Maturation of active zone assembly by Drosophila Bruchpilot

Wernher Fouquet,1,2 David Oswald,1,2 Carolin Wichmann,1,3 Sara Mertel,1 Harald Depner,1 Marcus Dyba,4 Stefan Hallermann,5 Robert J. Kittel,3,5 Stefan Eimer,6 and Stephan J. Sigrist1,2

1Institute for Biology/Genetics, Free University Berlin, 14195 Berlin, Germany
2BioImaging Center and 3Institute for Clinical Neurobiology, Universität Würzburg, 97078 Würzburg, Germany
3Research & Development, Leica Microsystems CMS GmbH, 68165 Mannheim, Germany
4Carl Ludwig Institute for Physiological, Medizinische Fakultät, Universität Leipzig, 04103 Leipzig, Germany
5European Neuroscience Institute and Center for Molecular Physiology of the Brain, 37077 Göttingen, Germany

SYN

Synaptic vesicles fuse at active zone (AZ) membranes where Ca2+ channels are clustered and that are typically decorated by electron-dense projections. Recently, mutants of the Drosophila melanogaster ERC/CAST family protein Bruchpilot (BRP) were shown to lack dense projections (T-bars) and to suffer from Ca2+ channel-clustering defects. In this study, we used high resolution light microscopy, electron microscopy, and intravital imaging to analyze the function of BRP in AZ assembly. Consistent with truncated BRP variants forming shortened T-bars, we identify BRP as a direct T-bar component at the AZ center with its N terminus closer to the AZ membrane than its C terminus. In contrast, Drosophila Liprin-α, another AZ-organizing protein, precedes BRP during the assembly of newly forming AZs by several hours and surrounds the AZ center in few discrete punctae. BRP seems responsible for effectively clustering Ca2+ channels beneath the T-bar density late in a protracted AZ formation process, potentially through a direct molecular interaction with intracellular Ca2+ channel domains.

Introduction

The arrival of action potentials mediates Ca2+ influx through strategically localized clusters of voltage-operated Ca2+ channels at the synaptic active zone (AZ) membrane. Ca2+ triggers exocytosis of synaptic vesicles, and tight coupling between release-ready vesicles and Ca2+ channels seems important for efficient neurotransmitter release (Neher and Sakaba, 2008). AZs are further characterized by macromolecular cytomatrices named dense bodies (Zhai and Bellen, 2004; Sikou et al., 2007). The role of these electron-dense specializations and of AZ-enriched proteins in the assembly of the AZ and/or the synaptic vesicle exo-endocytosis cycle is under intense investigation (Owald and Sigrist, 2009). Protein architectures constituting and controlling dense bodies remain to be revealed, and their contributions to AZ assembly in general and Ca2+ channel clustering in particular must be defined genetically.

W. Fouquet and D. Oswald contributed equally to this paper.
Correspondence to Stephan J. Sigrist: stephan.sigrist@fu-berlin.de

Abbreviations used in this paper: au, arbitrary units; AZ, active zone; BRP, Bruchpilot; Cac, Cacophony; DGluR, Drosophila glutamate receptor subunit; DLiprin-α, Drosophila Liprin-α; EMS, ethyl methyl sulfonate; FS, freeze substitution; HPF, high pressure freezing; IP, immunoprecipitation; mStraw, mStrawberry; NMJ, neuromuscular junction; PSD, postsynaptic density; PSF, point spread function; STED, stimulated emission depletion; UAS, upstream activator sequence.

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densities were compromised within AZs (Kittel et al., 2006). However, whether BRP performs an essential signaling role in T-bar formation or whether the protein itself is an essential building block of T-bars remained to be clarified. Thus, we entered into a structure-function analysis of BRP.

mAb Nc82 maps toward the C-terminal end of BRP

mAb Nc82 is derived from a Drosophila head extract–directed library (Hofbauer et al., 2009) and allowed the first identification of the BRP protein. mAb Nc82 is a widely used marker in Drosophila, both for neuropil in general and for AZs in particular (Wucherpfennig et al., 2003; Kittel et al., 2006; Wagh et al., 2006). Previously, we had loosely mapped the epitope of Nc82 to the region between aa 635 and the end of the 1,740-aa BRP protein (based on cDNA AT09405; Wagh et al., 2006). To define the Nc82 epitope more precisely, various BRP fragments (Fig. 1 A and Table I) were ectopically expressed in wing discs using dpp-Gal4 (Fig. 1 B). In this manner, the mAb Nc82 epitope (hereafter BRPNc82) could be mapped to the region between aa 1,227 and 1,740. Additionally, an antibody directed against an N-terminal peptide (BRPN-Term antibody; aa 62–75; Fig. 1 B) was produced.

The predicted lengths in aa of the UAS-BRP fragments (relative to full-length BRP [1,740 aa]) are shown.

Table I. BRP-reexpressing constructs

<table>
<thead>
<tr>
<th>BRP fragments</th>
<th>Start (aa)</th>
<th>End (aa)</th>
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</thead>
<tbody>
<tr>
<td>Domain 1 [D1]</td>
<td>1</td>
<td>320</td>
</tr>
<tr>
<td>Domain 2 [D2]</td>
<td>268</td>
<td>617</td>
</tr>
<tr>
<td>Domain 3 [D3]</td>
<td>473</td>
<td>1,226</td>
</tr>
<tr>
<td>Domain 4 [D4]</td>
<td>1,152</td>
<td>1,740</td>
</tr>
</tbody>
</table>

family are generic AZ proteins. In mice, CAST/ERC proteins have been shown to localize to AZs of various synapses and to bind other AZ proteins such as RIM (Rab3a-interacting molecule) and Liprin-α (Ohkuma et al., 2002; Wang et al., 2002; Ko et al., 2003; Deguchi-Tawarada et al., 2004). In Caenorhabditis elegans, the CAST/ERC family member ELKS (glutamine-, leucine-, lysine-, and serine-rich protein) appears to operate genetically downstream of Syd-2/Liprin-α during the assembly of AZs at vulval synapses (Dai et al., 2006; Patel et al., 2006). Recently, the CAST/ERC family member Bruchpilot (BRP), a coiled-coil rich protein of nearly 200 kD, was identified via its localization to Drosophila AZs. Mutants of brp lacked T-bars, and Ca<sup>2+</sup> channels were mislocalized at AZs, leading to inefficient vesicle release and changes in synaptic short-term plasticity (Kittel et al., 2006; Wagh et al., 2006).

In this study, we provide evidence that BRP takes up an elongated conformation and is a direct component of the T-bar. The N terminus of BRP is found superimposed on the Ca<sup>2+</sup> channel clusters at the AZ center. BRP and Cac arrive at an advanced stage of the protracted synapse assembly process, and both proteins interact in vitro. In contrast, a further AZ-organizing protein, Drosophila Liprin-α (DLiprin-α), localizes to a different subcompartment of the AZ and enters nascent AZs substantially earlier than BRP. Thus, the assembly of the T-bar is instructed by BRP, which seems essential for clustering higher numbers of Ca<sup>2+</sup> channels at an advanced stage of AZ maturation.

