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Quantitative super-resolution imaging of Bruchpilot distinguishes active zone states

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The precise molecular architecture of synaptic active zones (AZs) gives rise to different structural and functional AZ states that fundamentally shape chemical neurotransmission. However, elucidating the nanoscopic protein arrangement at AZs is impeded by the diffraction-limited resolution of conventional light microscopy. Here we introduce new approaches to quantify endogenous protein organization at single-molecule resolution in situ with super-resolution imaging by direct stochastic optical reconstruction microscopy (dSTORM). Focusing on the Drosophila neuromuscular junction (NMJ), we find that the AZ cytomatrix (CAZ) is composed of units containing ~137 Bruchpilot (Brp) proteins, three quarters of which are organized into about 15 heptameric clusters. We test for a quantitative relationship between CAZ ultrastructure and neurotransmitter release properties by engaging Drosophila mutants and electrophysiology. Our results indicate that the precise nanoscopic organization of Brp distinguishes different physiological AZ states and link functional diversification to a heretofore unrecognized neuronal gradient of the CAZ ultrastructure.
A major challenge facing the scientific exploration of brain function is the accurate interpretation of structure–function relationships. The synaptic active zone (AZ) is a specialization within the presynaptic terminal, which is functionally defined as the site of neurotransmitter release and can be morphologically identified by the proteinaceous AZ cytomatrix (CAZ). At AZs, complex molecular interactions deliver the speed, precision and plasticity unique to neurotransmission. The CAZ is believed to provide a structural platform for these interactions. In electron microscopy (EM), the CAZ is revealed as an electron-dense structure, whose morphology varies between different types of neurons and between individual synapses formed by the same partner cells. Importantly, activity-dependent structural variations of the CAZ can occur at individual synapses in a dynamic manner and appear to correlate with functional properties of transmitter release. The mechanistic coupling of molecular composition, CAZ structure and neurotransmission, however, remains largely elusive.

Despite a gradually emerging comprehensive protein catalogue, we still lack basic information describing how the nanoscopic organization of proteins gives rise to synaptic function. In essence, this is because of the diffraction-limited resolution of light microscopy that has hindered access to the spatial nanodomain in a physiologically relevant context. In recent years, super-resolution imaging methods have emerged that enable far-field fluorescence microscopy with spatial resolutions approaching virtually EM. Here, super-resolution imaging by single-molecule photoactivation or photoswitching and position determination (localization microscopy) captures an outstanding single-molecule photoactivation or photoswitching and position because it reconstructs the super-resolved image from temporally separating fluorescence emission from single fluorophores within a diffraction-limited area. The position of single-molecule fluorescence signals can then be precisely determined (localized) by fitting of a two-dimensional (2D) Gaussian function to the point-spread function. The localizations of all emitters are finally used to reconstruct a super-resolution image of all emitters is finally used to reconstruct a super-resolution image of endogenous Brp protein copies in their native environment by direct stochastic optical reconstruction microscopy (dSTORM). Drosophila mutants were used to analyze different AZ states, and electrophysiology was applied to functionally calibrate super-resolution images. Our results demonstrate that functional information on neurotransmission is provided by the nanoscopic organization of Brp.

### Results

#### Localization microscopy of the CAZ nanostructure.

To obtain detailed structural information on the CAZ in situ, we used dSTORM at the glutamatergic larval Drosophila neuromuscular junction (NMJ). The CAZ was recognized with a monoclonal antibody (mAb Brp) directed against a C-terminal epitope of Brp. To optimize structural resolution, we used a secondary F(ab')2 fragment labelled on average with 1.3 Cy5 fluorophores. With an IgG size of 8–10 and ~4 nm for the F(ab')2 fragment, we estimate ~13 nm for the spatial dimensions of the antibody–fluorophore complex. Localization microscopy relies on temporally separating fluorescence emission from single fluorophores within a diffraction-limited area. The position of single-molecule fluorescence signals can then be precisely determined (localized) by fitting of a two-dimensional (2D) Gaussian function to the point-spread function. The localizations of all emitters are finally used to reconstruct a super-resolution image of all emitters is finally used to reconstruct a super-resolution image of endogenous Brp protein copies in their native environment by direct stochastic optical reconstruction microscopy (dSTORM)

