 au, n = 335; dsyd-1 rescue pre: 46.71 ± 1.60 au, n = 513; dsyd-1 rescue post: 81.25 ± 3.54 au, n = 344; control × dsyd-1: P < 0.001; control × dsyd-1 rescue pre: P < 0.001; control × dsyd-1 rescue post: P < 0.001; dsyd-1 × dsyd-1 rescue pre: P < 0.001; dsyd-1 × dsyd-1 rescue post: P > 0.05; dsyd-1 × dsyd-1 rescue × dsyd-1 rescue post: P < 0.001). (H) Integrated DGluRIIB signal (control: 33.88 ± 0.66 au, n = 1,064; dsyd-1: 66.85 ± 2.09 au, n = 667; dsyd-1 rescue: 36.31 ± 0.87 au, n = 830; control × dsyd-1: P < 0.001; control × dsyd-1 rescue pre: P > 0.05; dsyd-1 × dsyd-1 rescue: P < 0.001). (I) Integrated DGluRIIB signal (control: 46.40 ± 0.99 au, n = 934; dsyd-1: 23.85 ± 0.60 au, n = 783; dsyd-1 rescue: 35.46 ± 0.89 au, n = 770; control × dsyd-1: P < 0.001; control × 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 rescue: 1.24 ± 0.08, n = 6; control × dsyd-1: P < 0.001; control × dsyd-1 rescue: P > 0.05; dsyd-1 × dsyd-1 rescue: P < 0.001). 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.

(Fig. 5, A and B; and Fig. 7, A–C, arrowheads). This enlargement was rescued after presynaptic (using the motoneuron driver ok6-GAL4; Fig. 7, C and G) but not postsynaptic (using the muscle driver G14-GAL4; Fig. 7 G) expression of UAS–dsyd-1 in either DSyd-1 or DLiprin-α. These two GluR types differ in their single-channel properties, and GluR composition also controls the morphological size of the NMJ and the number of individual synaptic contacts (DiAntonio, 2006). DGluRIIA levels were dramatically increased at dsyd-1 mutant NMJs, but were restored to normal levels after pre- and postsynaptic expression of UAS–dsyd-1 in embryos (Fig. 7, D–F and H). However, the levels of DGluRIIB were specifically reduced (Fig. 7, D–F and I), shifting the ratio between the two GluR types (Fig. 7 J).

As DGluRIIA complexes show higher single-channel conductance than DGluRIIB complexes (Schmid et al., 2008), one might expect enlarged miniature excitatory junctional currents (mEJCs) at dsyd-1 NMJs. In Fig. 4 B, we show that mean mEJC amplitudes are not changed between dsyd-1 mutants and controls. However, in histogram plots, a moderate tendency toward elevated frequencies of large mEJCs was observable for dsyd-1 mutants (unpublished data). DGluRIIA-dominated NMJs also show slow decay kinetics (Schmid et al., 2008). Indeed, the mEJC decay $\tau$ recorded from dsyd-1 mutant cells was increased by a mean of 12% compared with controls ($n = 9$ cells each, $P = 0.006$, Mann-Whitney test), whereas the eEJCs decay $\tau$ was $\sim 48\%$ larger ($n = 9$ cells each, $P = 0.008$, Mann-Whitney test; compare with the traces in Fig. 4 A).

Collectively, parallel to its function of blocking overgrowth of presynaptic AZs, DSyd-1 has a specific function in restricting the size and defining the composition of postsynaptic GluR fields.

**DLiprin-α is needed for the pre- but not the postsynaptic phenotype of dsyd-1 mutants in embryos.**

Our behavioral analysis indicated that DSyd-1 shares functions with DLiprin-α, but that DSyd-1 also executes DLiprin-α–independent functions.

If both proteins solely acted in the same pathway, double mutant combinations should show similar phenotypes as single mutants. To test this, *dliprin-α; dsyd-1* double mutants were established. Although *dsyd-1* and *dliprin-α* single mutants survived to adulthood, double mutants were embryonic lethal, again indicating that the functions of both proteins do not fully overlap.

We asked whether the embryonic lethality of *dsyd-1; dliprin-α* might be due to an inability of the double mutant to form AZs and synapses altogether. Thus, ultrastructural analysis of high-pressure frozen/freeze-substituted (Fouquet et al., 2009) embryos was performed. T bars and planar synaptic membrane contacts were found in *dsyd-1* and *dliprin-α* single mutants, as well as in *dliprin-α; dsyd-1* double mutant embryos (Fig. 8 A). Hence, synapse formation including T bar assembly can in principle proceed in the absence of both proteins. We therefore consider DSyd-1 and DLiprin-α not to be structurally essential for AZ formation but rather to promote this process (Fig. 8 A).
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 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—by 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-short<sub>GFP</sub> 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) was observed (Fig. 9 A). DSyd-1 clearly and invariably preceded BRP at newly forming release sites (Fig. 9 A, arrowheads).

**We noted whether both factors would reciprocally influence their accumulation whether both factors would reciprocally influence their...**

**Newly formed AZs were usually decorated with both DLiprin-α and DGluRIID, 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...**

**We questioned whether both factors would reciprocally influence their distribution and AZ localization (Fig. 10, A–E). As expected...**
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.

