Naked Dense Bodies Provoke Depression

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At presynaptic active zones (AZs), the frequently observed tethering of synaptic vesicles to an electron-dense cytomatrix represents a process of largely unknown functional significance. Here, we identified a hypomorphic allele, *brp^{nude}*, lacking merely the last 1% of the C-terminal amino acids (17 of 1740) of the active zone protein Bruchpilot. In *brp^{nude}*, electron-dense bodies were properly shaped, though entirely bare of synaptic vesicles. While basal glutamate release was unchanged, paired-pulse and sustained stimulation provoked depression. Furthermore, rapid recovery following sustained release was slowed. Our results causally link, with intramolecular precision, the tethering of vesicles at the AZ cytomatrix to synaptic depression.

Introduction

The rate at which sensory information can be transmitted across synapses is an important factor that limits rapid and precise information processing in the nervous system. The mechanisms that operate in the presynaptic terminals to release neurotransmitter at AZs at high repetition rates are currently intensely investigated (Atwood and Karunanithi, 2002; Zucker and Regehr, 2002; Kidokoro et al., 2004; Neher and Sakaba, 2008). AZs are decorated with electron-dense material of varying shape and size depending on synapse type and species (Zhai and Bellen, 2004).

Recently, the CAST/ERC-family member Bruchpilot (BRP), a coiled-coil rich protein of nearly 200 kDa, was identified via its localization to *Drosophila* AZs. BRP was shown to be crucial for cytomatrix formation, proper Ca²⁺ channel clustering within AZs, and efficient neurotransmitter release at the *Drosophila* neuromuscular junction (Kittel et al., 2006; Wagh et al., 2006). Furthermore, BRP is a direct component of the electron-dense cytomatrix (T-bar) and adopts an elongated conformation, with its N terminus (N-term) facing Ca²⁺ channels at the membrane and its C-term reaching into the cytoplasm (Fouquet et al., 2009).

Materials and Methods

Genetics. Larvae were raised at 25°C [cacophony (Cac) RNA interference (RNAi) larvae and their controls were raised at 29°C] in bottles or on apple agar plates (for high-pressure freezing), and the following genotypes were used: Bruchpilot studies: *brp^{nude}* or *brp^{1,3}* or *brp^{5,45}/df(2R)BSC29*, *ok6-GAL4* (Fouquet et al., 2009), controls: *+/ok6-GAL4*. Cacophony images: *ok6-GAL4*, *UAS-Cac^{GFP}/+* and *brp^{nude}/df(2R)BSC29*, *ok6-GAL4*; *UAS-Cac^{GFP}/+*. Cacophony RNAi studies: *UAS-Cac* RNAi (Transformant ID 5551) (Dietzl et al., 2007)/+; *elav-GAL4/+*, control: *elav-GAL4/+*.

Stimulated emission depletion microscopy. Stimulated emission depletion (STED) microscopy experiments were performed essentially as described by Kittel et al. (2006), using the commercially available Leica TCS STED microscope (Leica Microsystems). Secondary antibodies were conjugated to the Atto 647N dye (AttoTech). Atto 647N fluorophores were excited at a wavelength of 635 nm and depleted at 750 nm. Images were acquired using APD detectors within a range of 645–715 nm. Image processing was performed using the Inspector software (Max-Planck Innovation) by applying a linear deconvolution at single STED slices.

To assess Ca²⁺ channel clustering, neuromuscular junctions were stained with M- α -GFP (Invitrogen) and G- α -M-Atto647N to visualize Ca²⁺ channels (Cac^{GFP}). Additionally, serving as a reference, postsynaptic glutamate receptors were labeled with primary Rb- α -DGluRIID and secondary Sheep- α -Rb-Cy3 (Invitrogen) antibodies and imaged via conventional confocal microscopy. From such images, AZs were selected that appeared planar to the optical slice (i.e., the AZ membrane is parallel to the focal slice). With Mathematica 5.0 (Wolfram Research), each channel (Cac^{GFP} and GluRIID) of each image of a planar AZ was automatically fitted with a two-dimensional Gaussian function. The peak of these Gaussians was used to align the Cac^{GFP} signal either with itself or with the GluRIID signal for subsequent averaging. The average images were again fitted with two-dimensional Gaussian functions and both horizontal and vertical intensity profiles through the peak of the two-dimensional Gaussian functions were averaged and fitted with one-dimensional Gaussian functions (see Fig. 2B).

