The Bruchpilot cytomatrix determines the size of the readily releasable pool of synaptic vesicles

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Synaptic vesicles (SVs) fuse at a specialized membrane domain called the active zone (AZ), covered by a conserved cytomatrix. How exactly cytomatrix components intersect with SV release remains insufficiently understood. We showed previously that loss of the Drosophila melanogaster ELKS family protein Bruchpilot (BRP) eliminates the cytomatrix (T bar) and declusters Ca\(^{2+}\) channels. In this paper, we explored additional functions of the cytomatrix, starting with the biochemical identification of two BRP isoforms. Both isoforms alternated in a circular array and were important for proper T-bar formation. Basal transmission was decreased in isoform-specific mutants, which we attributed to a reduction in the size of the readily releasable pool (RRP) of SVs. We also found a corresponding reduction in the number of SVs docked close to the remaining cytomatrix. We propose that the macromolecular architecture created by the alternating pattern of the BRP isoforms determines the number of Ca\(^{2+}\) channel-coupled SV release slots available per AZ and thereby sets the size of the RRP.

Introduction

At the presynaptic side of synapses, neurotransmitter release is initiated by the depolarization-induced opening of voltage-gated presynaptic Ca\(^{2+}\) channels that are concentrated at defined release sites within the presynaptic active zone (AZ; Rettig and Neher, 2002; Gundelfinger et al., 2003; Murthy and De Camilli, 2003; Jin and Garner, 2008; Sigrist and Schmitz, 2011). Synchronous release can be explained by short-lived, so-called nanodomains of elevated [Ca\(^{2+}\)], which build up and decay rapidly around these Ca\(^{2+}\) channels (Meinrenken et al., 2002; Bucurenciu et al., 2008; Neher and Sakaba, 2008). Whether discrete synaptic vesicle (SV) binding sites allowing for nanodomain-coupled release exist and what their molecular organization might look like remain unknown. Of note, an electron-dense cytomatrix referred to as presynaptic density or cytomatrix of the AZ (hereafter short cytomatrix; Gundelfinger et al., 2003), has been found to be associated with the intracellular face of the AZ membrane and is meant to coordinate SV fusion at the AZ (Südhof, 2012). Electron-dense cytomatrices directly superimpose on the intracellular parts of voltage-gated Ca\(^{2+}\) channels, as indicated by electron tomography of frog (Harlow et al., 2001).

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and Drosophila melanogaster (Fouquet et al., 2009) neuromuscular junctions (NMJs).

Clearly, genetic analysis of AZ components is critical for establishing precise causal relations between the molecular, structural, and functional features of AZs. Over the last years, an evolutionarily conserved complex of protein components highly enriched at AZs was identified: Munc13s (Wojcik and Brose, 2007), Rab3-interacting molecules (RIMs; Mittelstaedt et al., 2010), RIM-binding proteins (Liu et al., 2011), and α-Liprins (Spangler and Hoogenraad, 2007), as well as ELKS family proteins (Hida and Ohtsuka, 2010). Using superresolution light microscopy, we previously showed that ELKS family protein Bruchpilot (BRP) forms the electron-dense AZ cytomatrix (T bar) in Drosophila (Kittel et al., 2006; Fouquet et al., 2009). In brp-null mutants, T bars were lost, and AZ Ca$^{2+}$ channels declustered (Kittel et al., 2006). In response, basal-evoked fusion of SVs was severely impaired. Importantly, the residual release was asynchronous, and short-term plasticity changed to atypical paired-pulse facilitation.

Here, we demonstrated a direct function of the BRP-based cytomatrix in controlling the number of readily releasable SVs. We characterized the two major isoforms of BRP with 190- and 170-kD apparent sizes by biochemical analysis and generated isoform-specific alleles and tagged genomic constructs. Electron and superresolution fluorescence microscopy showed that BRP-190 and BRP-170 contribute to T-bar cytomatrix assembly to a similar extent and alternate in a circular array. Mutants in both alleles still formed cytomatrices but of reduced size. These smaller cytomatrices still allowed normal Ca$^{2+}$ channel clustering; however, baseline transmission was reduced nonetheless. The vesicular release probability determined by variance–mean analysis was also unchanged, indicating a different cause for the transmission defect in these alleles than in brp nulls. Instead, variance–mean analysis and back extrapolation of quantal contents both revealed a reduction in the size of the readily releasable pool (RRP) of SVs here. Correspondingly, the number of SVs docked close to the remaining cytomatrix found by EM was reduced. Thus, we suggest a novel role for BRP isoforms in creating a stereotypic arrangement of the cytomatrix that defines the number of Ca$^{2+}$ nanodomain–coupled release slots available per AZ, a function which can be separated from Ca$^{2+}$ channel clustering.

**Results**

**The brp locus expresses two isoforms of 190 and 170 kD**

As previously reported (Wagh et al., 2006), Western blots of Drosophila adult head extracts probed with the monoclonal antibody (AB) NC82 (from here on termed BRP$^{C\text{-Term}}$, epitope position shown in Fig. 1 D) show two bands of apparent 190 (BRP-190)- and 170 (BRP-170)-kD sizes (Fig. 1 A). Notably, another previously generated AB (Fouquet et al., 2009), anti-BRP$^{N\text{-Term}}$, raised against a peptide encoded in the previously annotated exon cluster CG12933 (Fig. 1 D), recognized only BRP-190 (Fig. 1 A), indicating that BRP-170 might lack the CG12933 encoded protein sequence. To further address this, we used anti-BRP$^{C\text{-Term}}$, anti-BRP$^{N\text{-Term}}$, and a third AB, anti-BRP$^{D2}$ (Fouquet et al., 2009), raised against a sequence encoded by CG30336 (Fig. 1 D), for immunoprecipitations (IPs). Silver staining of gels loaded with these IPs showed that anti-BRP$^{N\text{-Term}}$ AB only precipitated BRP-190 (Fig. 1 B, arrows), whereas anti-BRP$^{D2}$ and anti-BRP$^{C\text{-Term}}$ precipitated both isoforms (Fig. 1 B, arrows and arrowheads, respectively). Mass spectrometry analysis of anti-BRP$^{C\text{-Term}}$ IPs found that the 190-kD band matched peptides encoded by CG12933, CG30336, and CG30337, identical to the protein to be expected from the reported cDNA (Wagh et al., 2006). In contrast, the 170-kD band contained the sequences of CG30336 and CG30337 but not of CG12933. Using cDNA library screening, we identified a partial clone defining a transcription start site for the BRP-170 encoding mRNA in between CG12933 and CG30336, indicating that the 170-kD isoform is transcribed from a second, independent promoter. This is compatible with the results from the IP, as the anti-BRP$^{D2}$ and anti-BRP$^{C\text{-Term}}$ epitopes are shared by both isoforms, whereas the anti-BRP$^{N\text{-Term}}$ epitope is specific to BRP-190 (Fig. 1 D). The BRP-190–specific sequence encoded by CG12933 contains stretches highly homologous to other ELKS family proteins (Fig. 1 E).

**Generation of isoform-specific brp alleles**

To investigate the functions of these two BRP isoforms, we produced isoform-specific mutants. Candidate chromosomes displaying reduced fitness and eclosion rate over previously established null alleles of brp (brp$^{69}$ and brp$^{66}$) were screened by chemical mutagenesis (Fouquet et al., 2009). Thus, we isolated an allele (brp$^{190}$; Fig. 1 D) leading to a premature STOP codon at aa 261 of BRP-190 (Q to STOP). Western blots of brp$^{190}$ fly heads confirmed that this mutation eliminates BRP-190 but leaves BRP-170 intact (Fig. 1 C). Next, we mobilized the P element transposon line d09839, located 0.6 kb upstream of the first exon of the transcript encoding BRP-170 (Fig. 1 D). Imprecise excision led to an allele with a ~5.9-kb deletion including the first exon of BRP-170 (brp$^{170}$; Fig. 1 D). Western blots of brp$^{170}$ fly heads confirmed that this mutant specifically removes BRP-170 without any major effect on the expression level of BRP-190 (Fig. 1 C). For further analysis, brp$^{170}$ and brp$^{190}$ were outcrossed into the wild-type background (Canton-S) for six generations. Different from brp-null alleles (Kittel et al., 2006; Fouquet et al., 2009), both isoform-specific alleles survived to adulthood when placed over deficiency.