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.

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.
We here provide evidence that DSyd-1 is required to properly target DLiprin-α to AZs. In the absence of DSyd-1, DLiprin-α distributes unevenly at NMJ terminals, sparing many AZs. Thus, we provide direct evidence that the RhoGAP DSyd-1 operates upstream in AZ assembly in vivo: DSyd-1 seemingly stalls DLiprin-α to developing AZs in order to allow for the AZ nucleation function of DLiprin-α to effectively operate.

DLiprin-α seems to be a direct substrate of DSyd-1 (Fig. 10 G). Our data imply that other presynaptic substrate proteins of DSyd-1 might exist at nascent synapses, a finding that is unexpected based on analysis of AZ formation in C. elegans. Therefore, we deduce from our findings that presynaptic DSyd-1 (but apparently not DLiprin-α) plays an important role in shaping the PSD assembly. Embryos and larval mutants for dsyd-1, and importantly, diliprin-α; dsyd-1 double mutant embryos (the double mutant is embryonic lethal), showed increased overall amounts of postsynaptic GluRs, whereas diliprin-α single mutant embryos (Fig. 8) and larvae did not (not depicted). These increased amounts of GluRs in dsyd-1 mutants vanished after presynaptic reexpression of UAS–dsyd-1 cDNA.

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).

Despite enlarged receptor fields and specifically elevated DGlurRIIA 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 dglurRIIB-deficient (and thus GlurRIIA 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 these ectopic pools of DLiprin-α provoke the aberrant accumulation of electron densities in dsyd-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 dsyd-1 embryos were no longer present in dsyd-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 dsyd-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; Nieratschker et al., 2009). Furthermore, mutants for the serine/threonine kinase unc-51I have recently been shown to suffer from BRP targeting defects (Wairkar 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 GTPase has been shown to be crucial for effective nucleation of BRP at AZs (Graf et al., 2009). In an interesting parallel to dsyd-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 dsyd-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 (Giaigtzoglou 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 BRPα2ε (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 dithiothreitol, carboxymethylated using iodoacetamide, and digested with trypsin (Betschinger et al., 2003). Peptides were extracted with formic acid [FA] and separated by nanoflow high-performance liquid chromatography (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 protein bands were visualized using SYPRO red (Invitrogen). The elution and control lanes contained, i.e., immunoprecipitation with mouse IgG 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 0.15% FA (Sigma-Aldrich). The sample volumes were adjusted to the sample amount. The dissolved samples were subjected to a nano-LC coupled electrospray ionization tandem MS using an orthogonal quadruple time-of-flight mass spectrometer (Q-Tof Ultima; Waters). The nano-LC system was equipped with a C18 pepMap100 column (75 µm ID, 3 µm, 100 Å; Dionex) running with a flow rate of 180 nl/min. The buffers used were as follows: buffer A (H2O and 0.1% [vol/vol] FA) and buffer B (80% [vol/vol] acetonitrile and 0.1% [vol/vol] FA). The gradient applied was 90% (vol/vol) buffer A to 55% (vol/vol) buffer B in 60 min, 55% (vol/vol) buffer A to 10% (vol/vol) buffer B 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) comprising 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
dsyd-1 constructs were obtained by PCR on pUAS/dsyd-1 (see the Molecular cloning paragraph) and cloned into pGADT7 and pGBK7 (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)/−Leu/−Trp medium plates. After growing for 2–3 d, at least 10 clones each were analyzed on SD/−Ade/−His/−Leu/−Trp/X-a-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, pGBK7T-p53 was cotransformed with pGAD7 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.). dsyd-1 mutants (dsyd-1ns1.4, eliminating the complete dsyd-1 and partially deleting the 3′ hept loop) and dsyd-1ns1.2, eliminating the complete dsyd-1 loop and partially deleting the 5′ (thre- chelatase) loop; and 2 ex3.4, eliminating the 5′ (thre- chelatase) loop; and 2 ex3.4 and 2 ex3.15, eliminating the complete dsyd-1 loop and partially deleting the 3′ hept loop) were constructed and validated by genomic PCR according to Parks et al. (2004). For dilpirina, dilpirinaPUBIC/dilpirinaDilpirina2.5 (Kauffman et al., 2002) were used. dilpirinaPUBIC/dilpirinaDilpirina2.5 and dilpirinaDilpirina2.5; dsyd-1ns1.4 and dsyd-1ns1.2 were kept using the T12; CyO/GFP; TM3GFP complement balancer (Eissenberg et al., 2005).