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Electron microscopy. Electron microscopy and conventional embedding was performed as previously described (Fouquet et al., 2009) on late third-instar *Drosophila* larvae. The number of docked vesicles was determined along the whole synapse showing the typical close apposition of electron-dense presynaptic and postsynaptic membrane. We counted only vesicles without any discernible distance between vesicle and AZ membrane.

Electrophysiology. Two-electrode voltage-clamp (TEVC) recordings of EPSCs were obtained at room temperature from late third-instar male *Drosophila* larvae (ventral longitudinal muscle 6, segments A2 and A3), essentially as previously described (Kittel et al., 2006). For the TEVC experiments with *brp^{nude}*, *brp^{1,3}*, and *brp^{5,45}* mutants, both mutants and control animals carried a copy of *ok6-GAL4* to ensure comparability with a previous study (Kittel et al., 2006). The composition of the extracellular hemolymph-like saline (HL-3) was as follows (in mM): NaCl 70, KCl 5, MgCl₂ 20, NaHCO₃ 10, trehalose 5, sucrose 115, HEPES 5, CaCl₂ 1 or as indicated, and pH adjusted to 7.4. Recordings were made from cells with an initial resting membrane potential between -50 and -70 mV (holding potential at -60 mV) using intracellular electrodes with resistances of 10 – 32 M Ω , filled with 3 M KCl. Train stimulation protocols consisted of 100 pulses applied at 60 Hz. The recovery was assessed by evoking APs at (in ms following the last pulse in the train): 25, 50, 100, 200, 500, 1000, 2000, 5000, 10,000, 20,000, 50,000, and 100,000 (Wu et al., 2005). EPSCs reflect the compound response to stimulation of both motoneurons innervating muscle 6, and care was therefore taken to ensure their stable recruitment. Infrequently observed recruitment failures were linearly interpolated. Only cells that recovered at least 70% of their initial EPSC amplitude following tetanic stimulation were included in the analysis. The recordings were analyzed with pClamp 9 (Molecular Devices), and the peak amplitude was determined as the difference between the peak value of the EPSC and the baseline value before onset of that EPSC. The steady-state EPSC amplitudes (see Fig. 3D) were evaluated for each experiment as the average of the last 25 EPSC amplitudes in the train and normalized to the average steady-state EPSC amplitude of the corresponding control for either *brp* or *Cac* RNAi experiments. The time constants of the fast and slow components of recovery (see Fig. 3E,F) were determined from monoexponential functions fitted to the average fast and slow component of recovery and weighted with the errors of the average data with Igor Pro 6.1 (Wavemetrics). The estimated errors of the fit parameters were taken from Igor Pro (which are based on the square roots of the diagonal elements of the covariance matrix). From the time constants, the SEs, and the number of experiments, statistical comparisons were performed with Student's *t* test, and the error bars of the normalized values were calculated according to Gaussian error propagation.

Statistical analysis. The nonparametric Mann–Whitney rank sum test was used for statistical analysis if not stated otherwise. The data are reported as mean $\pm$ SE, *n* indicates the sample number, and *p* denotes the significance ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

Results

Impaired vesicle tethering at AZs of a Bruchpilot mutant (*brp^{nude}*) lacking the last 17 C-terminal amino acids

In a chemical [ethyl methyl sulfonate (EMS)] mutagenesis screen, a novel *brp* allele was identified with a premature STOP codon at amino acid position 1724 (*brp^{5,38}*, hereafter *brp^{nude}*) of 1740 aa. While in the previously published mutants *brp^{5,45}* and *brp^{1,3}* a significant proportion of the C-terminal amino acids are absent (50% and 30%, respectively; STOP codons at position 867 or 1390) (Fouquet et al., 2009), *brp^{nude}* lacks only the last 1% (Fig. 1A,C). Nevertheless, all three mutants showed significantly reduced survival rates and motor abilities as adult flies (Fig. 1B).