**BRP-190 and -170 coexpress within the AZs of neuromuscular terminals**

The glutamatergic NMJ synapses of Drosophila larvae are well suited for the integrated analysis of AZ function, and several brp alleles have been extensively studied here (Kittel et al., 2006; Fouquet et al., 2009; Hallermann et al., 2010). At control NMJs (muscle 6/7) contained with anti-BRP$^{N\text{-Term}}$ and anti-BRP$^{C\text{-Term}}$, individual AZs were colabeled by both ABs (Fig. 2 A, left), as expected (Fouquet et al., 2009; Miskiewicz et al., 2011). NMJs in brp$^{190}$ larvae lacked BRP$^{N\text{-Term}}$ AB staining completely (Fig. 2 A, right), consistent with Western results (Fig. 1 C). In contrast, brp$^{170}$ NMJs had rather normal BRP$^{N\text{-Term}}$ labeling.
of either BRP isoform did not result in a change of NMJ size as quantified by HRP staining, which labels all neuronal membranes (control: 100.0 ± 5.7; brp<sup>170</sup>: 88.8 ± 3.5, P = 0.1560 vs. control; brp<sup>190</sup>: 100.6 ± 5.1, P = 0.9036 vs. control and P = 0.1368). Of note, BRPC-Term staining was reduced by ~50% compared with controls in both mutants, indicating that both isoforms might contribute to the AZ cytomatrix in similar amounts (Fig. 2 C). Lack of either BRP isoform did not result in a change of NMJ size as quantified by HRP staining, which labels all neuronal membranes (control: 100.0 ± 5.7; brp<sup>170</sup>: 88.8 ± 3.5, P = 0.1560 vs. control; brp<sup>190</sup>: 100.6 ± 5.1, P = 0.9036 vs. control and P = 0.1368).

Figure 1. The brp locus encodes two major isoforms. (A) Western blots of Drosophila adult head extracts probed with anti-BRP<sub>C-Term</sub> AB (short AB) and anti-BRP<sub>N-Term</sub> AB. Black line indicates that intervening lanes have been spliced out. (B) Silver gel of IPs conducted on Drosophila adult head extracts with the indicated ABs. Arrows represent the two BRP isoforms (top arrows: BRP-190; bottom arrows: BRP-170). (C) Western blot of Drosophila adult head extracts of the indicated genotypes probed with anti-BRP<sup>190</sup> and anti-Tubulin (Tub). (D) Schematic representation of brp genomic locus. BRP-190 and BRP-170 have distinct N-terminal sequences (magenta, BRP-190; green, BRP-170) but share the same C-terminal exons (black). AB epitopes are indicated in yellow. brp<sup>170</sup> allele: ethyl methyl sulfonate–induced point mutation from CAG (Glutamine [Q]) to TAG, leading to a premature STOP at aa 261 (encoded by the BRP-190–exclusive exon 3). brp<sup>170</sup> was generated by imprecise excision of P<sub>element</sub> P(XP)<sub>d09839</sub> deleting the region indicated by the dashed lines. Genomic rescue construct (brp<sup>rescue</sup>) encompassing the whole locus including two neighboring genes. (E) Protein sequence alignment (MAFFT with BLOSUM62 scoring matrix) between Drosophila BRP-190 and BRP-170, mouse ERC1 (NCBI Nucleotide Database accession no. NP_835186) and ERC2 (NCBI Nucleotide Database accession no. NP_808482), and Caenorhabditis elegans ELKS1 (NCBI Nucleotide Database accession no. NP_500329). Numbering is based on BRP-190. Similarity in amino acid identity is indicated by a color code (dark gray, 100%; gray, 80–100%; light gray, 60–80%; white, <60% similarity). Term, terminal.
vs. brp\(^{170}\); \(n = 27\) NMJs in nine larvae/genotype; unpublished data). For a BRP-independent estimate of AZ numbers, we stained for Drosophila RIM-binding protein (DRBP; Fig. 2 D), which closely associates with BRP at AZs (Liu et al., 2011), and glutamate receptors (GluRIID; Qin et al., 2005), which are postsynaptically matched to BRP-positive presynapses (not depicted; for an example, see Fig. S2 A). The number of both DRBP\(^{C-Term}\) (Fig. 2, D and E, quantification) and GluRIID (Fig. 2 F quantification) and not depicted) dots per NMJ were normal, indicating that there is no change in the AZ number in either of the BRP isoform mutants. Anti-DRBP\(^{C-Term}\) staining intensity was slightly reduced in both isoform mutants (control: 100.0 ± 2.6; brp\(^{170}\); 85.4 ± 2.7, \(P = 0.0004\) vs. control; brp\(^{190}\); 81.5 ± 3.0, \(P < 0.0001\) vs. control and \(P = 0.2993\) vs. brp\(^{170}\); \(n = 27\) NMJs in nine larvae/genotype), consistent with the partial dependency of DRBP on BRP presence we observed previously (compare with Fig. S7 in Liu et al., 2011). GluRIID label intensity was not changed compared with controls (control: 100.0 ± 7.2; brp\(^{170}\); 100.0 ± 6.2, \(P = 0.7880\) vs. control; brp\(^{190}\); 99.7 ± 4.7, \(P = 0.4781\) vs. control and \(P = 0.2993\) vs. brp\(^{170}\); \(n = 18\) NMJs in six larvae/genotype), differing from brp nulls, which showed increased GluRIID intensity (Kittel et al., 2006).

To explore the contribution of BRP-190 and BRP-170 to the overall BRP cytomatrix, we wanted to directly and individually visualize both BRP isoforms on the level of individual AZs. At its very N terminus, BRP-170 contains only 9 aa not included within BRP-190. Despite our efforts, we failed to produce a working AB against this short stretch. Thus, we generated a
genomic rescue construct (brp\textsuperscript{rescue}) based on P(acman) technology (Venken et al., 2006), encompassing the whole locus, including two neighboring genes (Fig. 1 D). This construct rescued brp-null alleles to adult vitality and fertility. Because we could previously show that the N terminus of BRP binds to Ca\textsuperscript{2+} channels (Fouquet et al., 2009) and the C terminus is important for SV binding (Hallermann et al., 2010), we chose a low conserved region in exon 13 to insert an EGFP or mCherry tag (Fig. 1 D, XFP). We then introduced the STOP codon mutation giving rise to brp\textsuperscript{A190} into the brp\textsuperscript{rescue,GFP} construct (hereafter called BRP-170\textsuperscript{GFP}). Similarly, a deletion mimicking brp\textsuperscript{A170} was introduced into the brp\textsuperscript{rescue,Cherry} transgene (hereafter called BRP-190\textsuperscript{Cherry}). Both transgenes, when expressed in either wild-type or brp-null mutant background, appeared as bands of expected size (unpublished data) in Western blots. Expression of these constructs also rescued the lethality of brp-null alleles (unpublished data).

We previously developed protocols to in vivo live image individual NMJ terminals in intact larvae (Rasse et al., 2005; Schmid et al., 2008). Live imaging of larvae coexpressing BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} (in wild-type background) revealed that all NMJ AZs contained both isoforms (Fig. 3 A). Western blots of larval body wall preparations (containing NMJs) indicating an equal abundance of both isoforms (Fig. S1, A and B, quantification). We sought to address the way these forms integrate into the AZ cytomatrix and what role they might play for structure and function there.