**Results**

The AZ protein BRP was recently shown to be crucial for efficient neurotransmission at Drosophila NMJs. Presynaptic AZs missing BRP lacked dense bodies (T-bars), and Ca<sup>2+</sup> channel
Structure-function analysis of BRP in T-bar formation

So far, the analysis of BRP function was based on the brp<sup>69</sup> allele in which most of the protein-coding sequence (corresponding to aa 283–1,740) is deleted (Fig. 1 C, green). As previously reported, T-bars were missing at brp<sup>69</sup> AZs (Fig. 2, compare A and B), and the BRP<sup>Nc82</sup> label (Fig. 2, A and B) was absent (Kittel et al., 2006). The BRP<sup>N-Term</sup> label was also completely absent (Fig. 2 B), indicating that the predicted residual protein (corresponding to aa 1–282) is unstable or at least does not localize to the NMJ. To ensure that brp<sup>69</sup> reflects a true null phenotype, we produced the deletion mutant brp<sup>6.1</sup> (Fig. 1 C, green) in which all genomic sequences of brp were removed (see Materials and methods). This led to a complete loss of BRP<sup>Nc82</sup>/BRP<sup>N-Term</sup> labels and T-bars, whereas some traces of residual electron-dense material appeared at the same frequency as in brp<sup>69</sup> (Kittel et al., 2006; unpublished data). As brp<sup>6.1</sup> and brp<sup>69</sup> (Kittel et al., 2006) behaved identically in all aspects, our previous analysis based on brp<sup>69</sup> reflected a true null situation.

BRP is a large protein (1,740 aa). To enter into a structure-function analysis of BRP in T-bar assembly, additional brp alleles were looked into. First, a piggyBac-transposon insert (brp<sup>c04298</sup>; Fig. 1 C, blue; Bellen et al., 2004) located toward the middle of the locus was characterized. At brp<sup>c04298</sup> NMJs, the BRP<sup>Nc82</sup> label was absent, whereas the BRP<sup>N-Term</sup> label was dramatically reduced (Fig. 2 C). Comparable with our observations...
for brp^{60} (Kittel et al., 2006), electron microscopic analysis of brp^{04298} showed a complete lack of T-bars (Fig. 2 C), and only traces of electron-dense material remained at AZ membranes. Thus, as this allele is a site-specific insertion but not a deletion (which in principle might eliminate control elements of genes other than brp), this allele provides further proof that BRP is essential for T-bar assembly. However, as the molecular alterations of brp^{04298} cannot be predicted easily, we sought to analyze aa point mutations in brp. To do so, a chemical mutagenesis screen (ethyl methyl sulfonate [EMS]) selecting for reduced viability or larval lethality over brp-null alleles was performed.

The brp^{5.45} allele is characterized by a stop codon at aa position 867 (~50% protein length), which leads to pupal lethality over brp null with weak escapers (Fig. 1 C). As expected, the BRPN^{Nc82} label was absent from brp^{5.45} NMJs. Although the number of BRPN^{N-Term} clusters was reduced over the whole NMJ (Fig. S1 A), those remaining in brp^{5.45} were slightly smaller, although of comparable intensity as in controls (Fig. 2 D; and Fig. S1, B and C). Despite extensive analysis, T-bars were not detected at brp^{5.45} NMJs (Fig. 2 D).

The EMS allele brp^{1.3} delivered paralyzed adult escapers over brp null as the result of a premature stop codon at aa 1,390 (generating a protein 523 aa longer than predicted for brp^{5.45}; Fig. 1 C). Although the number of BRPN^{N-Term} clusters was reduced to ~40% (Fig. S1 A), their sizes and intensities were comparable with controls. At the same time, the BRPN^{Nc82} label was absent (Fig. 2 E). T-bar–like structures were observed at brp^{1.3} NMJs (Fig. 2 E), although at lower frequency than in controls (not depicted). However, upon closer inspection, the T-bar–like structures typically appeared truncated (Fig. 2).

For EM, conventional room temperature embedding procedures, including aldehyde fixation and dehydration of the tissue (Fig. 2, A–E), are prone to shrinkage artifacts. To use an alternative conservation method for the analysis of brp^{1.3}, we introduced high pressure freezing (HPF)/freeze substitution (FS) EM (Gray et al., 2006; Rostaing et al., 2006; Sikou et al., 2007) to larval NMJs. With HPF/FS, NMJ tissue appeared well preserved, as judged by the smooth membrane surfaces of, for example, mitochondria (Fig. S2, A and B) or presynaptic boutons (not depicted). Furthermore, electron-dense structures appeared taller, which was likely caused by a reduced loss of material during HPF/FS embedding (e.g., the synaptic cleft; Fig. 2 F). Unlike T-bars visualized in conventionally embedded tissues, HPF/FS-processed T-bars were characterized by filamentous elements at their distal ends (Fig. 2 F, arrowheads). At brp^{1.3} NMJs, HPF/FS EM (similarly to our observations obtained with standard EM) typically revealed shortened T-bars (Fig. S2 C, quantification). In conclusion, elimination of aa 1,390–1,740 of BRP did not prevent the formation of T-bar–like assemblies per se. However, these assemblies were significantly smaller than in controls. This result is in line with the assumption that BRP operates as a building block shaping the T-bar.

To both confirm and extend our results, BRP-encoding cDNA fragments (C-terminally GFP tagged for in vivo visualization) were expressed in motoneurons of brp^{60} larvae (Fig. 2, G and H). As expected (Kittel et al., 2006), full-length BRP localized to AZs and restored T-bar formation (not depicted). The C-terminally truncated fragment D1-3GFP (BRPD1-3GFP; Δ aa 1,227–1,740; Fig. 1 A and Table I) localized to presynaptic sites but was not sufficient for T-bar formation (Fig. 2 G). Thus, expression of BRPD1-3GFP further suggests that the C-terminal region of BRP (distal of aa 1,226; Table I) is important for T-bar formation.

By expressing D2-4GFP (BRPD2-4GFP; Δ aa 1–267; Fig. 1 A and Table I), the role of the N-terminal region of BRP was tested. The expressed protein was found close to individual AZs (Fig. 2 H). Although T-bars were never observed, small electron-dense aggregates (clearly smaller than T-bars) localized to the AZ membrane at high frequency (Fig. 2 H). Thus, the N-terminal region of BRP seems important for the formation of proper, full-sized T-bars, whereas the C-terminal region is required for the assembly of T-bars by itself. Thus, the entire BRP protein appears to take part in configuring the T-bar structure.
Our measurements were performed using sandwiches of primary antibodies and labeled secondary antibodies. For distances in the double-digit nanometer range, the size of individual Ig molecules (used for the detection of epitopes) might be relevant. Thus, we sought to independently validate the distance between BRP N and C termini. To do so, BRPD1-4GFP was expressed in the brp69 background, and the distance between the BRP C terminus (endogenous GFP fluorescence) and the N terminus (BRPN-Term antibody) was determined (Fig. 4 A). Again, $\approx 70$ nm was measured. Finally, the center to center distance between BRP N-Term and CacGFP was measured as $\approx 60$ nm (Fig. 4 A). Collectively, we conclude that the BRPNc82 and BRPN-Term epitopes are segregated along an axis perpendicular to the AZ membrane.