#### Azulymphores are accessible.

This provides insight into biological function because it reconstructs the super-resolved image from temporally separating fluorescence emission from single fluorophores within a diffraction-limited area. The position of single-molecule fluorescence signals can then be precisely determined (localized) by fitting of a two-dimensional (2D) Gaussian function to the point-spread function. The localizations of all emitters are finally used to reconstruct a super-resolution image of all emitters is finally used to reconstruct a super-resolution image of endogenous Brp protein copies in their native environment by direct stochastic optical reconstruction microscopy (dSTORM)

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### Neurotransmitter release is controlled by a multi-step process of vesicle delivery and molecular maturation at the AZ.

Neurotransmitter release is controlled by a multi-step process of vesicle delivery and molecular maturation at the AZ to generate fusion-competent synaptic vesicles. The availability of such readily releasable vesicles (RRVs) and their calcium-dependent probability of fusion fundamentally determine synaptic plasticity. The precise spatial organization of AZ constituents shapes such basic elements of presynaptic function and contributes to use-dependent synaptic plasticity. Here, we set out to test whether a quantitative analysis of the CAZ ultrastructure could provide information on these functional properties.

To this end, we focused specifically on Bruchpilot (Brp), a major structural and functional component of the CAZ in Drosophila. Brp performs a dual function of clustering Ca2+ channels and concentrating synaptic vesicles at neuromuscular AZs. By promoting excitation–secretion coupling, Brp supports efficient transmitter release, shapes synaptic plasticity and participates in certain forms of learning. Using STED (stimulated emission depletion) microscopy, previous work has provided an ultrastructural description of the orientation of Brp in the CAZ. Building upon this basic understanding, we sought to extract quantitative data on the number and precise spatial arrangement of Brp molecules within AZs.

The present study puts forward a novel approach to extract protein counts from macromolecular assemblies at single-molecule resolution. We demonstrate this procedure by estimating endogenous Brp protein copies in their native environment by direct stochastic optical reconstruction microscopy (dSTORM)

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Quantifying the substructural organization of Brp in the CAZ. To attain further quantitative information on the molecular AZ architecture, we developed an approach to count Brp molecules in their native environment. Owing to the single-molecule sensitivity of dSTORM, fluorophore localizations can be used not only to extract information about the distribution of individual molecules, but crucially, also to approximate the absolute number of protein copies. Since the number of localizations measured for a fluorophore-labelled antibody is influenced by its nano-environment, reference experiments using different antibody concentrations had to be conducted at the CAZ to unequivocally correlate localization counts with the number of underlying Brp protein epitopes (Fig. 3; see Methods section).

First, titrations were performed with secondary antibodies to unravel the number of localizations detected for a single Cy5-labelled secondary antibody (2ndary Ab-Cy5) bound to the primary antibody within the CAZ (Fig. 3b). In order to reliably identify the CAZ at low 2ndary Ab-Cy5 concentrations (high dilutions), mAb BrpNc82 was co-stained with Alexa Fluor 488 (2ndary Ab-A488). Next, the concentration of the primary antibody was titrated to estimate saturation of Brp epitopes (Fig. 3c). And finally, the number of secondary antibodies bound to each primary antibody was estimated. To obtain this information, a low concentration of mAb BrpNc82 was combined with a normal concentration of 2ndary Ab-Cy5. Comparing the number of Cy5 localizations per putative Brp epitope with those of a single 2ndary Ab-Cy5 (Fig. 3b) provided an approximation of 1.59 2ndary Ab-Cy5 per mAb BrpNc82. To visualize the CAZ in this experiment, co-staining was performed with an antibody directed against an N-terminal Brp epitope (rabbit-BrpN-term plus 2ndary Ab-A488 anti-rabbit).