A “circular array” of alternating BRP-190 and -170 clusters forms the AZ cytomatrix

Because of the small size of AZ cytomatrices (200–300 nm; diffraction-limited resolution of conventional microscopy >200 nm), the distribution of both isoforms within each individual AZ could not be further resolved with conventional confocal microscopy. Previously, we had been using stimulated emission depletion (STED) microscopy (Hell, 2007) to show that BRP takes up an elongated conformation with its N terminus closer to the AZ membrane than its C terminus (Fouquet et al., 2009). The C terminus appeared donut shaped when imaged at an ~100-nm resolution (Kittel et al., 2006). Using higher STED resolution (<70 nm), this further dissolved into a circular array of single “clusters,” each cluster probably representing a bundle of BRP filaments (Fouquet et al., 2009; Liu et al., 2011). Here, we used a two-color STED microscope providing 50-nm resolution in the focal plane (Bückers et al., 2011) to image individual AZs labeled with BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} in a wild-type background. Both isoforms were found in an alternating pattern of discrete, nonoverlapping clusters in planar (Fig. 3 B) and vertical (Fig. 3 C) views, also apparent in intensity distribution profiles. We counted the number of BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} dots at planar-imaged AZs (also with reversed secondary ABs as a control; Fig. S1, C and D) to get a more quantitative view of the AZ composition (Fig. 3 D, counting example shown on the left). This revealed that most AZs contain four to five dots of each BRP isoform (Fig. 3 E). For quantification of the alternating pattern, we counted the transitions from one puncta to the next going clockwise around planar-imaged AZs with line profiles shifted in such a way to achieve clear separation of peaks. Here, transitions with color changes (BRP-170\textsuperscript{GFP} to BRP-190\textsuperscript{Cherry} and BRP-190\textsuperscript{Cherry} to BRP-170\textsuperscript{GFP}) were counted as change, transitions without color change (BRP-170\textsuperscript{GFP} to BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} to BRP-190\textsuperscript{Cherry}) were counted as same, and transitions to or from punctae with overlapping signals (inseparable BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} peaks) were counted as not applicable (N/A; overlap; Fig. 3 F, left). In BRP-190\textsuperscript{Cherry} versus BRP-170\textsuperscript{GFP}, we observed a rate of 82% change transitions and only 10% same transitions (Fig. 3 F, right). 8% of the transitions were N/A (Fig. 3 F, transition 3). As a control for more overlap, we used BRP-190\textsuperscript{Cherry} versus BRP\textsuperscript{C-Term} (example AZs shown in Fig. S1 F). Here, the same quantification led to only 17% of transitions changing color but 83% N/A. Similarly, BRP-190\textsuperscript{Cherry} versus BRP-170\textsuperscript{GFP} had 4% of all punctae overlapping, whereas BRP-190\textsuperscript{Cherry} versus BRP\textsuperscript{C-Term} had 67% overlap (Fig. 3 G). Also, when put in brp-null background, BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} showed an alternating pattern, excluding a major influence of unlabeled endogenous BRP on the pattern (Fig. S1 E). Thus, BRP-170\textsuperscript{GFP} and BRP-190\textsuperscript{Cherry} seem to integrate into the AZ cytomatrix in a stoichiometric manner forming a circular array of alternating clusters.

Smaller AZ cytomatrices after eliminating either BRP-190 or -170

We previously showed using EM that the T bar found at Drosophila AZs is absent in brp-null mutants (Kittel et al., 2006; Fouquet et al., 2009). To preserve physiological ultrastructure, we first applied high-pressure freezing (HPF)/freeze substitution (FS) EM (HPF EM; Fouquet et al., 2009; Jiao et al., 2010; Südhof, 2012). In both brp isoform mutants, T bars appeared clearly smaller when compared with controls (Fig. 4 A; see also tomography Videos 1, 2, and 3). HPF EM displays T bars as filamentous structures, whereas in conventional embedded samples, including an initial aldehyde fixation step, T bars appear in a more compact manner and therefore easier to quantify (Südhof, 2012). In random sections of conventional embedded NMJ synapses (Fig. 4 B), T-bar height was slightly reduced only in brp\textsuperscript{A170} mutants (Fig. 4, B and C, quantification), whereas the widths of pedestal (Fig. 4, B [arrowheads] and D [quantification]) and platform (Fig. 4, B [arrows] and E [quantification]) were reduced in both mutants. Thus, both isoforms are needed to form proper-sized T bars.

AZs of brp\textsuperscript{A170} mutants were contained with anti-BRP\textsuperscript{N-Term} and anti-BRP\textsuperscript{C-Term} and subjected to STED. As expected, N- and C-terminal labels were clearly separated in vertical views at the control AZs (Fig. 5 A, top left inset). Although N- and C-terminal epitopes remained separated, AZs in brp\textsuperscript{A170} mutants appeared smaller than those of the controls in both vertical and planar views (Fig. 5 A, insets and example AZs marked with arrowheads [vertical] and arrows [planar] in overviews). Because brp\textsuperscript{A190} mutants have no BRP\textsuperscript{N-Term} staining left (Fig. 2 A), we instead used an AB against the C terminus of DRBP, which localizes very similarly to the N terminus of BRP (Fig. 5 B). AZs in brp\textsuperscript{A190} mutants, similar to brp\textsuperscript{A170} mutants, appeared smaller compared with the controls in both vertical and planar orientation
Figure 3. Superresolution analysis of BRP isoform distribution at NMJ AZs. (A) Live imaging in intact third instar larvae expressing BRP-170GFP and BRP-190Cherry. (B and C) Images of individual AZs labeled with BRP-170GFP and BRP-190Cherry acquired with a two-color STED microscope (~50 nm xy resolution compared with ≥200-nm resolution of a confocal microscope). Dotted lines define the position of the intensity distribution profiles (right). (D) The number of AZs vs. the number of dots in the images. (E) Mean number of dots per AZ for BRP-170GFP and BRP-190Cherry. (F) Transitions [%] between BRP-190 and BRP-170 for different regions of AZs. (G) Puncta with overlap [%] for different regions of AZs.

1 = change
2 = same
3 = N/A (overlap)
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Ultrastructural analysis of brp isoform mutants. (A) Representative T bars taken from random ultrathin sections from controls, brp<sup>170</sup>, and brp<sup>190</sup> mutant third instar larvae after HPF embedding. (B) Examples of electron micrographs of T bars in controls, brp<sup>170</sup>, and brp<sup>190</sup> mutants third instar larvae after conventional embedding. T-bar pedestal (arrowheads) and platform (arrows) are indicated. (C) The T-bar height (pedestal plus platform) was reduced in brp<sup>170</sup> mutants compared with controls, whereas no difference could be detected in brp<sup>190</sup> mutants (control: 55.8 ± 1.4 nm; brp<sup>170</sup>: 54.7 ± 1.4, P = 0.98 vs. control; brp<sup>190</sup>: 45.7 ± 1.4 nm, P < 0.001 vs. control and P < 0.001 vs. brp<sup>170</sup>; n = as indicated in the bars). (D) T-bar pedestals were significantly thinner in both isoform mutants compared with controls (control: 85.5 ± 9.8 nm; brp<sup>170</sup>: 56.0 ± 4.9, P < 0.01 vs. control; brp<sup>190</sup>: 53.7 ± 4.4 nm, P < 0.01 vs. control and P = 0.93 vs. brp<sup>170</sup>; n = as indicated in the bars). (E) T-bar platforms were significantly thinner in both isoform mutants compared with controls (control: 176.5 ± 11.8 nm; brp<sup>170</sup>: 94.0 ± 5.8 nm, P < 0.0001 vs. control; brp<sup>190</sup>: 105.8 ± 8.2 nm, P = 0.0004 vs. control and P = 0.3573 vs. brp<sup>170</sup>; n = as indicated in the bars). All panels show mean values and errors bars representing SEMs. **, P ≤ 0.01; ***, P ≤ 0.001; n.s., P > 0.05, Mann–Whitney U test.

Figure 4.