The BRP N terminus displays a confined distribution close to the AZ membrane

We proceeded to study the molecular organization of AZs at the Drosophila NMJ with STED microscopy to obtain improved optical resolution in xy coordinates. Previously, it was demonstrated that BRP$^{Nc82}$ forms doughnut-shaped structures when visualized at AZs arranged planar to the optical axis (Kittel et al., 2006). BRP$^{Nc82}$ doughnuts were reproduced from planar AZs (Fig. 5, A [arrow] and B) with a resolution displaying an
and BRPNc82 was similar to that observed at control AZs (Fig. 5 E). This suggests that individual BRP molecules can adopt an elongated conformation.

The N terminus of BRP overlays the Ca²⁺ channels at the AZ core

How does the molecular architecture of BRP relate to Ca²⁺ channels at AZs? Ca²⁺ channel spots (CacGFP) imaged at standard confocal resolution were found to cocenter with the BRPN-Term label and BRPNc82 doughnuts at planar AZs. At vertical AZs, the CacGFP signal localized toward the AZ membrane relative to both BRPN-Term (Fig. 5 C) and BRPNc82 (Fig. 5, F and G). When imaged with STED resolution, Ca²⁺ channels consistently localized to small, typically slightly elliptical patches (~100–150 nm effective point-spread function (PSF) of 80-nm full-width half-maximum. Other than BRPNc82, BRPN-Term did not show a doughnut-shaped distribution when imaged with STED (Fig. 5 D). The combination of STED resolution (confined to one channel in our experiments) for BRPNc82 and confocal resolution for BRPN-Term revealed a polarized and funnel-like distribution of BRP epitopes (Fig. 5, A–C). Notably, the BRPNc82 signal did not appear fully continuous but instead consisted of discrete foci (Fig. 5, B and F) within an overall circular array. In an additional experiment, we expressed full-length BRPD1-4 (Wagh et al., 2006) in brp69 mutants. The distance between BRPN-Term and BRPNc82 was similar to that observed at control AZs (Fig. 5 E). This suggests that individual BRP molecules can adopt an elongated conformation.
Figure 5. STED analysis of AZ organization at Drosophila NMJ synapses. (A) Overview of a bouton stained for BRP<sub>N-Term</sub> (confocal; magenta) and BRP<sub>Nc82</sub> (STED; green) showing planar (arrow) and vertical (arrowhead) AZs. (B and C) Magnifications of individual planar (left) and vertical (right) AZs stained for BRP<sub>N-Term</sub> (STED) and BRP<sub>N-Term</sub> (confocal; B) and BRP<sub>N-Term</sub> (STED) and CacGFP (confocal; C). (D) Mean normalized planar BRP<sub>N-Term</sub> (magenta) and BRP<sub>Nc82</sub> (green) arrangement shown with STED resolution (BRP<sub>N-Term</sub>, n = 14; BRP<sub>Nc82</sub>, n = 47). (right) The merge superimposed with the intensity profile along one axis through the midpoint for BRP<sub>N-Term</sub> (magenta) and BRP<sub>Nc82</sub> (green) is shown. Error bars indicate ± SEM. (E) BRP<sub>Nc82</sub> (STED) and BRP<sub>N-Term</sub> (confocal) after expression of full-length BRP cDNA in brp<sup>69</sup> background (BRP<sup>D1-4</sup>). (F and G) Individual planar (left) and vertical (right) AZs stained for BRP<sub>Nc82</sub> (STED) and CacGFP (confocal; F) and CacGFP (STED) and BRP<sub>Nc82</sub> (confocal; G). All images were deconvolved using Imspector software. Bars: (A) 1 µm; (G) 100 nm.
map,” DLiprin-α<sub>GFP</sub> was expressed in motoneurons and visualized via αGFP stainings with STED microscopy (Fig. 6 A). DLiprin-α<sub>GFP</sub> revealed substructures beyond the diffraction limit of confocal microscopy. (B–D) STED images of an individual AZ. Discrete dots of DLiprin-α<sub>GFP</sub> are arranged at the AZ edge (magenta, BRP<sub>Ned2</sub> [B] and DGluRIID [C and D]). Left, planar AZ; right, vertical AZ. B and C show controls, and D shows brp<sup>69</sup>. (E) Single confocal slices of control (left) and dliprin-α (right) junctions labeled for BRP<sub>Ned2</sub> with STED resolution. Atypical clusters of BRP doughnuts are observed at dliprin-α mutant NMJs. Bars: (A) 1.5 μm; (D) 100 nm; (E) 1 μm.

**DLiprin-α localizes to discrete compartments surrounding the AZ center**

Liprin-α localizes to the AZ and has been shown to be important for the formation of AZs in both Drosophila and C. elegans (Kaufmann et al., 2002; Dai et al., 2006; Patel et al., 2006). To extend our “AZ map,” DLiprin-α<sub>GFP</sub> was expressed in motoneurons and visualized via αGFP stainings with STED microscopy (Fig. 6 A). DLiprin-α localized to presynaptic AZs opposite DGluRIID-positive PSDs. However, as opposed to BRP, DLiprin-α clustered somewhat lateral from the AZ center. STED resolution revealed that DLiprin-α formed discrete “quantal” clusters at the edge of a single AZ co-labeled with BRP<sub>Ned2</sub> (Fig. 6 B) or DGluRIID (Fig. 6 C).

Discrete DLiprin-α clusters were still observable at brp<sup>69</sup> NMJs, suggesting that the presence of BRP is not essential for
the recruitment of DLiprin-α to the AZ (Fig. 6 D). However, the localization of BRP, as imaged with STED, appeared aberrant at dliprin-α NMJs (Fig. 6 E). Strikingly, individual BRP doughnuts seemed interconnected, which is directly consistent with the previous observation of complex, multi–T-bar AZs at dliprin-α mutant NMJs (Kauffmann et al., 2002).

**BRP and Ca⁺⁺ channels accumulate late during AZ assembly**

So far, we have provided evidence that BRP operates as an essential building block of the T-bar. Notably, fast assembly of T-bars might drive experience-dependent changes of synaptic transmission in the fly central nervous system (Brandstatter et al., 1991; Rybak and Meinertzhagen, 1997). Thus, to learn about the T-bar assembly process in the frame of synapse reorganization, we visualized BRP accumulation in vivo during the developmental formation of individual synapses (Rasse et al., 2005; Fuger et al., 2007; Schmid et al., 2008). Previously, we found that neuromuscular accumulation of glutamate receptors (as DGluRIIAs) in PSDs typically form at a distance from existing PSDs and then grow over several hours before reaching a final mature size (Rasse et al., 2005; Schmid et al., 2008). Thus, for the analysis of AZ assembly in vivo, DGluRIIA was coimaged to serve as a reference point for our temporal analysis (Fig. 7, A, B, and D; and Table II).