Taking these considerations into account allows for quantitative image analysis and delivers an estimate of 137 ± 29 (s.e.m.) Brp molecules per CAZ-unit (conversion factor of molecules per localization: 0.134 ± 0.028 s.e.m.; see Methods section). Correspondingly, ~7 Brp molecules are recognized per multiprotein cluster (52.2 ± 0.7 localizations s.e.m., n = 2,102 clusters). According to this calculation, Brp proteins would assemble as polarized rod-like heptamers via coiled-coils to form a multiprotein filament. The plausibility of this stoichiometry can be appraised by comparison with other filamentous protein structures, for example, seven subfibrils form intermediate filaments of ~10 nm diameter.

Interestingly, the image analysis described that ~26% of Brp localizations in CAZ-units are not clearly grouped into clusters (Fig. 2d, black triangles). Taking the image background into account (only 78 ± 7 localizations per μm² s.e.m.), we estimate that <1% of localizations within CAZ-units (~10⁶ localizations per μm²) are caused by unspecific labelling. This indicates that a substantial fraction of Brp molecules are not part of macro-molecular filaments.

In localization microscopy, labelling density, in addition to localization precision, critically determines the ability to resolve spatial features of a given structure. Thus, the high density of Brp molecules at the CAZ and the strong affinity of the antibodies form the basis for the high spatial resolution in dSTORM experiments. While epitope shielding may well introduce an error to our approximation of Brp protein and cluster numbers, the tight correlation with EM data lends strong support to our quantitative approach.

Ultrastructural analysis of different AZ states. Brp reorganizations are involved in synaptic plasticity operating on time scales ranging from milliseconds to days. Such plastic rearrangements could, in principle, involve changes in Brp
protein number per CAZ\textsuperscript{34} or the spatial orientation of Brp within the CAZ\textsuperscript{17}. To test our quantitative imaging approach, we analysed different AZ states by using two previously investigated Drosophila mutants with altered Brp organization.

The small vesicle-associated GTPase Rab3 regulates the enrichment of Brp at AZs. At Rab3 mutant (\textit{rab}\textsuperscript{3\#}) larval NMJs, altered synaptic transmission is accompanied by fewer Brp-positive synapses, although these display greatly enlarged Brp aggregates\textsuperscript{34}. The hypomorphic mutant, \textit{brp}\textsuperscript{nude}, lacks merely the last 17 C-terminal amino acids of Brp (that is, \textasciitilde1% of the entire protein; Fig. 3a). While the overall CAZ structure is left intact, \textit{brp}\textsuperscript{nude} T-bars display a strikingly reduced vesicle tethering capacity that leads to slowed vesicle recruitment and short-term depression of neurotransmitter release\textsuperscript{17}.

In line with previous work, we used confocal microscopy to confirm that \textit{rab}\textsuperscript{3\#} NMJs contain fewer Brp-positive AZs.


(~35% of controls; control: 472 ± 43 s.e.m., n = 14 NMJs; rab3rup: 164 ± 15, n = 14, rank sum test P < 0.001; Fig. 4e and Table 1; ref. 34) and then applied dSTORM to study the nanoscopic organization of Brp at individual AZs. In rab3rup mutants, the CAZ was significantly enlarged (control: 0.120 ± 0.006 μm² s.e.m., n = 16 NMJs; rab3rup: 0.212 ± 0.01 μm², n = 11, rank sum test P < 0.001; Fig. 4f and Table 1) and contained more Brp molecules (control: 1,257 ± 89 localizations s.e.m., n = 16 NMJs; rab3rup: 1,999 ± 98, n = 11, rank sum test P < 0.001; Fig. 4f and Table 1). Electron micrographs have described a high concentration of T-bars at a subpopulation of rab3rup AZs, which likely correspond to those AZs that are Brp-positive. In addition, dSTORM resolved a complex organization of the large rab3rup CAZ, often lacking a clearly distinguishable modular composition (Fig. 4, enlarged boxed regions, lower panel). CAZ-units could therefore not be unequivocally identified at rab3rup AZs.