In planar orientation, both brp isoform mutants showed a reduced number of BRPC-Term dots per AZ (Fig. 5, A and B, planar insets). Although the majority of wild-type AZs had four and five dots per AZ, in both isoform mutants, this was shifted to two to three dots per AZ (Fig. 5 C). Regularly, AZs with just one cluster of BRPC-Term were found in mutants but not controls. Accordingly, mean numbers of dots per AZ were reduced from approximately four to less than three in both isoform mutants (Fig. 5 D). Interestingly, the mean number of dots per wild-type AZ obtained for BRP-190 and BRP-170 each (mean number: 4.5/AZ; Fig. 3, D and E) is similar to the number of dots obtained by BRPC-Term AB staining (mean number: 4.1/AZ), which recognizes both isoforms. Together with the alternating pattern observed for BRP-170<sup>GFP</sup> and BRP-190<sup>Cherry</sup>, this suggests that each BRP dot recognized by the BRPC-Term AB is built up of one cluster of BRP-170<sup>GFP</sup> and BRP-190<sup>Cherry</sup>. Overall, STED and EM analyses agree that both BRP isoforms localize to AZs independently of each other but cannot fill in for each other in the formation of a proper AZ molecular architecture. After losing one of the isoforms, the number of BRP clusters per AZ dropped, and the typical pentagonal gestalt no longer formed. Next, we were interested in which functional consequences these ultrastructural changes might have.

Reduction of baseline transmission in the absence of BRP-190 or -170

We were curious whether synaptic transmission might be affected by the reduced cytomatrix size. We performed two-electrode voltage clamp (TEVC) recordings on larval body wall muscles of third instar larvae to analyze potential physiological effects after losing either of the BRP isoforms. The mean evoked excitatory junctional current (eEJC) amplitude was decreased in both mutants by 20–30% (control: −107.1 ± 6.0, n = 22, brp<sup>170</sup>: −80.5 ± 5.3, P < 0.01; n = 22; brp<sup>190</sup>: −73.3 ± 3.3, n = 19, P < 0.001; one-way analysis of variance [ANOVA] Tukey’s posttest; Fig. 6 A) with a more pronounced effect in the brp<sup>190</sup> mutant. At the same time, kinetics of evoked synaptic responses were not altered (Fig. 6 B).

The size of spontaneous vesicle fusion events (miniature excitatory junctional currents [mEJCs]) was unaltered (Fig. 6 C).
Figure 5. **STED analysis of brp isoform mutants.** (A and B) Overviews of two-color STED images of control and brp$^{\Delta170}$ (A) or brp$^{\Delta190}$ (B) mutant NMJs stained with the indicated antibodies. (Insets) Magnifications of individual vertical (arrowheads) and planar (arrows) oriented AZs. The asterisk in the vertical inset in B marks beginning of a second AZ close to the centered one. (C) Number of BRPC-Term dots per AZs (x axis) plotted against the number of AZs with that number of dots (y axis) in control, brp$^{\Delta170}$, and brp$^{\Delta190}$ mutants. (D) Quantification of the mean number of BRPC-Term dots per AZ. Control: 4.1 ± 0.1; brp$^{\Delta170}$: 2.8 ± 0.2, P < 0.0001 versus control; brp$^{\Delta190}$: 2.7 ± 0.2, P < 0.0001 versus control and P = 0.7506 versus brp$^{\Delta170}$; Mann–Whitney U test; n (AZs) is indicated in the bars and is identical to C. Error bars represent SEMs.
however, mEJCs were more frequent in \textit{brp}^{A170} (Fig. 6 D). mEJC kinetics were identical to controls for both mutants (Fig. 6 E).

Notably, although basal synaptic transmission was reduced in \textit{brp}^{A170} and \textit{brp}^{A190} (Fig. 6 A), short-term plasticity analyzed with standard protocols was not changed, unlike other \textit{brp} alleles analyzed up to now (Kittel et al., 2006; Hallermann et al., 2010). Depression kinetics at 10-Hz stimulation for 10 s were identical to controls, as was obvious from normalized response amplitudes (Fig. 6 F). Additionally, no difference in paired-pulse ratios at a 10– or 30-ms interstimulus interval (ISI) was detected (Fig. 6 G).

The release deficit in \textit{brp}-null mutants can likely be attributed mainly to Ca$^{2+}$ channel declustering (Kittel et al., 2006). Given that we did not detect any changes in short-term plasticity in either of the isoform mutants with standard protocols, we wondered whether Ca$^{2+}$ channel mislocalization could be observed. The $\alpha$1 subunit Cacophony (Cac) is the only representative of the mammalian Ca$_{\alpha}2.1/2.2$ family in \textit{Drosophila}. In cac-$null$ mutants, evoked SV release at the NMJ is almost absent, leading to embryonic lethality (Kawasaki et al., 2002; Hou et al., 2008). We generated a genomic Cac construct (Venken et al., 2008) labeled with GFP at the C terminus, similar to the upstream activator sequence-Cac-GFP previously used (Kawasaki et al., 2004). In \textit{brp}^{null} background, Cac-GFP signals appeared declustered as expected (unpublished data). In neither of the \textit{brp} isoform mutants (Fig. S2 A), however, were any Ca$^{2+}$ channel clustering defects observed. Instead, Cac-GFP intensities measured over the whole NMJ, number of dots, and mean dot size were all similar to controls (Fig. S2, B–D). Thus, the release deficit observed in \textit{brp} isoform mutants cannot be attributed to declustering of Ca$^{2+}$ channels. As mentioned earlier in this paper, tight local organization of Ca$^{2+}$ channels and the release apparatus is crucial for the spatiotemporal coupling of Ca$^{2+}$ influx to SV release and thus determinative of the release probability of single SVs. Ca$^{2+}$ channel declustering in both \textit{brp}- and \textit{drbp}-null mutants goes along with delayed synaptic transmission that is consequent of imperfect synchronization of AP-triggered SV release. Lack of either of the two main BRP isoforms does not decluster Ca$^{2+}$ channels and, therefore, does not influence the tight spatiotemporal control of release. This is reflected by the unchanged kinetics of evoked release (Fig. 6 B) as well as the normal short-term plasticity (Fig. 6, F and G) found in both mutants.

**BRP isoforms define the number of readily releasable SVs**

To further address the question how the reduced size of the BRP scaffold affects synaptic transmission, we made use of variance–mean analysis of synaptic responses (Clements and Silver, 2000; Sakaba et al., 2002; Silver, 2003). Thus, we determined the mean amplitude and variance of eEJCs in TEVC mode at Ca$^{2+}$ concentrations between 0.5 and 6 mM and calculated vesicular release probabilities at the used Ca$^{2+}$ concentrations applying parabolic fits (see Materials and methods; Fig. 7, A [an example recording of a control cell] and B [the according fit]; and Fig. S3, A and B, examples for all genotypes). The vesicular release probability was indeed unchanged in both isoform mutants compared with controls over the whole range of concentrations tested (Fig. 7 C).

Apart from the vesicular release probability, the amount of release-ready vesicles determines how many quantal units get exocytosed per arriving action potential. By the performed variance–mean analyses, we could also determine the size (n) of the RRP of SVs. In fact, RRP sizes were reduced by half in \textit{brp}^{A190} and, to a lesser extent, in \textit{brp}^{A170} mutants (control: 627.6 ± 92.0, n = 8; \textit{brp}^{A170}: 554.4 ± 99.0, n = 8, P > 0.05; \textit{brp}^{A190}: 317.7 ± 53.3, n = 8, P < 0.05; one-way ANOVA Tukey’s posttest; Fig. 7 D). Mean quantal size was unchanged (Fig. S3 C).

We used a high-frequency stimulation protocol of 100 Hz for 300 ms to assess the RRP size with an additional independent assay (Fig. 7 E). Here, RRP sizes were determined by back extrapolation of cumulative quantal contents of responses. Again, both mutants showed fewer release-ready SVs, again with a significant difference of pool size in \textit{brp}^{A190} compared with the control (control: 395 ± 29, n = 21; \textit{brp}^{A170}: 319 ± 25, n = 20 , P > 0.05; \textit{brp}^{A190}: 289 ± 24, n = 14 , P < 0.05; one-way ANOVA Tukey’s posttest; Fig. 7 F).