At neuromuscular junctions of *brp^{nude}*, the reactivities of antibodies directed against the C-term (BRP^{Nc82}; Wagh et al., 2006) and the N-term (BRP^{N-term}) (Fouquet et al., 2009) of BRP are still present (Fig. 1D), and the number of AZs per junction is normal (quantified as BRP^{Nc82} spots per junction; 553 ± 27 and 518 ± 28

for control and *brp^{nude}*; $n_{\text{junction}} = 6$ and 6 ; $p = 0.24$). Furthermore, high-resolution light microscopic images (STED microscopy) (Hell, 2007) of BRP^{Nc82} acquired simultaneously with confocal images of BRP^{N-term} appeared unchanged compared to controls. Especially, the previously described ring-like distribution of Nc82 reactivity (Kittel et al., 2006) is preserved at *brp^{nude}* synapses (Fig. 1D). The normal distribution of C- and N-terminal signals at *brp^{nude}* AZs indicates that the missing 1% of BRP does not alter the overall structure of the cytomatrix. In fact, an electron microscopic analysis of the ultrastructure of *brp^{nude}* synapses revealed normal amounts of AZ cytomatrix and an ordinary height and platform length of dense bodies (T-bars) (Fig. 1E,F). Furthermore, the diameter of the synaptic vesicles as well as the total number of synaptic vesicles per bouton section were unchanged in *brp^{nude}* (diameter: 36 ± 1.7 and 35 ± 0.3 nm for control and *brp^{nude}*, $n_{\text{vesicles}} = 220$ and 243 (10 larvae each), respectively; $p = 0.9$; number: 114 ± 24 and 131 ± 7 SV/ μm^2 for control and *brp^{nude}*, $n_{\text{bouton-section}} = 6$ and 6 , respectively; $p = 0.2$). Finally, the number of docked vesicles per AZ section was not significantly different between *brp^{nude}* and controls (1.8 ± 0.2 and 1.4 ± 0.3 for control and *brp^{nude}*, $n_{\text{AZ}} = 21$ and 24 , respectively; $p = 0.4$) (see Fig. 1G; for definition of docked vesicles, see Materials and Methods). However, T-bars at *brp^{nude}* AZs were bare of vesicles (Fig. 1E). A quantification of the number of vesicles within three shells of each 50 nm thickness surrounding the AZ revealed a significant reduction in the average number of vesicles near the T-bar at *brp^{nude}* synapses (Fig. 1G). These data indicate that while the basic ultrastructure of the cytomatrix is unaltered at *brp^{nude}* AZs, vesicle tethering is specifically impaired.

Normal Ca²⁺ channel clustering and basal release at *brp^{nude}* synapses

Using STED microscopy, we tested whether Ca²⁺ channel clustering is affected at *brp^{nude}* synapses. STED images of GFP-labeled Ca²⁺ channels (Cac^{GFP}) were acquired simultaneously with confocal images of postsynaptic glutamate receptors (GluRIID) to identify the position of AZs (opposite GluRIID patches) independently of Ca²⁺ channel clustering (Fig. 2A). Fitting Gaussian functions to the intensity profiles of average images revealed a normal Ca²⁺ channel distribution at *brp^{nude}* synapses (Fig. 2B). Comparable results were obtained when the Cac signals were aligned with themselves. With this method, the delocalization of Ca²⁺ channels in *brp* null mutants (Kittel et al., 2006) could clearly be resolved (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Thus, we isolated a *brp* allele lacking only the last 1% of C-terminal amino acids, which severely affects vitality but not the clustering of AZ Ca²⁺ channels.

Next, we tested the functional consequences of defective vesicle tethering. EPSCs evoked at 0.2 Hz at *brp^{nude}* synapses did not differ in terms of their peak amplitude, rise time, or decay time constant from those of controls (Fig. 2C). In *brp^{1,3}* and *brp^{5,45}* mutants, with poorly clustered Ca²⁺ channels (Fouquet et al., 2009), the EPSC amplitudes were reduced to $30 \pm 8\%$ ($n = 4$) and $10 \pm 8\%$ ($n = 12$) of control amplitudes, respectively (Fig. 2C). The amplitude of miniature EPSCs was not significantly affected in any of these three mutants (Fig. 2D). These data indicate that basal release (at 0.2 Hz) requires Ca²⁺ channel clustering, but not vesicles tethered to the cytomatrix.