In contrast, brp^nude NMJs contained normal numbers of Brp-positive AZs (brp^nude: 397 ± 25, n = 13, rank sum test P = 0.254 versus control; Fig. 4e and Table 1; ref. 17) and displayed a modular arrangement of Brp into units within the CAZ (Fig. 4, enlarged boxed regions upper panel). Interestingly, dSTORM revealed a decrease in the area of the CAZ at brp^nude AZs (0.097 ± 0.005 μm² s.e.m., n = 13 NMJs, rank sum test P = 0.005 versus control; Fig. 4f and Table 1). This structural property had heretofore not been recognized using high-resolution imaging via STED or EM. Despite this decrease in size, the average brp^nude CAZ contained normal numbers of Brp molecules (1,129 ± 104 localizations s.e.m., n = 13 NMJs, rank sum test P = 0.28 versus control; Fig. 4f and Table 1). This prompted us to investigate the

Figure 4 | Variable nano-organization of Brp in the CAZ. (a,b) Overview of brp^nude and (c,d) rab3rup NMJs stained against Brp. Enlarged boxed regions (left panels epifluorescence and right panels dSTORM) demonstrate the ordered arrangement of brp^nude CAZs, with Brp immunoreactivity largely confined to the CAZ margins, and the disordered nanoscopic organization of greatly enlarged rab3rup CAZs. (e-g) Quantification of imaging data acquired with confocal (e, rank sum test versus controls (n = 14 NMJs); brp^nude (n = 13) P = 0.254; rab3rup (n = 14) P < 0.001) and localization microscopy (f, rank sum test versus controls (n = 16 NMJs): brp^nude (n = 13) P = 0.005, rab3rup (n = 11) P < 0.001; g, rank sum test versus controls (n = 16 NMJs): brp^nude (n = 13) P = 0.28, rab3rup (n = 11) P < 0.001). (g) En face views of individual CAZ-units were aligned according to their centres of mass and the radial density distributions of Brp localizations were plotted (dark lines: average and shaded area: s.e.m.). Compared with controls (black), the Brp epitope was distributed more narrowly in brp^nude (grey) CAZ-units. Scale bars, 2 μm (a-d) and 500 nm (enlarged boxed regions).

Table 1 | Structure of AZs.

| Treatment               | Confocal Area (μm²) | dSTORM Localizations | Number of Brp molecules |
|-------------------------|--------------------|----------------------|-------------------------|
| Control CAZ             | 472 ± 43 (n = 14 NMJs) | 0.120 ± 0.006 (n = 16 NMJs) | 1,257 ± 89               | 168 ± 34 |
| brp^nude CAZ            | 397 ± 25 (n = 13 NMJs) | 0.097 ± 0.005 (n = 13 NMJs) | 1,129 ± 104              | 151 ± 35 |
| rab3rup CAZ             | 164 ± 15 (n = 14 NMJs) | 0.212 ± 0.01 (n = 11 NMJs) | 1,999 ± 98               | 268 ± 58 |

AZ, active zone; Brp, Bruchpilot; CAZ, active zone cytomatrix; dSTORM, direct stochastic optical reconstruction microscopy; NMJ, neuromuscular junction. Confocal microscopy was used to approximate the number of AZs per NMJ (via their Brp-positive CAZ), and super-resolution imaging by dSTORM was engaged to quantify ultrastructural properties of the CAZ and to estimate Brp protein copies. Data are presented as mean ± s.e.m.
spatial organization of Brp in more detail. Because the arrangement of Brp within individual brp

**Functional properties of AZ states.** Next, we performed electrophysiological recordings to obtain mechanistic descriptions of AZ function in both mutants. During low levels of activity, synaptic transmission is fairly normal at both rab3

**Dissecting structure–function relationships.** Building upon the mutant analyses of different AZ states, we sought to link quantitative information on the CAZ ultrastructure with functional properties of neurotransmitter release. In view of the modelling results, the number of Brp-positive AZs per NMJ (~35% of controls in rab3

![Figure 5](https://example.com/figure5.png)

**Figure 5** | Electrophysiological characterization of different AZ states. (a) Representative traces (normalized amplitude of the first eEPSC) and average data of two-electrode voltage clamp recordings from larval NMJs show similar depression of brp

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resolve a clear gradient of both CAZ size and the number of Brp molecules per CAZ along the Ib neuron (Fig. 7). With the largest values at terminal boutons, this structural diversity closely matches the functional gradient\(^35,39\).