**T-bar pedestal close SVs specifically reduced in \textit{brp} isoform mutants**

It appears that the reduction in the size of the AZ cytomatrix we observed scales with the reduction in the number of RRP SVs, as determined by electrophysiological analysis. As mentioned previously in this paper, physiological data show that Ca$^{2+}$ sensors on the SVs get into a very close physical proximity to the Ca$^{2+}$ channels. Our previous analysis had shown that Ca$^{2+}$ channels are clustered directly underneath the BRP N termini, at the T-bar pedestal (Kittel et al., 2006). In \textit{brp}^{null} background, T bar pedestals (Fig. 7 G, left) of a particularly large AZ cytomatrix (Fig. 7 G, red) where SVs tethered to the distal parts of the cytomatrix can be clearly detected (Fig. 7 G, gold). Most importantly, we regularly observed SVs clustered directly at the base of the T bar (Fig. 7 G, right, yellow and arrows in virtual section). Thus, the BRP cytomatrix might define SV binding and release sites (“slots”) in tight proximity to the Ca$^{2+}$ channels, allowing the coupling of SVs to Ca$^{2+}$ nanodomains. In EM serial section reconstructions, both mutants showed a significant decrease in those SVs found in close proximity to both the plasma membrane and the T-bar pedestal (Fig. 7 H).

In \textit{brp}^{null} mutants, lacking the last 17 aa, T bars are bare of SVs in EM (Hallermann et al., 2010). When we counted the distally tethered SVs and SVs lateral to T bars using three 50-nm concentric shells in random ultrathin sections (similar to Hallermann et al., 2010), no reduction could be observed in the \textit{brp} isoform mutants (Fig. S4). Collectively, these findings suggest a reduced amount of RRP SVs in the AZs with reduced size of the BRP cytomatrix, probably reflecting a decreased number of slots in which SVs could bind to achieve nanodomain coupling to Ca$^{2+}$ channels.

**Discussion**

An elaborate protein cytomatrix covering the AZ membrane is meant to facilitate and control the SV release process. Quantitative analysis of neurotransmitter release has provided evidence
Figure 6.  Electrophysiological characterization of brp isoform mutant NMJs. (A) Example traces and quantification of evoked excitatory junctional currents (eEJC) recorded in 1.5 mM Ca\(^{2+}\) at 0.2 Hz. n is as indicated in the bars. (B) Rise time and the decay time constants (\(\tau\)) of eEJC\s were unchanged (rise time: control, 1.23 ± 0.06, n = 21; brp\(^{170}\), 1.40 ± 0.07, P > 0.05, n = 19; brp\(^{190}\), 1.37 ± 0.06, P > 0.05, n = 19; \(\tau\): control, 6.74 ± 0.17; brp\(^{170}\), 6.80 ± 0.19, P > 0.05; brp\(^{190}\), 7.21 ± 0.30, P > 0.05; one-way ANOVA Tukey’s posttest). (C, left) Example traces of mEJC\s. (right) mEJC amplitude was unchanged in both brp isoform mutants (control: −0.93 ± 0.04, n = 19; brp\(^{170}\), −0.86 ± 0.04, P > 0.05, n = 16; brp\(^{190}\), −0.95 ± 0.03, P > 0.05, n = 16; one-way ANOVA Tukey’s posttest). (D) mEJC frequency was increased in brp\(^{170}\) mutants (control: 0.74 ± 0.08, n = 19; brp\(^{170}\), 1.47 ± 0.20, P < 0.01, n = 16; brp\(^{190}\), 1.03 ± 0.13, P > 0.05, n = 16; one-way ANOVA Tukey’s posttest). (E) mEJC kinetics were not changed...
that the number of SV release sites per AZ might be fixed (Clements and Silver, 2000). Although these sites are thought to be located in close proximity to presynaptic Ca\textsuperscript{2+} channels, ultrastructural and molecular information is largely missing here (Haucke et al., 2011). Potentially, specific interactions between SVs and certain cytomatrix components might be involved. Here, we provide evidence that the BRP-based cytomatrix plays a role in defining the number of readily releasable SVs, possibly by offering morphological and molecular-determined “release slots.”

**BRP alleles isolate discrete functionalities of the presynaptic cytomatrix**

Previously, we characterized the role of BRP based on null alleles, which result in a complete absence of AZ cytomatrix (T bar), partially declustered Ca\textsuperscript{2+} channels, and likely as a direct consequence, reduced vesicular release probability (Kittel et al., 2006). In contrast, in the analysis of BRP isoform-specific mutants, we here neither observed any Ca\textsuperscript{2+} channel clustering deficits nor changes in vesicular release probability.

We have previously found a binding site between the intracellular C terminus of the Cac Ca\textsuperscript{2+} channel and an N-terminal stretch of BRP, which is unique to BRP-190 (Fouquet et al., 2009). That solely losing BRP-190 is not sufficient to affect Ca\textsuperscript{2+} channel clustering could possibly be explained by the presence of redundant binding sites within BRP-170. Ca\textsuperscript{2+} channel clustering might well be a collective feature of the cytomatrix, and Ca\textsuperscript{2+} channels likely use multiple simultaneous interactions with several cytomatrix proteins to anchor within the AZ membrane.

In fact, RIM-binding protein family proteins at rodent and Drosophila AZs bind Ca\textsuperscript{2+} channels, and loss of the only RIM-binding protein in Drosophila results in partial loss of Ca\textsuperscript{2+} channels from AZs (Liu et al., 2011). RIM-binding protein levels at AZ were slightly but significantly reduced in the BRP isoform mutants. Clearly, it remains a possibility that RIM-binding protein is a major scaffold determinant of the release slots and that e.g., subtle mislocalizations of RIM-binding protein might in part contribute to the BRP isoform mutant phenotype. The brp-null phenotype we now interpret as a “catastrophic event” in which a complete loss of this large scaffold protein leads to a severe decrease of cytomatrix avidity (potentially mediated via a loss of RIM-binding protein) below a critical level, resulting in a “collapse” of the normal cytomatrix architecture. Thus, functionalities associated with discrete regions of BRP and RIM-binding protein can apparently be masked when the BRP-based AZ scaffold is completely eliminated.

As mentioned previously in this paper, the distal cytomatrix in brp\textsuperscript{null} is bare of SVs (model in Fig. 8, brp\textsuperscript{null}) in EM, and SV replenishment is defective, resulting in short-term depression (and not facilitation as in brp nulls). However, no change of short-term plasticity could be detected in the brp isoform alleles with the same analyses, consistent with neither a change in Ca\textsuperscript{2+} channel clustering (Fig. S2) nor in SV clustering at the distal cytomatrix (Fig. S4). Nevertheless, a basal release deficit was observed, which can be explained by a reduction in the size of the readily releasable vesicle pool (Fig. 7), assigning an additional function to the BRP cytomatrix.

**Cytomatrix architecture and local environments for SV release slots**

Release-ready SVs are meant to be molecularly and positionally primed for release. Important factors are the equipment with or the attachment to the proteins of the core release machinery (Rizo and Südhof, 2012) and the localization of the SV in proximity to the Ca\textsuperscript{2+} source. At the Drosophila NMJ, SV release is insensitive to slow Ca\textsuperscript{2+} buffers such as EGTA (Kittel et al., 2006); therefore, SVs are thought to be spatially tightly coupled to Ca\textsuperscript{2+} channels (nanodomain coupling; Eggermann et al., 2012). As we find Ca\textsuperscript{2+} channels localized directly underneath the T-bar pedestal composed of the N-terminal region of BRP (Fouquet et al., 2009), release-ready SVs might well correspond to the SVs that we find docked at the pedestal of the T bar and thus in very close proximity to the Ca\textsuperscript{2+} channels. This in turn is in agreement with BRP itself being important for defining the number of release-ready SVs determined by electrophysiology and EM (Fig. 7).