Figure 1. Impaired vesicle tethering at AZs of a *bruchpilot* mutant (*brpnude*) lacking the last 17 C-terminal amino acids. **A**, Chemical induction of premature stop codons resulted in truncated versions of the BRP protein (BRP; dark blue; boxes indicate coiled-coil domains) of ~99% (*brpnude*; gray), of ~70% (*brp^{1.3}*; green), and of ~50% length (*brp^{5.45}*; blue). Below, Illustration of the BRP protein (blue) within the AZ (red: Ca²⁺ channels; gray: synaptic vesicle) and the corresponding positions of the truncation (arrows) (Fouquet et al., 2009). **B**, All three BRP mutants (*brpnude*, *brp^{1.3}*, *brp^{5.45}*) showed severely impaired survival rates and walking skills. **C**, In the *brpnude* allele, a single base mutation at the C-terminal position 1724 leads to a premature stop codon and a BRP protein (BRP^{nude}) lacking the last 17 of 1740 aa. **D**, STED images of neuromuscular junctions stained with a C-terminal BRP antibody (green; BRP^{Nc82}) and simultaneous confocal images of an N-terminal BRP antibody (magenta; BRP^{N-term}). The distribution of both antibody signals appears unaltered at *brpnude* synapses (right) compared to controls (left). Arrows and arrowheads indicate planar and vertical AZs, respectively. **E**, Examples of conventionally embedded AZs of control and *brpnude*. Note fewer tethered vesicles in *brpnude*. **F**, Height and length of the platform of the electron-dense cytomatrix (T-bar) for control and *brpnude*. **G**, The number of docked vesicles per AZ section for control and *brpnude* (n = 22 and 25 AZs, respectively) and the number of vesicles found within three shells (see inset) each of 50 nm thickness surrounding the AZ (n = 20 and 31 AZs, respectively). Scale bars: **D**, 1 μm; **E**, 200 nm.

Tethering vesicles to the active zone dense body prevents synaptic depression

To investigate the impact of vesicle tethering on short-term plasticity, we analyzed synaptic transmission during paired-pulse stimulation (Fig. 3A). Strikingly, the amplitude of the second EPSC was significantly reduced at short interpulse intervals of 10 and 30 ms (Fig. 3B). This depression decayed with a time constant of 19 ms. Next, synaptic transmission was analyzed during a train of 100 stimuli at 60 Hz (Fig. 3C) (Hallermann et al., 2010). The depression during the train was stronger in *brpnude* as quantified

by a reduction in the steady-state EPSC amplitude at the end of the train to 35 ± 3% compared to 52 ± 5% in controls (n = 21 and 20 for control and *brpnude* mutants, respectively) (Fig. 3C,D). Consistent with the altered paired-pulse ratio, the amplitude of the second EPSC in the train was already significantly reduced to 80 ± 10% at *brpnude* synapses (n = 21 and 20).

If sustained release is impaired in *brpnude* mutants because fewer vesicles are tethered to the cytomatrix, then sustained release in *brp^{1.3}* and *brp^{5.45}* mutants, both of which show severely impaired cytomatrices (Fouquet et al., 2009), should be im-

Figure 2. Normal Ca^{2+} channel clustering and basal release at *brp^{nude}* synapses. **A**, GluRIID labels (magenta, confocal) were used to quantify the Ca^{2+} channel (Cac^{GFP}, green, STED) clusters in control animals (top row) and *brp^{nude}* mutants (below). The Cac signals at synapses that appeared ideally planar ($n = 16$ each) were averaged after alignment with the postsynaptic GluRIID signal (right panels) or themselves (center panels). While the example images were scaled up individually, all four averages were scaled up with the same factor. **B**, Intensity profiles of the average Cac images in black for controls and in gray for *brp^{nude}* mutants with SE bars and the corresponding Gaussian fits (standard deviations given by σ) in gray. **C**, Examples of EPSCs elicited at 0.2 Hz in control (black), *brp^{nude}* (gray), *brp^{1.3}* (green), and *brp^{5.45}* mutants (blue; average of 10 each) in 1.0 mM Ca^{2+} . The average peak EPSC amplitudes and rise and decay kinetics were normal in *brp^{nude}* mutants. In *brp^{1.3}* and *brp^{5.45}* mutants EPSC amplitudes were reduced and rise times increased ($n = 22, 18, 4$, and 12 for control, *brp^{nude}*, *brp^{1.3}*, and *brp^{5.45}*, respectively). **D**, Example traces of mEPSCs of control (black), *brp^{nude}* (gray), *brp^{1.3}* (green), and *brp^{5.45}* mutants (blue). The average mEPSC amplitudes were normal in *brp^{nude}*, *brp^{1.3}*, and *brp^{5.45}* mutants ($n = 6, 6, 4$, and 6 for control, *brp^{nude}*, *brp^{1.3}*, and *brp^{5.45}*, respectively). Scale bar in **A**, 250 nm.