Larvae coexpressing two fluorescently tagged synaptic proteins were imaged (Fig. 7), and quantitative data were obtained to analyze the temporal sequence of protein arrival at developing AZs. For a given larval NMJ, two in vivo images were acquired with a time interval of 12 h. Sites were regarded as new synapses if protein labels exceeded the mean background by a factor of 2.5 at the second (t = 12 h) but not at the first time point (t = 0 h). This way, a temporal sequence of molecular AZ assembly was extracted.

We first compared BRP and DGluRIIA accumulation. For visualization of BRP, we used a fragment of the protein (BRP-short), which delivered a label that fully matched the label of endogenous BRP (Schmid et al., 2008). When BRP-short was examined with STED, doughnuts were detected that resembled those found with BRPΔC (unpublished data). As previously described (Schmid et al., 2008), the accumulation of DGluRIIA clearly preceded BRP arrival in vivo (Fig. 7 A and Table II). Moreover, all postsynaptic DGluRIIA accumulations eventually incorporated presynaptic BRP, demonstrating that DGluRIIA accumulation reliably indicates the formation of new synapses.

As described in the previous section, presynaptic DLiprin-α localization seems to be largely independent of BRP (Fig. 6 D), which is compatible with DLiprin-α functioning upstream of BRP in AZ assembly. Consistently, DLiprin-α incorporation invariably preceded BRP accumulation (Fig. 7 C and Table II).

BRP is crucial for either the initial formation or the maintenance of Ca⁺⁺ channel clusters. Thus, we analyzed Cac localization at individual developing AZs. Cac and BRP appeared highly correlated, and the timing of Cac accumulation at AZs was typically very close to the advent of BRP with a slight tendency of Cac to precede BRP (Fig. 7 E and Table II).

Collectively, we show that newly forming AZs, similar to PSDs (Rasse et al., 2005), are small to begin with and then increase in size over many hours in vivo before accumulating detectable levels of BRP and reaching a final mature size at developing NMJs. This assembly process of individual new synapses is protracted over hours and is characterized by the contribution of pre- and postsynaptic proteins in a defined, overlapping sequence. DLiprin-α appears to be a very early player involved in initializing AZ assembly, whereas BRP, together with Cac, follows only after postsynaptic DGluRIIA incorporation is already detectable (Fig. 7, A and D).

**BRP controls Ca⁺⁺ channel accumulation at maturing AZs**

If Cac slightly precedes BRP during assembly, how can the Cac-clustering defects described in brpΔ9 (Kittel et al., 2006) be explained? To exclude allele-specific effects, we first scored Cac clustering at AZs (opposite DGluRIID receptor fields in the brp alleles brpΔ1, brpΔ9, brpΔ4298, and brpΔ1.3 (Fig. 8 A). A Cac-clustering defect identical to that found in brpΔ9 was observed in the full-deletion brpΔ1. As expected, brpΔ4298 also showed an identical clustering defect (Fig. 8 A). Previously, we provided evidence that the Cac delocalization in brpΔ9 is responsible for reduced neurotransmitter release. In this study, we took the opportunity to test the influence of BRP on neurotransmission independently of brpΔ9 and recorded from brpΔ4298 NMJs. All electrophysiological features of brpΔ4298, including the alterations of short-term plasticity connected to defective Ca⁺⁺ channel clustering, were similar to

<table>
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<th>Coexpressed proteins (A × B)</th>
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<th>B before A</th>
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<tr>
<td>BRP × DGluRIIA</td>
<td>BRP+/IIA−</td>
<td>BRP+/IIA−</td>
<td>BRP−/IIA−</td>
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<td>17/39 (44%)</td>
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<tr>
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<td>42/62 (68%)</td>
<td>7/62 (11%)</td>
<td>13/62 (21%)</td>
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Quantification of the relative accumulation of the indicated synaptic proteins at newly forming AZs (Δt = ~12 h). A synaptic site was scored positive (+) or negative (−) for a specific protein depending on whether protein fluorescence signals exceeded the mean background level by >2.5-fold. For example, when comparing BRP and DGluRIIA (at synapses forming newly over 12 h), 44% were positive for both proteins, 0% for BRP only, and 56% for DGluRIIA only.

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reaching their fully mature size. Thus, we wondered whether BRP may be more important for maturation and potentially less so for initialization of Cac clustering. To address this, we visualized Cac in intact, living *brp* mutant larvae together with DGluRIIA as a reference for synapse maturation (Fig. 8 B). In *brp* larvae, nascent synapses, identified by their small DGluRIIA accumulations, seemed to accumulate Cac at normal density (Fig. 8, B and C). However, larger, more mature synapses those observed in *brp* (Fig. S3; Kittel et al., 2006). Thus, several independent alleles clearly demonstrate that loss of BRP results in defective clustering of Cac at the AZ, which in turn provokes defects in transmitter release.

Notably, the mislocalization of Cac is not absolute, but instead, a certain degree of Cac remained clustered at AZs in *brp* null. As our in vivo imaging experiments show (Fig. 7), AZs go through a long, protracted assembly process before finally reaching their fully mature size. Thus, we wondered whether BRP may be more important for maturation and potentially less so for initialization of Cac clustering. To address this, we visualized Cac in intact, living *brp* mutant larvae together with DGluRIIA as a reference for synapse maturation (Fig. 8 B). In *brp* larvae, nascent synapses, identified by their small DGluRIIA accumulations, seemed to accumulate Cac at normal density (Fig. 8, B and C). However, larger, more mature synapses...
Alternatively, the Ca\textsuperscript{2+} channel–clustering defect in brp could be caused by impaired Cac transport. To address this, Cac trafficking dynamics were measured using FRAP experiments. Notably, Cac FRAP was slow, with a recovery half-time of \(\approx 12\) h (Fig. 8 D), which is similar to other synaptic membrane proteins such as DGluRIIA (Schmid et al., 2008). Importantly, however, Cac FRAP was essentially unaltered at brp boutons, indicating that the long-range transport of Cac to the AZ is not affected by the loss of BRP (Fig. 8 D). Thus, BRP seems to be directly required for Cac\textsuperscript{2+} channel clustering at AZs but not for its recruitment to the terminal. We aimed to further address the relation between T-bar assembly and Cac\textsuperscript{2+} channel clustering. Notably,
constructs in a yeast two-hybrid assay. Only the C-terminal domain of Cac and the N-terminal domain of BRP showed positive for interaction (Fig. 9 A). Thus, in agreement with our hypothesis concerning the orientation of BRP within the T-bar, the BRP N terminus may interact with the Cac C-terminal domain. To further investigate this interaction, we double transfected Schneider S2R+ cells with a GFP-tagged N-terminal construct of BRP (aa 1–617) and Myc-tagged cacC-Term. Western blotting shows the pull-down of the BRP construct in the anti-Myc IP at ~100 kD (arrow). The corresponding band is not detected in the control lanes (IgGs). The slightly different migration of the band in the input lane and in the IP lanes is the result of differences in sample buffer. MW, molecular weight. (C) Spatiotemporal model of AZ assembly and organization at Drosophila NMJs. SVs, synaptic vesicles.

brp1.3 (Fig. 8 A) showed partially restored Cac clustering. Moreover, those AZs positive for BRP-N-Term in brp1.3 corresponded to the AZs of restored Cac clustering (Fig. 8 E). Thus, AZ accumulation of the C-terminally truncated but N-terminally intact BRP protein seems to permit the assembly of a distally truncated T-bar, which still functions in clustering Cac.