**Discussion**

Here, we introduce a novel procedure to determine endogenous protein numbers in tissue by localization microscopy using standard labelling with primary antibodies and secondary F(\text{ab}')\(_2\) fragments. Correlating the nanoscopic organization of a single large protein with electrophysiological recordings enabled us to link the filamentous CAZ ultrastructure to neurotransmitter release properties.

Our data are in line with the observation that \(p_v\) scales with AZ size\(^40\). Studies at *Drosophila* NMJ and hippocampal synapses have also reported that larger AZs provide for more RRV\(_s^5,40,41\). Surprisingly, our analysis of the grossly enlarged rab\(_3\)\(_{rup}\) CAZ (Fig. 4, enlarged boxed regions, lower panel) that suffices to increase \(p_v\) via calcium channel clustering but fails to provide...
additional vesicle release sites. However, keeping in mind that release persists and N appears unaltered in brp-null mutants, Brp itself is unlikely the primary determinant of a release site. In any case, it will be of great interest to clarify why the wt CAZ is subdivided into Brp-positive CAZ-units, and to investigate whether this modular arrangement is matched by a complementary substructural organization of the opposing postsynaptic density.

Our results demonstrate that counting Brp proteins and quantifying their spatial distribution provides more detailed and precise information than merely measuring the CAZ area. This is exemplified by the small brp<sup>+/–</sup> CAZ that contains a normal number of Brp localizations and gives rise to an unchanged \( p_C \) (Figs 4f and 6b and Table 1). Instead, Brp localizations are more confined to the perimeter of brp<sup>+/–</sup> CAZ-units (Fig. 4g). Altered post-translational modification of Brp can promote vesicle tethering to the CAZ and provokes spreading out of AZ-filaments. Conversely, the small brp<sup>+/–</sup> CAZ correlates with deficient vesicle tethering and slow vesicle recruitment, leading to short-term depression (Figs 5 and 6)\(^{17}\). Hence, vesicle tethering may contribute to the shape of the CAZ by contorting proteinaceous filaments or, alternatively, the precise CAZ conformation affects its tethering ability. While this cannot be specified at present, either way the spatial organization of Brp provides information on vesicle reloading kinetics.

By engaging dSTORM, we discovered a heretofore unrecognized gradient of the CAZ ultrastructure along a glutamatergic neuron, concealed by the limited resolution of confocal microscopy. Importantly, this finding provides a mechanistic basis for the functional diversification of AZ\(^{35,39}\). A functional gradient has not been described for type Ia motorneurons. Moreover, AZs of Ia neurons reportedly possess a higher \( p_C \) than their Ib counterparts, although this functional feature is not matched by a larger number of T-bars per AZ\(^{28,29,43}\). Similarly, we detected comparable numbers of Brp localizations at type Ia and Ib CAZs (the Ia CAZ is in fact slightly smaller) and found no ultrastructural gradient in the Ia neuron (Supplementary Fig. 4). This consideration highlights that Brp is not the sole determinant of \( p_C \) and motivates the study of further AZ protein constituents. Intriguingly, for example, synaptic vesicle size differs between these two different glutamatergic neurons\(^{44}\).

The present investigation emphasizes how fundamentally different AZ states may be disguised by giving rise to similar facets of short-term depression (Fig. 5a). For descriptions of synaptic function, the degree of paired-pulse depression is routinely interpreted to reflect the magnitude of release probability. In light of these results and recent data from different synapses reporting fast vesicle reloading\(^{45}\), transient fusion\(^{46}\) and release site clearance\(^{47}\), analysing the CAZ nanostructure to test alternative interpretations of depression may well provide new insights into the molecular control of neurotransmission.