Light microscopic inspection of an AB directed against the C terminus of BRP, common to both isoforms (Fig. 1), with 50-nm STED resolution, typically revealed approximately five dots arranged as a circle or regular pentagon (Fig. 5). We labeled both isoforms individually and found that (a) both isoforms seem to localize with their C termini similarly toward the distal edge of the cytomatrix and (b) both isoforms typically form an identical number of dots per AZ (Fig. 3) similar to the number of dots observed with the BRP\textsuperscript{C-Term} AB recognizing both isoforms. Thus, the BRP isoforms seem to be arranged in neighboring but not overlapping clusters, forming a circular array (Fig. 8, wild-type model). Consistent with both BRP isoforms not overlapping in space, there was neither efficient co-IP between them (Fig. 1B, N-term AB lane) nor did elimination of one isoform substantially interfere with the AZ localization of the respective other isoform. Thus, BRP-190 and -170 seem to form discrete oligomers. The alternating pattern of BRP-190 and -170 appears to set a typical cytomatrix size, as both isoform mutants had a reduced T-bar width in EM (Fig. 4) and a
Figure 7. BRP isoform mutants have normal vesicular release probability but harbor fewer release-ready vesicles. (A) Control example of eEJC amplitudes recorded at the indicated Ca$^{2+}$ concentrations. (B) Parabolic fit of variance–mean analysis of example cell in A. (C) Vesicular release probabilities of brp$^{170}$ and brp$^{190}$ mutants at various Ca$^{2+}$ concentrations do not differ from controls (P $>$ 0.05 for all Ca$^{2+}$ concentrations; one-way ANOVA Tukey’s posttest).
reduced mean number of BRP dots per AZ (Fig. 5 and Fig. 8, brp(190) model). As this corresponded with a similar reduction in the number of SVs in the RRP (Fig. 7), this AZ architecture could set a typical number of Ca\(^{2+}\) nanodomain-coupled RRP slots possibly located between BRP clusters. However, beyond providing a discrete morphological architecture, the two BRP isoforms described here might harbor additional functionalities. The brp(190) phenotype was more pronounced than the brp(170), leaving the possibility that the highly conserved N terminus of BRP-190 (Fig. 1 E) promotes release by further mechanisms going beyond the points analyzed in this study. Future analysis will also have to address whether localization and regulation of additional cytomatrix and release components, such as RIM-binding protein (Liu et al., 2011), Unc-13 family proteins (Aravamudan et al., 1999), or RIM (Müller et al., 2012), contribute to the formation of release slots as well.

### Scaling between cytomatrix size and evoked release

Ultimately, functional differences between individual synaptic sites must be defined by variances in their molecular organization. Functional features of a synapse can be extracted electrophysiologically. Thereby, the number of Ca\(^{2+}\) channels was recently identified as a major determinant of the release probability of single vesicles, P\(_{\text{vr}}\), in rat calyces (Sheng et al., 2012). Furthermore, AZ size seems to scale with the overall likelihood of release from a given AZ (Holderith et al., 2012). Our results suggest that the BRP-based cytomatrix should be a general determinant of the release likelihood per AZ by establishing P\(_{\text{vr}}\), through Ca\(^{2+}\) channel clustering, as shown previously (Kittel et al., 2006), and, as shown here, by determining the size of the RRP. Our genetic results show that the cytomatrix can, in principle, control the RRP size independent of Ca\(^{2+}\) channel clustering. A coupled increase in the size of the T-bar cytomatrix together with increasing SV release was previously observed at NMJs compensating for loss of the glutamate receptor subunit GluRIIA (Reiff et al., 2002). Moreover, an increase in the number of release-ready SVs together with an increase in the amount of BRP was recently described as part of a homeostatic presynaptic response after pharmacological block of postsynaptic GluRIIA (Weyhersmüller et al., 2011). In line with this scenario, it was recently shown that lack of acetylation of BRP in elp3 mutants led to an increase in the complexity of the AZ cytomatrix along with an increase in RRP size (Miśkiewicz et al., 2011). Furthermore, in vivo imaging of synaptic transmission with single synapse resolution revealed that the likelihood of release correlates with the amount of BRP present at an individual AZ (Peled and Isacoff, 2011). This cytomatrix size–SV release scaling might be a general principle, as a correlation between the amount of SV exocytosis, measured by an optical assay, and the amount of the AZ protein Bassoon at individual synapses of cultured rat hippocampal neurons has also been observed.
Materials and methods

IPS and silver staining

Wild-type adult fly heads were mechanically homogenized in a buffer [B] containing 50 mM Tris/HCl, pH 8, 1% Naoxylol, 150 mM NaCl, and protease inhibitor [Complete; Roche] followed by incubation at 36°C for 30 min and, after addition of 0.1% Triton X-100, by incubation for another 30 min at 4°C. Tissue debris were isolated from the supernatant by repeated centrifugation (Owald et al., 2010). Supernatant was subjected to SDS-PAGE and samples were heated at 95°C for 5 min. After centrifugation for 5 min at 16,000 g, to pellet fly head debris, 10 µl of each sample (equivalent to one, two, three, and four larval body walls) were subjected to denaturing SDS-PAGE using a 6% Tris-Ci gel. Proteins were transferred onto a nitrocellulose membrane and probed with affinity-purified primary antibody anti-BRP (1:10,000). For detection, a secondary anti–rabbit IgG HRP-conjugated AB (Dianova) and an ECL detection system (GE Healthcare) were used.

Western blot analysis of larval NMJs

Larval body wall protein extraction was performed as quadruplicate as follows: 15 wild-type larva each were dissected as described previously (Gin et al., 2005) with minor modifications. Internal organs including the larval central nervous system were removed, and body walls were sheared manually into 20 µl of 2% Sds aqueous solution using a micropipettor fitting tightly into a 1.5-mI cup. 20 µl of 2x sample buffer (Laemmli, 1970) was added, and samples were heated at 95°C for 5 min. After centrifugation for 5 min at 16,000 g, to pellet fly head debris, 10 µl of each sample (equivalent to five head) was subjected to denaturing SDS-PAGE using a 6% Tris-Ci gel. Proteins were transferred onto a nitrocellulose membrane and probed with affinity-purified primary AB rabbit anti-BRP (1:10,000). For detection, a secondary anti–rabbit IgG HRP-conjugated AB (Dianova) and an ECL detection system (GE Healthcare) were used.

Western blot analysis of BRP isoform mutants

For flies protein extraction, 20 heads per genotype were sheared manually in 20 µl of 2% Sds aqueous solution using a micropipettor fitting tightly into a 1.5-mI cup. 20 µl of 2x sample buffer (Laemmli, 1970) was added, and samples were heated at 95°C for 5 min. After centrifugation for 5 min at 16,000 g, to pellet fly head debris, 10 µl of each sample (equivalent to five heads) was subjected to denaturing SDS-PAGE using a 6% Tris-Ci gel. Proteins were transferred onto a nitrocellulose membrane and probed with affinity-purified primary AB rabbit anti-BRP (1:10,000). For detection, a secondary anti–rabbit IgG HRP-conjugated AB (Dianova) and an ECL detection system (GE Healthcare) were used.

Fly stocks

All fly strains were reared under standard laboratory conditions. For larval genotypes, see Immunostainings.

Isolation of brp190 mutants

The brp190 allele was identified in a chemical mutagenesis screen with 25 mM ethyl methyl sulfonate by reduced fitness and eclosion rate when placed over previously established null alleles of brp (brp107 and brp104).

Generation of brp170 mutants

To test access to a specific elimination of the 170 kD isoform, we mobilized the P element transposon line D09839, located 0.6 kb upstream of the first exon of the transcript encoding BRP-170. The brp170 allele was generated through imprecise excision of the P element. P element transposon line D09839 was obtained from the Exelixis Collection at the Harvard Medical School.

Molecular cloning

Generation of P(acman)-BRP genomic constructs. The P(acman) constructs for BRP have a size of 83 kb and were cloned according to Venken et al. (2006). In brief, the bacterial artificial chromosome (BAC) clone RP81-1424J2 was obtained from BACPAC Resources Center was used as a template for cloning of the BRP genomic P(acman) construct. 5′ homology arm [left arm] flanked by Ascl–NolI was produced by PCR using primers 5′-GGCGGCGCGTGAAGGACATTTGC-3′ and 5′-GCGCTTACTTGCACTGCTCGT-3′. 3′ homology arm [right arm] flanked by Nol–PacI was produced by PCR using the primers 5′-GGCGGCGCGTCCATGCGAAGAAGTCCC-3′ and 5′-TAACTGAAACATGCCTCGGTAAG-3′.

The left arm and right arm PCR products were cut by PacI and Ascl and ligated into attB-P(acman)-ApR. A recombination event between the BAC and the attB-P(acman)-ApR-LARA entails the complete brp locus inclusive of the neighboring genes with an average of 70.1 kb (genomic region 5′-3,067,010-5,437,720) resulted in a P(acman)-BRP construct of 83.4 kb size.