To further explore this, we prepared larval NMJ AZs via HPF/FS. In this study, a gap between the AZ membrane and the T-bar pedestal (Fig. 8 F, arrows) was observed, where peg-like (Harlow et al., 2001) structures extended from the AZ membrane and distally contacted (or even slightly penetrated) the T-bar pedestal (Fig. 8 F, top, arrowheads). Such pegs were also observed at brp1.3 AZs (Fig. 8 F, middle, arrowheads) but not at AZ membranes of the brp69-null allele (Fig. 8 F, bottom). Thus, these data further support the notion that Cac clustering beneath the T-bar pedestal is restored at brp1.3 AZs. Moreover, the cytoplasmic domains of Ca2+ channels might well extend from the AZ membrane, possibly allowing for a direct interaction with BRP.

Possible direct interaction between the C terminus of Cac and the N terminus of BRP

To address a possible interaction between BRP and Ca2+ channels, we tested for protein–protein interactions in vitro. First, all intracellular loops of Cac were tested with BRP constructs in a yeast two-hybrid assay. Only the C-terminal domain of Cac and the N-terminal domain of BRP showed positive for interaction (Fig. 9 A). Thus, in agreement with our hypothesis concerning the orientation of BRP within the T-bar, the BRP N terminus may interact with the Cac C-terminal domain. To further investigate this interaction, we double transfected Schneider S2R+ cells with a GFP-tagged N-terminal construct of BRP (aa 1–617) and Myc-tagged cacC-Term. Western blotting shows the pull-down of the BRP construct in the anti-Myc IP at ~100 kD (arrow). The corresponding band is not detected in the control lanes (IgGs). The slightly different migration of the band in the input lane and in the IP lanes is the result of differences in sample buffer. MW, molecular weight. (C) Spatiotemporal model of AZ assembly and organization at Drosophila NMJs. SVs, synaptic vesicles.
Discussion

Efficient neurotransmission is believed to crucially depend on the structural and functional integrity of the presynaptic AZ compartment (Schoch and Gundelfinger, 2006). An ancestral set of AZ components is conserved between Drosophila, C. elegans, and mammals (Stryker and Johnson, 2007; Jin and Garner, 2008). The strong phenotype we observed at Drosophila NMJs in the absence of the ERC/CAST member BRP lacking T-bar–dense bodies and defective Ca\textsuperscript{2+} channel clustering (Kittel et al., 2006; Wagh et al., 2006), forms an entry point for studying AZ assembly, which is often complicated by redundant and cooperative interactions between AZ components (Jin and Garner, 2008).

BRP and dense body formation

We addressed whether BRP signals T-bar formation (without being a direct component of the T-bar) or whether the protein itself is an essential building block of this electron-dense structure. In this study, we provide evidence that BRP is a direct T-bar component. Immuno-EM identifies the N terminus of BRP throughout the whole cross section of the T-bar (Fig. 3, A and B), and genetic approaches show that a truncated BRP, lacking the C-terminal 30% of the protein’s sequence, forms truncated T-bars (Fig. 2, E and F). Immuno-EM and light microscopy consistently demonstrate that N- and C-terminal epitopes of BRP are segregated along an axis vertical to the AZ membrane and suggest that BRP is an elongated protein, which directly shapes the T-bar structure (Fig. 9 C).

In brp\textsuperscript{-}545 (predicted as aa 1–866), T-bars were not detected, whereas brp\textsuperscript{1–1,389} formed T-bar–like structures, although fewer and smaller than normal (Fig. 2, E and F; and Fig. S2 C). Moreover, the BRP\textsuperscript{91–307GFP} construct (1–1,226) did not rescue T-bar assembly. Thus, domains between aa 1,226 and 1,390 of BRP may also be important for the formation of T-bars. Clearly, however, the assembly scheme for T-bars is expected to be controlled at several levels (e.g., by phosphorylation) and might involve further protein components. Nonetheless, it is highly likely that the C-terminal half of BRP plays a crucial role.

BRP, the dense body, and Ca\textsuperscript{2+} channel clustering

As BRP represents an essential component of the electron-dense T-bar subcompartment at the AZ center, it might link Ca\textsuperscript{2+} channel–dependent release sites to the synaptic vesicle cycle (Neher and Sakaba, 2008). Interestingly, light and electron microscopic analysis has located CAST at mammalian synapses both with and without ribs (Tom Dieck et al., 2005; Deguchi-Tawarada et al., 2006; Siksou et al., 2007). Overall, this study is one of the first to genetically identify a component of an electron-dense synaptic specialization and thus paves the way for further genetic analyses of this subcellular structure.

The N terminus of BRP is found significantly closer to the AZ membrane than the C terminus, where it covers a confined area very similar to the area defined by the Cac\textsuperscript{GFP} epitope. Electron tomography of frog NMJs suggested that the cytoplasmic domains of Ca\textsuperscript{2+} channels, reminiscent of pegs, are concentrated directly beneath a component of an electron-dense AZ matrix resembling ribs (Harlow et al., 2001). In addition, freeze-fracture EM identified membrane-associated particles at flesh fly AZs, which, as judged by their dimensions, might well be Ca\textsuperscript{2+} channels (Feeney et al., 1998). We observed peg-like structures beneath the T-bar pedestal. Similar to fly T-bars, the frog AZ matrix extends up to 75 nm into the presynaptic cytoplasm. Based on the amount of cytoplasmic Ca\textsuperscript{2+} channel protein (Catterall, 1998), Harlow et al. (2001) concluded that Ca\textsuperscript{2+} channels are likely to extend into parts of the ribs. Thus, physical interactions between cytoplasmic domains of Ca\textsuperscript{2+} channels and components of ribs/T-bars might well control the formation of Ca\textsuperscript{2+} channel clusters at the AZ membrane. However, a short N-terminal fragment of BRP (aa 1–320) expressed in the brp-null background was unable to localize to AZs efficiently and consistently failed to restore Cac clustering (unpublished data).

The mean Ca\textsuperscript{2+} channel density at AZs is reduced in brp-null alleles. In vitro assays indicate that the N-terminal 20% of BRP can physically interact with the intracellular C terminus of Cac. Notably, we found that the GFP epitope at the very C terminus of Cac\textsuperscript{GFP} was closer to the AZ membrane than the N-terminal epitope of BRP (Fig. 4 A). It is conceivable that parts of the Cac C terminus extend into the pedestal region of the T-bar cytomatrix to locally interact with the BRP N terminus. This interaction might play a role in clustering Ca\textsuperscript{2+} channels beneath the T-bar pedestal.