paired, too. Indeed, investigations of sustained release in *brp^{1.3}* and *brp^{5.45}* mutants revealed stronger synaptic depression than in controls (supplemental Fig. S2A, C, available at www.jneurosci.org as supplemental material). To analyze whether the stronger depression in *brp^{1.3}* and *brp^{5.45}* mutants is simply the consequence of impaired Ca^{2+} channel density, we sought to compensate (“rescue”) the reduced Ca^{2+} channel density by increasing the extracellular Ca^{2+} concentration. In high Ca^{2+} (2 mM and 2.5 mM for *brp^{1.3}* and *brp^{5.45}* mutants, respectively), their EPSC amplitudes were comparable to those of controls in 1 mM Ca^{2+} . However, depression during sustained release was still pronounced (Fig. 3D; supplemental Fig. S2B, D, available at www.jneurosci.org as supplemental material).

Since vesicle recruitment critically depends on the intracellular spatiotemporal Ca^{2+} dynamics (Neher and Sakaba, 2008), we performed control experiments by reducing the density of Ca^{2+} channels genetically and tested whether subsequent elevations of extracellular Ca^{2+} could rescue sustained release. To this end, transgene-mediated RNAi directed against the $\alpha 1$ subunit of the *Drosophila* Ca^{2+} channel (Cac) was performed, resulting in a reduction of EPSC amplitudes by $\sim 50\%$. Extracellular Ca^{2+} was then elevated to 1.5 mM to obtain control EPSC amplitudes. However, in

contrast to *brp^{1.3}* and *brp^{5.45}* mutants, sustained release was unaltered by Cac RNAi (Fig. 3D; supplemental Fig. S2E, available at www.jneurosci.org as supplemental material).

Next, we addressed whether the enhanced depression during sustained high-frequency transmission at *brp^{nude}* synapses is accompanied by alterations in the kinetics of recovery from depression. Therefore, the recovery from synaptic depression after a train was investigated with test stimuli of increasing intervals following the train (Fig. 3E, F) (Wu et al., 2005; Haller et al., 2010). A biphasic recovery with time constants of $\tau_1 = 50$ ms and $\tau_2 = 6.1$ s was found in controls. In *brp^{nude}* mutants, the first component was slower (114 ms), while the second component was unaltered (5.6 s) (Fig. 3E, F). Consistently, the first component of recovery was also slower in *brp^{1.3}* and *brp^{5.45}* mutants with Ca^{2+} concentrations that rescued the basal EPSC amplitude. In contrast to the normal second component of recovery at *brp^{nude}* synapses, the second component of recovery in *brp^{1.3}* and *brp^{5.45}* mutants was also significantly slower (Fig. 3E, F; supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Finally, in experiments with Cac RNAi, which serve as a control for the approach of elevating Ca^{2+} , both components were normal (Fig. 3E, F; supplemental Fig. S2, available at www.jneurosci.org as supplemental material). In summary, these data indicate that the basal EPSC amplitude (cf. Fig. 2) as well as the second component of recovery rely on adequate Ca^{2+} channel clustering (impaired in *brp^{1.3}* and *brp^{5.45}*) and that high-frequency sustained release as well as the

first component of recovery also rely on proper vesicle tethering at the AZ (selectively impaired in *brp^{nude}*).

Discussion

To our knowledge, the specific impairment of vesicle tethering reported here delivers the first direct demonstration that efficient sustained release relies on the ability of the AZ to tether vesicles. While the overall AZ structure, including the distribution of Ca^{2+} channels, was unaffected, the impairment of vesicle tethering provoked pronounced synaptic depression and a slowed first component of recovery.

The C-terminal half of BRP consists of ~ 1000 aa of essentially contiguous coiled-coil sequence (Wagh et al., 2006), reminiscent of Golgi/ER-resident tethering factors such as, e.g., GM130 (Lupashin and Sztul, 2005). These coiled-coils typically form rod-like structures, where 100 aa residues extend ~ 15 nm when dimerized, and proteins such as Uso1p (Yamakawa et al., 1996) extend over 150 nm (Lupashin and Sztul, 2005). These rod-like proteins are believed to act before SNARE protein assembly by forming contacts between membranes at a distance, thereby increasing the specificity or efficiency of the initial at-