Genotypes used for in vivo imaging were (all from a w background): (a) ok6-GAL4, UAS-BRPshort/+; UAS-DSDyd-1/+; (b) ok6-GAL4, UAS-GFPDilpirinaDilpirina2.5/+; UAS-DSDyd-1/+; (c) ok6-GAL4, UAS-GFPDilpirinaDilpirina2.5/+; UAS-DSDyd-1/+; (d) ok6-GAL4, UAS-GFPDilpirinaDilpirina2.5/+; UAS-DSDyd-1/+.
and UAS-MitoGFP{ok6-GAL4}; dsyd-1°/dsydsym1°/dsydsym4°; (d) UAS-GFP{Dliprin-α}, UAS-BRP{short{Drosophila}/ok6-GAL4}; dsydsym1°/dsydsym4°; (e) UAS-GFP{Dliprin-α}, UAS-BRP{short{Drosophila}/ok6-GAL4}; dsydsym1°/dsydsym4°; (f) UAS-GFP{Dliprin-α}, UAS-BRP{short{Drosophila}/ok6-GAL4}; dsydsym1°/dsydsym4°. Genotypes used for Dliprin-α immunostainings were: ok6-GAL4/+; UAS-BRP{short{Drosophila}/ok6-GAL4}, UAS-Diprin-αf/+ (van Roessel et al., 2004). For Dsyd-1 immunostainings in the MB calyx, UAS-DaUASf/+; ok107-GAL4/+ was used.

**Antibody and Western blotting**

A rabbit serum against C-terminal SSGDSKNGSDEYDDIK was produced (Eurogentec). Serum was affinity purified with the same peptide.

**Molecular cloning**

As the partial clone (GenBank accession no. LD28013) was available, a Molecular cloning no. LD28013) was cut with XhoI and in vitro transcribed using T7 RNA polymerase. For this, the bps 1,183–2,933 not covered by LD28013 were

**In situ hybridization**

Whole-mount embryonic in situ hybridizations were performed essentially as described by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/). For the dsydsym1 sense RNA probe (Berkeley Drosophila Genome Project; available from GenBank/EMBL/DDJB under accession no. LD28013) was cut with Xhol and in vitro transcribed using T7 RNA polymerase. For antisense probes, LD28013 was cut with EcoRI, and SP6 RNA was in vitro transcribed.

**Image processing**

To measure the number of synapses per NMJ, first, the original stack was cut with Not and Xhol, and ligated. Bps 1,183–5,537 were amplified by elongation PCR from fly head genomic DNA using 5′-ATACGGCTTCAACCGGCTGAAGT-3′ and 5′-CGTGGCATCTCTTGCGG-3′. Fragments without introns were amplified via vent PCR. A: (A1) 5′-GAGCCGCGCGCCGCAGAAGC-3′ and (A2) 5′-GACTCTGGTCATCTCGTCGAGC-3′; B: (B1) 5′-TCCGACCCGGTGAATTCG-3′ and (B2) 5′-CCTGGAAAGAGTCTCGG-3′; C: (C1) 5′-CAAGAAGCGGCTGCTCGGGTGCTGCTGAACGAGTTCTCC-3′ and (C2) 5′-CTTGATACGCGTGGCGCAGA-3′; D: (D1) 5′-CCGGCGCGGCGGTACGGTGTCCG-3′ and (D2) 5′-TCCGATCATCGTGTCGAGCAGGAC-3′. Confocal stacks were processed with ImageJ software (National Institutes of Health). The segment of single synapses was reassigned to a value of “255.” This binary mask was then projected onto the original unmodified image using the “min” operation from the ImageJ image calculator. The synapses of the resulting image were counted onto the original unmodified image using the STED images were quantified using ImageJ. BRPNc82 size quantification was performed as described in proceeding paragraph, whereas

**Quantifications of AZ/PSD number, size, and intensity**

All images for synapse quantification from fixed samples were acquired using the same microscope settings. Control and mutant dissections were stained in the same vial.

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the “analyze particles” tool was applied within a predefined region of interest surrounding single PSDs. The sum of the area of all BRP-positive 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 MitoSP signal intensity of NMJs, 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 dsyd-1(ok6-GAL4/++; dsyd-1), dsyd-1 rescue (ok6-GAL4/+/dsyd-1, UAS–dsyd-1(1mau/dsyd-1)), and controls (w1118; ok6-GAL4/+), as well as diliprin-α and controls (w1118), 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 trehalose, 115 mM sucrose, 5 mM Heps, 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.

Freeze substitution and embedding were performed in acetone in a freeze-substitution device (EM AFS; Leica). In brief, freeze substitution was performed at −70°C (−5°C/h) to −20°C and incubated for 16 additional hours before being warmed to −5°C (−5°C/h) to 4°C. The samples were washed in acetone and warmed to room temperature. They were then embedded in epon (see Roosendaal et al., 2006; Sikou et al., 2007).

Subsequently, 55–65-nm (gray/silver) sections were cut using an ultramicrotome (EM Ultracut 6; Leica). Sections were collected on formvar-coated 100 mesh grids. Sections were dried and poststained with uranyl acetate and lead citrate as described previously (Schmidt et al., 2006). Micrographs were taken with a 1024 × 1024 charge-coupled device (CCD) detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems Microscopes), and images were obtained from dissected (CCD) detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems Microscopes) and rapidly transferred to liquid nitrogen for storage.

Reconstructions.

For 3D-reconstructions of larval T bars (w1118 vs. dSyd-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 eclosure 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 DSyd-1 with peptides identified via MS highlighted in red. Fig. S2 deals with the distribution of SVs and mitochondria in dSyd-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


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.
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, while 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.