Clearly, additional work will be needed to identify the contributions of discrete protein interactions in the potentially complex AZ protein interaction scheme. Our study should pave the way for a genetic analysis of spatial relationships and structural linkages within the AZ organization. Moreover, we aim to integrate our findings in the framework of mechanisms for Ca\textsuperscript{2+} channel trafficking, clustering, and functional modulation (Cao et al., 2004; Evans and Zamponi, 2006; Catterall and Few, 2008).

Timing of AZ assembly, Ca\textsuperscript{2+} channel accumulation, and synapse maturation

Our imaging assays allowed a temporally resolved analysis of AZ assembly in vivo (Fig. 7). BRP is a late player in AZ assembly, arriving hours after DLiprin-\textalpha and also clearly after the postsynaptic accumulation of DGlurRIIA. Accumulation of Cac was late as well, although it slightly preceded the arrival of BRP, and impaired Cac clustering at AZs lacking BRP became apparent only from a certain synapse size onwards (Fig. 8, B and C). In this study, we report that new AZs, similar to PSDs (Rasse et al., 2005), form at sites distant from preexisting ones and grow to reach a mature, fixed size. Thus, the late, BRP-dependent formation of the T-bar seems to be required for maintaining high Ca\textsuperscript{2+} channel levels at maturing AZs but not for initializing Ca\textsuperscript{2+} channel clustering at newly forming sites. As the dominant fraction of neuromuscular AZs is mature at a given time point, the overall impression is that of a general clustering defect in brp mutants. In reverse, it will be of interest to further differentiate the molecular mechanisms governing early Ca\textsuperscript{2+} channel clustering.

Pre- to postsynaptic communication via neurxin–neurelin (Missler et al., 2003; Li et al., 2007; Zeng et al., 2007) interactions might well contribute to this process. A further candidate involved in early Ca\textsuperscript{2+} channel clustering is the Fusellous protein, which was recently shown to be crucial for proper Cac localization at AZs (Long et al., 2008).
In summary, during the developmental formation of *Drosophila* NMJ synapses, the emergence of a presynaptic dense body, which is involved in accumulating Ca
sup+ channels, appears to be a central aspect of synapse maturation. This is likely to confer mature release probability to individual AZs (Kittel et al., 2006) and contribute to matching pre- and postsynaptic assembly by regulating glutamate receptor composition (Schmid et al., 2008). Whether similar mechanisms operate during synapse formation and maturation in mammals remains an open question.

**Outlook: dense body architectures and synaptic plasticity**

In this study, we concentrated on developmental synapse formation and maturation. The question arises whether similar mechanisms to those relevant for AZ maturation might control activity-dependent plasticity as well and whether maturation-dependent changes might be reversible at the level of individual synapses. Notably, experience-dependent, bidirectional changes in the size and number of T-bars (occurring within minutes) were implied at *Drosophila* photoreceptor synapses by ultrastructural means (Brandstatter et al., 1991; Rybak and Meinertz-Hagen, 1997). Moreover, at the crayfish NMJ, multiple complex AZs with double-dense body architecture were produced after stimulation and were associated with higher release probability (Wojtowicz et al., 1994). In fact, a recent study has correlated the ribbon size of inner hair cell synapses with Ca
sup+ microdomain amplitudes (Frank et al., 2009). Thus, a detailed understanding of the AZ architecture might provide a prediction of functional properties of individual AZs.

**Materials and methods**

**Genetics**

All fly strains were reared under standard laboratory conditions (Sigrist et al., 2003). Either w
sup1118 or w
sup1118 males with a chromosome +2R were used as background for transgenesis (BestGene Inc.). Molecular cloning in detail

**Chemical mutagenesis**

The EMS screen was performed according to standard protocols. In brief, isogenic w
sup1118 males were mutagenized with 25 mM EMS solution and the EMS screen was performed according to standard protocols. In brief, either w
sup1118 was used as background for transgenesis (BestGene Inc.). Molecular cloning in detail

**Generation of brp deletions**

brp chromosomal deletions were constructed using the Flp recombinease system as previously described (Parks et al., 2004). The different parental lines were provided by the Exelixis collection at Harvard Medical School (Boston, MA).

The following genotypes were used for the ectopic expression of the BRP constructs (Fig. 1 A; Fig. 2, G and H; and Fig. 5): for wing disc expression, upstream activator sequence (UAS)-BRP
subGFP, UAS-dpp-Gal4; UAS-BRP
sub2-GFP, UAS-dpp-Gal4, UAS-BRPN1.3GFP-dpp-Gal4. For expression of BRP constructs at NMJ in the brp mutant background, UAS-BRPN1.3GFP; Df(2R)BSC29/brp, ok6-Gal4, Df(2R)BSC29/brp, ok6-Gal4, UAS-BRPN1.3GFP/+. Df(2R)BSC29/brp, ok6-Gal4, UAS-BRPN1.3GFP/+. Df(2R)BSC29/brp, ok6-Gal4, UAS-BRPN1.3GFP/+. For morphology, 55–60-nm (gray silver) sections, and for immunocytochemistry, 85-nm (silver gold) sections were cut using an EM Ultracut 6 (Leica). Sections were collected on formvar-coated 100 mesh grids. For transmission EM, sections were fixed and poststained with uranyl acetate and lead citrate as described previously (Schmid et al., 2006). For immunocytochemistry, grids were placed onto double-stained 100 mesh grids, and immunolabeling started immediately. Immunocytochemistry was performed as described previously (Rostaing et al., 2006; Siksou et al., 2007). Rb-BRP
sub568 (1:500) and M-BRPN-Term (1:500) antisera were provided by E. Buchner, Universität Würzburg, Würzburg, Germany) were used. Ultrastructure was analyzed using a transmission electron microscope (Philips 902A; Carl Zeiss, Inc.) operated in bright field mode.

Conventional room temperature embedding was essentially performed as described previously (Wagh et al., 2006). Images were obtained from dissected preparations of third instar larvae (NMJ 6/7; segments A2/A3). Instead of 1-h fixation in 1% osmium tetroxide, the fixation was performed in 1% osmium tetroxide and 0.8% K
subFeC
subn in 0.1 M cacodylate buffer. After infiltration in epon resin, muscles were cut out (six animals for each genotype) and embedded in a single block. For Tbar size quantification, Tbars or residual Tbars were taken from vertical A2s. The electron density of the Tbar was measured from the AZ membrane to the Tbar platform if present (height) or along the AZ membrane (width).