In order to estimate the number of Brp molecules in the CAZ, several hurdles had to be overcome. In general, localization microscopy in combination with photoactivatable fluorescent proteins\(^{10,12}\) appears to represent the method of choice for quantification purposes because fluorescent proteins offer the distinct advantage of specific stoichiometric labelling of target molecules. On the other hand, we were interested in endogenous protein levels. Therefore, overexpression of fusion proteins could not be deployed, and substitution of native proteins by transgenic variants that display wt expression and function remains challenging. In addition, misfolded fluorescent proteins and those that cannot be photoactivated or photobleached already after only a few excitation/emission cycles, and thus emit an insufficient number of photons, withdraw themselves from detection and localization\(^{14,49}\). Besides localization microscopy, stepwise stochastic photobleaching of fluorophores upon illumination with light can be used to determine protein numbers\(^{50}\). Stepwise photobleaching is, however, limited to low protein numbers because the likelihood of missed events increases exponentially with the number of molecules\(^{51}\).

As an alternative approach, we evaluated the use of standard immunocytochemistry, that is, organic fluorophores and antibodies for quantification of endogenous protein levels. Here, challenges to be accepted include epitope accessibility, antibody affinity and multiple localizations owing to on/off switching on expanded time scales. Brp proteins appear to oligomerize as coiled-coils to form elongated, polarized filaments\(^{20}\). Therefore, it is conceivable that such a structural organization leads to a separation of epitopes along the filament circumference, promoting antibody accessibility despite a high density of Brp proteins (Supplementary Fig. 5). That said, differences in steric hindrance may exist at the level of individual AZs and in different genotypes. Furthermore, since epitopes can be shielded or lost during fixation, the determined number of Brp proteins might well represent a lower estimate.

Organic fluorophores exhibit certain advantageous characteristics, such as higher brightness and photostability than fluorescent proteins. Thus, in combination with the fact that each fluorophore can be localized multiple times, a higher percentage of accurately localized fluorophores can potentially be achieved. Nonetheless, the difficulty of extracting reliable information on how often a fluorophore-labelled antibody is localized remains, especially because the photoswitching performance of fluorophores is sensitively influenced by their local environment\(^{52}\). As a consequence, isolated fluorophores located outside of the investigated cellular compartment cannot be used as reference. Therefore, we developed an elaborate but secure two-colour method for identifying individual fluorophore-labelled antibodies in the structure of interest in order to determine the typical number of localizations. However, certain photophysical effects that depend, for example, on local fluorophore densities and photoswitching characteristics can never be completely ruled out in quantitative localization microscopy experiments. Nevertheless, by titrating primary and secondary antibodies, the described procedure delivers reliable estimates for the number of accessible protein epitopes per CAZ.

A major challenge facing the field of super-resolution microscopy is the development of analytical tools to quantify data sets and to help provide biological interpretations.\(^{30}\) The implementation of clustering algorithms provided an objective description of the distribution pattern of Brp molecules within the CAZ and revealed the organization of Brp into supramolecular clusters. Considering their structural properties, these clusters likely correspond to the multiprotein CAZ-filaments observed in EM\(^{27}\) (STED microscopy displays \( \sim 9 \) ‘dots’ per AZ\(^{41}\)). Why are the clusters elliptical? We speculate that an answer can be provided by the arrangement of fluorophores around CAZ-filaments in space. When CAZ-units are viewed on face (optical axis perpendicular to AZ membrane), filaments bent outwards could be viewed at a right angle to their long axis at the level of the mAb Brp<sub>NC82</sub> epitope (Fig. 3a and Supplementary Fig. 5). In the images, the filament diameter (~10 nm) only contributes to the separation of encircling fluorophores in x and y. Hence, the largest separation will be seen for Cy5 molecules located at opposite sides of the filament and aligned with the CAZ-unit circumference (Supplementary Fig. 5).

Intriguingly, the quantitative analysis described a substantial population of un-clustered Brp proteins in the CAZ. It will be of great interest to investigate the biological significance of this.
comprehensive molecular blueprint of the AZ.