Generation of modified and tagged P(acman)-BRP constructs. The insertion of the Δ190 point mutation and the Δ170 deletion of the alternative promoter and first exon were cloned according to the Counter Selection BAC Modification kit obtained from Gene Bridges GmbH company. The Δ190 point mutation was introduced at aa 261 of the brp locus corresponding to the brpΔ190 allele. To delete the brpΔ170, the 170 kD–specific promoter and the first exon were removed resulting in a 1,111-bp deletion. The rps-neomycin (neo) template DNA was used to generate selectable cassettes. The constructs consisted of 50-bp homology regions and a sequence for amplification of the rps-neo counter selection cassette. The selectable cassettes were generated in a proof reading PCR with Taq polymerase (New England Biolabs, Inc.) and primers pairs: EGFP/mCherry, 5′-CAGAAAGGCGCAACTGCTTACAC-3′ and 5′-CAGAAAGGCGCAACTGCTTACAC-3′; and 

CTGTAGGCCAAGGGAATGGGTG-3′ and 5′-CAGAAAGGCGCAACTGCTTACAC-3′.

For this, a single point mutation was introduced through a primer. To delete the BRP-170, the 170 kD–specific promoter and first exon were cloned according to the Counter Selection BAC Modification kit obtained from Gene Bridges GmbH company. The Δ190 point mutation was introduced at aa 261 of the brp locus corresponding to the brpΔ190 allele. To delete the brpΔ170, the 170 kD–specific promoter and the first exon were removed resulting in a 1,111-bp deletion. The rps-neomycin (neo) template DNA was used to generate selectable cassettes. The constructs consisted of 50-bp homology regions and a sequence for amplification of the rps-neo counter selection cassette. The selectable cassettes were generated in a proof reading PCR with Taq polymerase (New England Biolabs, Inc.) and primers pairs: EGFP/mCherry, 5′-CAGAAAGGCGCAACTGCTTACAC-3′ and 5′-CAGAAAGGCGCAACTGCTTACAC-3′.

For this, a single point mutation was introduced through a primer. To delete the BRP-170, the 170 kD–specific promoter and first exon were cloned according to the Counter Selection BAC Modification kit obtained from Gene Bridges GmbH company. The Δ190 point mutation was introduced at aa 261 of the brp locus corresponding to the brpΔ190 allele. To delete the brpΔ170, the 170 kD–specific promoter and the first exon were removed resulting in a 1,111-bp deletion. The rps-neomycin (neo) template DNA was used to generate selectable cassettes. The constructs consisted of 50-bp homology regions and a sequence for amplification of the rps-neo counter selection cassette. The selectable cassettes were generated in a proof reading PCR with Taq polymerase (New England Biolabs, Inc.) and primers pairs: EGFP/mCherry, 5′-CAGAAAGGCGCAACTGCTTACAC-3′ and 5′-CAGAAAGGCGCAACTGCTTACAC-3′.
Generation of genomic CacGFP flies. The attP(Pacman) BAC clone CH321-60D21 containing the genomic region of Cac was obtained from the BACPAC Resources Center. The C-terminal EGFP tag was incorporated according to Venken et al. (2008) using PL452-CEGFp-KanH (Drosomycin plasmid 19178) and the following primers: 5’-AGAGTGGTGTAACCCCTC- GACACTCCGATCGATGAAAGGATTTGCGGACCACTCCGAC- ATTCTGACATC-3’ (forward) and 5’-AACGACATCGGCAAGCCCTGTACGTCCTGATGC-3’ (reverse). After sequencing, the construct was injected into an attP site containing fly strain PBac(y[+]/+attP-3B)VK00033; Bloomington Drosophila Stock Center line #9750) using the services of BestGene Inc.

Immunostainings

Dissections and immunostainings were performed as described before (Qin et al., 2005). All larvae were raised at 25°C on a semi-defined medium (Bloomington Drosophila Stock Center recipe) and the following genotypes were used: control, Canton-S; (Bloomington Drosophila Stock Center recipe) and the following genotypes containing fly strain (PBac{y[+]attP-3B}VK00033; Bloomington Drosophila Stock Center line #9750) using the services of BestGene Inc. Dissections and immunostainings were performed as described before (Fouquet et al., 2009; Liu et al., 2011). Live imaging, confocal imaging, and image processing were essentially performed as previously described (Owald et al., 2010). Two-color live imaging included, and p-value denotes the significance according to one-way ANOVA with Tukey’s multiple comparison posttest. In the figures, the level of significance is marked with asterisks: * P < 0.05; ** P < 0.01; and *** P < 0.001.

Variance-mean analysis

The current amplitude at different extracellular Ca2+ concentrations of 0.5–6 mM (≥20 eEJCs each) was recorded. For a binomial model, the mean amplitude of synaptic responses is given by $I = NP$, where $P$ is the vesicular probability of release at a certain extracellular Ca2+ concentration. The variance $Var(I)$ is $NP(1 - P)$ was calculated by $Var(I) = (1/(n - 1)) * \left( I - \mu \right)^2$. The variance-mean plots were fitted for each individual cell with $Var(I) = P/N$. The vesicular release probability $P$ was adjusted with the experimentally determined coefficient of variation of 0.3 (Pawluk et al., 2004) according to Clements and Silver (2000). The data are reported as means ± SEM, $n$ indicates the number of cells examined and included, and p-value denotes the significance according to one-way ANOVA with Tukey’s multiple comparison posttest. In the figures, the level of significance is marked with asterisks: * P < 0.05; ** P < 0.01; and *** P < 0.001.

Cumulative postsynaptic current analysis

30 consecutive postsynaptic responses were recorded at a stimulation frequency of 100 Hz. eEJC amplitudes were measured from peak to baseline directly before the onset of the response. Quantal content of each response was calculated by division of the amplitude by the mean quantal size of the respective genotype. Release-ready vesicles were determined by back extrapolation of cumulative quantal contents. The data are reported as means ± SEM, $n$ indicates the number of cells examined and included, and p-value denotes the significance according to one-way ANOVA with Tukey’s multiple comparison posttest. In the figures, the level of significance is marked with asterisks: * P < 0.05; ** P < 0.01; and *** P < 0.001.

EM

For conventional embedding, third instar larvae were dissected and fixed in 4% PFA and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, washed three times for 5 min in sodium cacodylate buffer,
and postfixed on ice for 1 h with 1% osmium tetroxide and 0.8% KFeCn in 0.1 M sodium cacodylate buffer. After washing in sodium cacodylate buffer and distilled water (three 5-min washing steps), the samples were stained en bloc with 1% uranyl acetate in distilled water on ice. Samples were dehydrated in ascending ethanol concentrations at RT and infiltrated in Epon resin. After infiltration, the muscles 6/7 of segment A2/3 of two to five larval larvae were cut out and placed parallel to each other in an embedding mold. Blocks were polymerized for 48 h at 70°C. After conventional embedding, 65–70-nm serial sections were obtained and collected on Formvar-coated copper slot grids. Sections were postfixed and poststained with uranyl acetate/lead citrate following standard protocols. Micrographs were taken with an electron microscope (JEM 1011; JEOL) equipped with a camera (Orius 1200A; Gatan) using the DigitalMicrograph software package (Gatan). Serial 3D reconstructions were conducted using the 3D reconstruct software (Fila, 2005).

For HPF embedding, about three to five Drosophila late second/early third instar larvae were placed in aluminum specimen carrier of 200-µm depth (type A; BalTec), filled with yeast paste, and covered with a lid. The samples were frozen immediately in an HPF machine (HPM100; Leica) and rapidly transferred to liquid nitrogen for storage. Cryosubstitution was performed in an AFS (Leica) in anhydrous acetone with 1% EMD Millipore water, 1% glutaraldehyde, and 1% osmiumtetroxide at −90°C for 10 h. The samples were slowly (5°C/h) warmed to −20°C and incubated for an additional 12 h before being warmed (10°C/h) to 20°C. The samples were washed with acetone and incubated with 0.1% uranylacetate in anhydrous acetone for 1 h at RT. After rinsing, the samples were incubated in 30% Epon/70% acetone for 4 h followed by 70% Epon/30% acetone overnight. They were incubated twice in 100% Epon for 2 h before being embedded. 60–65-nm sections were cut using an ultramicrotome (RMC Power Tome XL; Boeckeler). Sections were collected on Formvar-coated 100 mesh grids. Sections were poststained with 2% uranylacetate for 30 min and lead citrate for 3 min. Micrographs were acquired on a microscope [Tecnai Spirit; FEI].