**Molecular cloning**

For expression constructs of DLiprin-α-GFP, a 3.6-kb fragment of pOT2 LD27334 was subcloned into pBluescript KS(+) (Agilent Technologies) using the Sall and EcoRI restriction sites inserted by PCR primers and subsequently double-strand sequenced. The insert was excised and inserted into pENTR4 (Invitrogen) via Sall and NotI sites. The final expression construct of DLiprin-α-GFP was obtained using the Gateway system (Invitrogen). In brief, pENTR4 DLiprin-α was recombined with pETG (a *Drosophila* pUAST Gateway vector developed in the laboratory of T. Murphy, The Carnegie Institution of Washington, Baltimore, MD).

The BRP constructs used for expression in *Drosophila* flies and *Drosophila* Schneider cell culture were obtained by PCR using the corresponding cDNA as template (brp cDNA; Wagh et al., 2006) and cloned into pEENTER, a modified version of pENTR4 using the Spel and Asp7181 restriction sites. The pUbiP Gateway destination vectors, used for coexpression experiments in S2R+ cell culture, were obtained from A. Herzog (Max Planck Institute, Göttingen, Germany). These contained a ubiquitin promoter and either an N- or C-terminal addition of a Gateway destination vector.

Yeast two-hybrid constructs for cac and brp were obtained by PCR using the corresponding cDNA as template (cac cDNA was provided by R.W. Ordway, The Pennsylvania State University, Philadelphia, PA; brp cDNA; Wagh et al., 2006) and cloned into the bait vector pGBK7 (Clontech laboratories, Inc.) or the prey vector pGAD77 (Clontech laboratories, Inc.) using the restriction sites introduced with the PCR primers.

Vectors used for fusion protein expression in Drosophila Schneider cell culture were made from respectively modified pENTR4 clones containing truncated brp or truncated cac using the Gateway system.

**Molecular cloning in detail**

**pENTER.** The multiple cloning site of pEENTER (Invitrogen) was modified by oligonucleotide annealing between two primers with a 5'-phosphate modification (MWG-Biotech AG): 5'-CATGGGAACTAGTCGCCGGCGCCGCCGCGGCGGCGG.
CCGCGTGACCGGC-3' and 5'-TGGGCTGAACTCCGGGCACTGGG-CCGCGGCGGGCTAGTTCG-3'. The annealed oligonucleotides were ligated into a previously cut pENTR4 vector using the NcoI and Xhol restriction sites. This modification resulted in a loss of the cccB gene and a new multiple cloning site.

**pTWMstrawberry**. In short, the *Drosophila* Gateway vector pTWG containing an EGFP tag placed downstream of the Gateway cassette was used as a template to replace the EGFP by mStraw (pStraw; pRE662, provided by R.Y. Tsien, University of California, San Diego, La Jolla, CA). For general information about the *Drosophila* Gateway vector collection, please visit http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html. 

**pENTR4** and pENTR. All final plasmids of pENTR4 or pENTER were constructed by double-strand sequence confirmed before any Gateway recombination with destination vectors.

**pENTR4 Dliprin-α**. 5'-GAGGTGAACGAGACAGTGGCCTCTTGCTCAAGA-3' and 5'-GGAAATCGCTTCTAGTCCATGACCCAAACGCTA-3', recombined with pTWG (Terminal EGFP tag).

**pENTER BRP D1-2 (aa 1–6,17)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCTGCTCTTTCCGCATCCGACG-3', recombined with pUbi::pFAEGFP (C-terminal EGFP).

**pENTER BRP D1-3 (aa 1,122,6)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCTGCTCTTTCCGCATCCGACG-3', recombined with pTWG (C-terminal EGFP tag).

**pENTER BRP D2-4 (aa 269–1,740)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCCTTGGCGCTTTCCTCCATGGTC-3', recombined with pTWG (Terminal EGFP tag).

**pENTER BRP D2-5 (aa 269–1,740)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCCTTGGCGCTTTCCTCCATGGTC-3', recombined with pENTR (C-terminal EGFP tag).

**pENTER BRP D2-6 (aa 269–1,740)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCCTTGGCGCTTTCCTCCATGGTC-3', recombined with pENTR (C-terminal EGFP tag) and pTWMstrawberry.

**pENTER Cac C terminus (aa 1,420–1,848)**. pGADT7 Cac C terminus (aa 1,420–1,848).

**pGADT7 BRP D2 (aa 269–617)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCCTTGGCGCTTTCCTCCATGGTC-3', recombined with pENTR (C-terminal EGFP tag) and pmStraw.

**pGADT7 BRP D2 (aa 1–320)**. 5'-GAGTTAAGATGCATGGGTCAAGGGCCAGCTTACATTGCCGC-3' and 5'-GTCGCTAGTCCTTGGCGCTTTCCTCCATGGTC-3', recombined with pENTR (C-terminal EGFP tag) and pmStraw.

In short, the *Drosophila* Schneider 2 cells were provided by A. Herzig and cultured at 25°C in an ambient atmosphere in Schneider’s Drosophila medium (BioWest) supplemented with 10% FCS + 2 mM L-glutamine, 100 mM phenylthiocarboline, and 100 µg/ml streptomycin (Invitrogen). Medium was exchanged every 3–4 d. Cells were split every 10–14 d. Cell co-transfection was conducted using the Effectene transfection reagent kit (Qiagen). Culture lysis was performed with lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol (vol/vol), 1% NP-40 (vol/vol), and 100 µg/ml streptomycin (Invitrogen). After incubation at room temperature, the coupled beads were thoroughly washed repeatedly and eluted by boiling in 40 µl of Laemmli buffer.

**Immunostainings** and **dissections** were performed as described previously (Qin et al., 2002). Larvae were incubated with antibody solutions overnight at 4°C. Larvae were mounted either in Vectashield (Vector Laboratories) or Triton X-100 (Sigma).

**Biochemistry**

*Drosophila* Schneider 2 cells were provided by A. Herzig and cultured at 25°C in an ambient atmosphere in Schneider’s Drosophila medium (BioWest) supplemented with 10% FCS + 2 mM L-glutamine, 100 mM phenylthiocarboline, and 100 µg/ml streptomycin (Invitrogen). Medium was exchanged every 3–4 d. Cells were split every 10–14 d. Cell co-transfection was conducted using the Effectene transfection reagent kit (Qiagen). Culture lysis was performed with lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol (vol/vol), 1% NP-40 (vol/vol), and 100 µg/ml streptomycin (Invitrogen). After incubation at room temperature, the coupled beads were thoroughly washed repeatedly and eluted by boiling in 40 µl of Laemmli buffer. 10 µl IP eluates and 30 µg whole cell lysates were subjected to denaturing SDS-PAGE using Tris-HCl NuPAGE 4–12% gradient gels and then transferred to a nitrocellulose membrane (Blot; Invitrogen). The membrane was probed with Rb-Brp-2 (1:500).