Figure 3. Tethering vesicles to the AZ dense body prevents synaptic depression. **A**, Examples of EPSCs elicited by a paired-pulse protocol with 30 ms interpulse interval in control (black) and *brp*^{nude} (gray). **B**, Average paired-pulse ratios plotted versus interpulse interval in 1.0 mM Ca^{2+} for control (black, $n = 12$) and *brp*^{nude} (gray, $n = 9$; asterisk indicates significant differences) superimposed with exponential fits (time constants are indicated). Inset, 0.6 mM Ca^{2+} . **C**, Average peak EPSC amplitudes during the 60 Hz train for control (black, $n = 21$) and *brp*^{nude} (gray, $n = 20$). Inset, Average amplitudes of the first 5 EPSCs of the train. The steady-state amplitude (average of EPSC amplitudes marked by the bracket) was significantly reduced in *brp*^{nude} mutants compared to controls. **D**, Steady-state amplitudes normalized to the steady-state amplitude of the respective control, for *brp*^{nude} (gray), *brp*^{1.3} (green), *brp*^{5.45} (blue), and *Cac* RNAi synapses (red) in extracellular Ca^{2+} concentrations that lead to normal ("rescued") basal EPSC amplitudes ($[\text{Ca}^{2+}]_{\text{rescue}}$) (see supplemental Fig. S2, available at www.jneurosci.org as supplemental material). **E**, Average peak EPSC amplitudes during the first rapid component of recovery after the train (τ_1) for control (black, $n = 21$) and *brp*^{nude} (gray, $n = 20$) fitted exponentially (lines). Time constants with SEs are indicated. Bottom, The time constants of the first component of recovery normalized to the corresponding time constant of the control (color code as in **D**). **F**, Corresponding average peak EPSC amplitudes during the second slower component of recovery after the train (τ_2) with corresponding average data.

tachment of vesicles (tethering) (Guo et al., 2000). We have provided morphological and functional evidence that BRP filaments tether vesicles, and thus further mechanistic comparisons between AZ and Golgi/ER trafficking, e.g., concerning the role of small GTPases, might well be informative.

The C-terminal half of BRP is very highly conserved in insects but not elsewhere (Wagh et al., 2006). Interestingly, the *Drosophila* genome does not appear to encode homologs of the vertebrate AZ components Piccolo and Bassoon (Wagh et al., 2006), which are key regulators of the vertebrate cytomatrix (Khimich et al., 2005). At central vertebrate synapses, CAST and Bassoon immunoreactivities (closer and further from the AZ membrane, respectively) were recently found to be associated with filaments that may connect vesicles to the AZ (Siksou et al., 2007). It is tempting to speculate that at AZs of central vertebrate synapses, CAST associates with coiled-coil domain proteins, such as bassoon, to perform the dual functions of Ca^{2+} channel clustering and vesicle tethering executed by the N-terminal and the C-terminal domains of BRP, respectively.

How synapses manage to repetitively release transmitter with high precision is intensely investigated. Vesicles tethered to

electron-dense bodies may represent a reservoir of vesicles required for sustained release (Zhai and Bellen, 2004). Consistent with this hypothesis, synaptic stimulation provokes depletion of vesicles tethered at dense bodies (LoGiudice et al., 2008; Jackman et al., 2009). While the supply of vesicles appears rate limiting during the train and the first component of recovery (Saviane and Silver, 2006), the maturation of vesicles closer to Ca^{2+} channels appears rate limiting during the second component of recovery [cf. *brp*^{1.3} and *brp*^{5.45} (Kittel et al., 2006; Fouquet et al., 2009)]. One may argue that the rapid component of depression observed at *brp*^{nude} synapses (Fig. 3*A,B*) could be partially attributed to fewer docked vesicles (though not significantly; cf. Fig. 1*G*) with a higher initial release probability. However, a functional estimation of the number of readily releasable vesicles using back-extrapolation from the cumulative EPSC amplitudes in the trains (Schneeggenburger et al., 1999) revealed similar numbers of readily releasable vesicles in *brp*^{nude} and controls (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Finally, we would like to point out that the C-term of BRP could be involved in endocytotic mechanisms, which have been shown to be crucial for sustained release (Koenig et al., 1998;

Kawasaki et al., 2000; Dickman et al., 2005; Hosoi et al., 2009; Yao et al., 2009). Novel techniques have begun to address the spatial organization of local vesicle reuse within active zones (Zhang et al., 2009). It will have to be clarified via which routes vesicles move within active zones and in which direction Bruchpilot steers their translocation.

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