For electron tomography on conventional embedded samples, 250-nm sections were acquired on a microscope (Tecnai Spirit; FEI). For electron tomography on HPF samples, 200-nm embedded sections of second instar larvae were applied to Formvar-coated copper 100 mesh grids and stained with 2% uranyl acetate and Reynold’s lead citrate. 10-nm gold beads were applied on both sides of the grid. Tilt series were acquired on a microscope [Tecnai Spirit] operated at 120 kV equipped with a charge-coupled-device camera (2K Eagle; FEI). Tilt series (a 56° with 1 or 2° tilt increments) were recorded at a nominal magnification of 30,000 at 3–5-µm defocus using Xplore3D (FEI). 3D reconstruction was performed using the IMOD software package (Fila, 2005), and the model was rendered using 3Dmod (The Boulder Lab for 3D Electron Microscopy).

Online supplemental material
Fig. S1 shows an additional Western blot of BRP isoforms with quantification performed using the IMOD 3dmod software (Kremer et al., 1996).

References


Figure S1. BRP isoform larval protein levels and additional analysis of distribution. (A) Western blots of Drosophila wild-type larval body walls for relative quantification of BRP isoform protein levels. Three independent experiments with two and three larvae loaded were conducted, and membranes were probed with anti-BRP. The two bands of 190- and 170-kD apparent sizes represent the BRP-190 and BRP-170 isoforms, respectively. (B) Quantification of the Western blots shown in A by densitometric readout revealed equal abundance of BRP-190 and BRP-170 in wild-type larval body walls (two larvae: BRP-190, 13.0 ± 2.3; BRP-170, 11.2 ± 1.1; P = 0.7; n = 3; three larvae: BRP-190, 15.9 ± 2.4; BRP-170, 16.6 ± 2.0; P = 1.0; n = 3; Mann–Whitney U test). Data are expressed in arbitrary units of intensity. Error bars represent SEMs. (C–F) Planar- and vertical-oriented AZs imaged with two-color STED resolution. Bottom images show the same channels displayed with the fire lookup table and with the maxima highlighted by the find maxima function of ImageJ (default settings). The respective third images show manual reconstructions of the punctae based on merges of the respective former two images. (C) BRP-170GFP stained with anti–mouse GFP and anti–mouse Atto647N, BRP-190Cherry stained with anti–rabbit dsRed and anti–rabbit Atto594 in wild-type background. (D) BRP-170GFP stained with anti–mouse GFP and anti–mouse Atto594, and BRP-190Cherry stained with anti–rabbit dsRed and anti–rabbit Atto647N in a wild-type background. (E) BRP-170GFP stained with anti–mouse GFP and anti–mouse Atto594 and BRP-190Cherry stained with anti–rabbit dsRed and anti–rabbit Atto647N in a brpnull background. (F) BRP-190Cherry stained with anti–rabbit dsRed and anti–rabbit Atto647N and BRP-C-Term (NC82) stained with anti–mouse Atto594 in a wild-type background.
Figure S2. Ca$^{2+}$ channel clustering is not affected in BRP isoform mutants. (A) Two boutons of NMJs on muscle 4 from third instar larvae with the indicated genotypes, labeled with the indicated ABs. (B) Quantification of Cac$^{GFP}$ intensity measured over the whole NMJ (HRP mask). Control, 100.0 ± 4.3; brp$^{A170}$, 101.3 ± 5.6, P = 0.9461 versus control; brp$^{A190}$, 95.8 ± 6.9, P = 0.1479 versus control and P = 0.1896 versus brp$^{A170}$; n = 20 NMJs in seven larvae/genotype. (C) Quantification of the number of Cac$^{GFP}$ dots measured over the whole NMJ (HRP mask). Control, 100.0 ± 4.9; brp$^{A170}$, 106.0 ± 6.2, P = 0.2559 versus control; brp$^{A190}$, 93.9 ± 7.1, P = 0.3104 versus control and P = 0.0658 versus brp$^{A170}$; n = 20 NMJs in seven larvae/genotype. (D) Quantification of Cac$^{GFP}$ dot size per AZ. Control, 0.12 ± 0.01; brp$^{A170}$, 0.11 ± 0.1, P = 0.3793 versus control; brp$^{A190}$, 0.10 ± 0.01, P = 0.2184 versus control and P = 0.5979 versus brp$^{A170}$; n = 20 NMJs in seven larvae/genotype. All quantifications except D are normalized to control. Error bars represent SEMs. n.s., P > 0.05, Mann–Whitney U test. n is as indicated in the bars.
Figure S3. Variance–mean analysis of BRP isoform mutants. [A] Example traces for Control, \( brp^{170} \), and \( brp^{190} \) \( (n \geq 20 \) traces each; \( Ca^{2+} \) concentrations as indicated). [B] Parabolic fits of variance–mean analysis of example cells in A. [C] Quantifications of quantal size determined by variance–mean analysis (control, 0.40 ± 0.07, \( n = 8 \); \( brp^{170} \), 0.38 ± 0.05, \( P > 0.05 \), \( n = 8 \); \( brp^{190} \), 0.52 ± 0.03, \( n = 8 \), \( P > 0.05 \); one-way ANOVA Tukey’s posttest). The graph shows mean values with errors bars representing SEMs, n.s., \( P > 0.05 \).
Video 1. **Control T-bar structure.** Electron microscope tomography reconstruction of wild-type larval NMJ terminals. Sections of second instar larvae (250 nm thick) were prepared by HPF/FS. Tilt series were acquired on a microscope (Tecnai Spirit) at a nominal magnification of 30,000 at 3–5-µm defocus using Xplore3D.

Video 2. **brpΔ170 T-bar structure.** Electron microscope tomography reconstruction of brpΔ170 larval NMJ terminals. Sections of second instar larvae (250 nm thick) were prepared by HPF/FS. Tilt series were acquired on a microscope (Tecnai Spirit) at a nominal magnification of 30,000 at 3–5-µm defocus using Xplore3D.

Video 3. **brpΔ190 T-bar structure.** Electron microscope tomography reconstruction of brpΔ190 larval NMJ terminals of control animals. Sections of second instar larvae (250 nm thick) were prepared by HPF/FS. Tilt series were acquired on a microscope (Tecnai Spirit) at a nominal magnification of 30,000 at 3–5-µm defocus using Xplore3D.

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**Figure S4. Cytomatrix distal and lateral SV distribution is not affected in BRP isoform mutants.** (A) The number of SVs within three shells each of 50-nm thickness surrounding the cytomatrix was counted. (B) Quantification of the number of SVs within three 50-nm shells surrounding the cytomatrix. -50 nm: control, 5.3 ± 0.3 (n [AZs] = 25); brpΔ170, 4.6 ± 0.3 (n [AZs] = 17); brpΔ190, 4.8 ± 0.3 (n [AZs] = 32); P = 0.3358, Kruskal–Wallis ANOVA. 51–100 nm: control, 7.9 ± 0.4 (n [AZs] = 25); brpΔ170, 7.6 ± 0.5 (n [AZs] = 17); brpΔ190, 6.4 ± 0.4 (n [AZs] = 32); P = 0.0727, Kruskal–Wallis ANOVA. 101–150 nm: control, 8.9 ± 0.5 (n [AZs] = 25); brpΔ170, 9.2 ± 0.8 (n [AZs] = 17); brpΔ190, 8.0 ± 0.5 (n [AZs] = 32); P = 0.3710, Kruskal–Wallis ANOVA. Error bars represent SEMs. n.s., P > 0.05.