**Image acquisition**

Conventional confocal images were acquired at room temperature with a 63× 1.4 NA oil objective suited in a confocal microscope (TCS-SP5; Leica). Images taken from fixed samples were from third instar larval NMJs 6/7 in segments A2 and A3. NMJs depicted in live experiments derive from muscles 26 and 27 in segments A2 and A3. The fluorescence detection was set with the acousto optical beam splitter between 500 and 530 nm for GFP and between 575 and 620 nm for mRFP and mStraw. Photomultiplier gain was set to 1,250 V. Images were excited using the 488-nm Ar-I laser line, whereas mRFP and mStraw were excited with a 561-nm diode-pumped solid-state laser. For STED images, the STED setup (TCS; Leica) was used in combination with a 100× 1.4 NA oil objective at 20°C (Leica). Dye (Atto647N; Atto-Tec) was excited with a pulsed laser at 635 nm and depleted with a laser adjusted to 760 nm (Mai Tai Ti:Sapphire; Newport Spectro-Physics). Detection of the Atto-647N was performed with avalanche photodiodes and optical filters permeable for light of wavelengths between 650 and 710 nm. Diode gain was continuously adjusted to 310 V. Excitation laser power varied according to the sample, but always ranged between 5.0 and 5.6 V.

**Immunostainings**

Conventional immuno-
Mowiol. The following antibody dilutions were used: Mg-Nc82 (provided by E. Buchner), 1:200; Ms-ΔGluRIIα (BD4D2; Developmental Studies Hybridoma Bank), 1:100; Rb-ΔGluRIIα, 1:250; Rbx-ΔGluRIID (Qin et al., 2005), 1:500; Ms-GFP (Invitrogen), 1:500; Rbx-GFP (Invitrogen), 1:500; and HRP-Cy5 (Dianova), 1:250. For standard immunostainings, secondary antibodies were diluted 1:500. For STED stainings, dye (Atto647N) conjugation to secondary antibodies giving sheep α-Mb647 and sheep α-HRP647N were performed according to producer protocols (http://www.atto-tec.com) and used 1:100. Embryos [118] and elav-Gal4, F1/1[13C129; Kawasaki et al., 2002] were staged by time (22–24 h after egg laying) and morphology, dissected, and stained as described for larvae.

Live imaging

The following strains were used for in vivo imaging experiments: for Ca2+ channels, ok-αGal4/+; Cα7598D, DGLuRIIαGFP/+ (control), Df(2R)BSC29, ok-αGal4/brp; Cα7598D, DGLuRIIαGFP/+ [brp mutant background]. For temporal analysis of AZ assembly, ok-αGal4/brp; Rbrp-shpr2/+; Df(2R)BSC29, ok-αGal4/brp; Dūp1pr-GFP/DGLuRIIαGFP, ok-αGal4/brp; Dūp1pr-GFP/DGLuRIIαGFP, ok-αGal4/brp; Dūp1pr-GFP/+; ok-αGal4/brp; Cα7598D/+; ok-αGal4/brp; Dūp1pr-GFP/+; Cα7598D/+.

Imaging of intact Drosophila larvae along with larval anaesthesia was performed as described previously (Rasse et al., 2005; Fuger et al., 2007; Schmid et al., 2008). For FRAP experiments, intense 488-nm laser light was applied inside a region of interest of ~10 μm of edge length (zoom, 25) bleaching both green and red fluorescent dyes. After an incubation of 12 h at 25°C, the junctions were imaged and compared with the prebleached pictures. Control regions were conserved at the junctions for internal control of intensity levels.

Image processing

Images were acquired using Application Suite Advanced Fluorescence (LAS-AF); Leica software.

Confocal imaging

Confocal stacks were processed with ImageJ software (National Institutes of Health). Single slices and confocal stacks were deconvolved using the ImageJ plug-ins iterative deconvolution and iterative deconvolution 3D, respectively (provided by B. Dougherty, OptiNav, Inc., Redmond, WA). To generate the PSF for deconvolution, the ImageJ plug-in diffraction PSF 3D (provided by B. Dougherty) was used. The PSF settings were adjusted according to our hardware parameters, emission wavelengths, and image dimensions. For analysis of FRAP data, intensity ratios between bleached areas and control nonbleached regions were retrieved. To compare several experiments, prebleached ratios were set to 1, and postbleached images were normalized accordingly. Recoveries were calculated by subtracting ratios generated immediately after the bleaching from ratios acquired 12 h after bleaching.

For quantification of areas and intensities, maximum projections, acquired with comparable confocal settings, were thresholded at 30 arbitrary units (au), and remaining areas were measured via the analyze particle function of ImageJ.

To obtain unbiased mean BRPshpr2 and BRPshpr4 distributions with STED resolution (Fig. 5 D), we used STED images of BRPshpr2-Atto647N (or BRPshpr4-Atto647N), which were simultaneously recorded with confocal images of the BΔGluRIIα-Alexa Fluor 488 (or BΔGluRIIα-Alexa Fluor 488). From these images, AIZs were selected, which appeared planar to the optical slice. The confocal channel of each image of a planar AZ was automatically aligned and subsequently averaged the corresponding STED signal. The averaged STED images [BRPshpr2 and BRPshpr4] were finally aligned according to the corresponding confocal counterpart. The BRPshpr2 signal was fitted with a single Gaussian (standard deviation = 52 nm), and the BRPshpr4 signal was fitted with the sum of two Gaussians (standard deviation = 48 nm and peak distance Δx = 189 nm).

STED imaging

STED images were processed using a linear deconvolution software integrated into the Inspector Software package (MaxPlanck Innovations GmbH). Regularization parameters ranged from 1e-6 to 1e-12. The PSF was generated by using a 2D Lorentzian function with its half-width and half-height fitted to the half-width and half-height obtained by images of 25-nm crimson beads conjugated to Atto647N.

Electrophysiology

Two electrode voltage clamp recordings were essentially conducted as previously described (Kittel et al., 2006). All experiments were performed on male third instar larval NMJs (muscle 6; segments A2 and A3) in HL3 (70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM Hepes, and 1 mM CaCl2, pH 7.2). Muscle cells had an input resistance >2 MΩ. Intracellular dyes were filled with 3 M KCl, and resistances ranged from 10–25 MΩ. Stimulation artifacts of evoked excitatory junctional currents were removed for clarity. Genotypes used were brpshpr2/+/Df(2R)BSC29 and w1118 as controls.

Statistics

Data were analyzed using the Mann-Whitney rank sum test for linear independent data groups (Prism; GraphPad Software, Inc.). Means are annotated ± SEM. Asterisks are used to denote significance (*, P < 0.05; **, P < 0.01; ***, P < 0.005; not significant, P > 0.05).

Online supplemental material

Fig. S1 shows a quantification of BRPshpr2 expression levels in several brp mutants. Fig. S2 shows a comparison of mitochondrial ultrastructure either preserved using conventional room temperature embedding with aldehyde fixation and dehydration or HF/FS along with Tbar size quantification of conventional and HF/FS-prepared control and brp–1 larvae. Fig. S3 shows amplitude and rise time of evoked excitatory junctional current recorded from brpshpr2 NMJs. Fig. S4 shows that dūp1pr AZs do not suffer from Cac-clustering defects. Fig. S5 shows that BRP clustering is not disturbed at cac AZs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200812150/DC1.

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