Fly stocks

Electrophysiology. In brief, two-electrode voltage clamp recordings (Axoclamp 900A amplifier, Molecular Devices) of eEPSCs (Vholding = −60 mV, stimulation artefact removed for clarity in figures) and mins (Vholding = −80 mV, 90 s recording) were made from muscle 6 (segments A2 and A3) at room temperature with intracellular electrodes (resistances of 10−20 MΩ, filled with 3 M KCl) essentially as previously reported14,15. The composition of the extracellular haemolymph-like solution (HL-3) was (in mM): NaCl 70, KCl 5, MgCl2 20, NaHCO3 10, trehalose 5, sucrose 115, HEPES 5 and CaCl2 1.5, pH adjusted to 7.2. Muscle cells with an initial membrane potential between −30 and −70 mV, and input resistances of ≥2 MΩ were accepted for analysis. Signals were sampled at 10 kHz, low-pass filtered at 1 kHz and analysed with Clampfit 10.2 (Molecular Devices). EPSCs were evoked by stimulating the innervating nerve (300 μs pulses typically at 10 V) via a suction electrode.

Ten eEPSCs were averaged for each cell pair by a three-pulse protocol and for low-frequency stimulation. Paired-pulse recordings were made at 0.2 Hz with interstimulus intervals of (in ms): 10, 30, 100, 300 and 1,000. Ten seconds of rest were afforded to the cell in between recordings. The amplitude of the second response in 10 ms inter-pulse recordings was measured from the peak to the point of interception with the extrapolated first response. High-frequency stimulation followed an established protocol14,15 consisting of 100 pulses applied at 60 Hz. The recovery was monitored by stimulating at increasing intervals following the train (in ms): 25, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000 and 100,000.

Modelling. STP modelling of 60 Hz trains and of the recovery thereafter was performed as previously described16,18. Two constrained STP models were used. First, a model with one pool of release-ready vesicles refilled from a finite supply pool was used (model 1). Model 1 is characterized by the following parameters: the Ca2+ dependence of facilitation, χ, that defines the release probability, N, the number of RRVs, N0, the number of vesicles in the supply pool from which the readily releasable pool is refilled; and k−1, k+1, k−0 and k0 the refilling rates of N and N0, respectively. In addition, we also used a model with two pools of release-ready vesicles and heterogeneous release probabilities (model 2). In model 2, a small pool of release-ready vesicles (N0) with a high release probability (p0,r) is refilled with a rate k0 from a larger pool (N1), which has a lower vesicular release probability (p1,r), and which, in turn, is refilled from a supply pool (N0). The refilling rates of these pools (k+, k−, k−0 and k0) are defined as in model 1. The refilling rates for p0,r and p1,r for model 2 were defined according to a biophysical Ca2+-dependent model of facilitation with one single free parameter (x)18,58. Both models had two additional free parameters: N for model 1, N0 for model 2 and k−1 for both models, resulting in three free parameters for each model. The remaining parameters were constrained to values previously estimated at the Drosophila NMJ19 with the facilitation parameter (χ) adjusted to 0.4 μm−1 to reproduce the initial facilitation observed here with an extracellular Ca2+ concentration of 1.5 mM.

Individual experiments including depression during 60 Hz train stimulation and the recovery from depression were fitted with either models 1 or 2 as previously described. The fits were judged by a visual inspection for both individual experiments of the different genotypes as mean ± s.e.m. in Fig. 6b.

Confocal imaging. Larvae were dissected in ice-cold HL-3, fixed for 10 min using 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and blocked for 30 min in PBT (PBS with 0.05% Triton X-100, Sigma) containing 5% normal goat serum (Jackson ImmunoResearch). Preparations were incubated with primary antibodies at 4 °C overnight. After one short and three ×20 min washing steps, the films were incubated with secondary antibodies for 2 h followed another three washing steps. The samples were mounted in Vectashield (Vector Laboratories) for confocal imaging or kept in PBT for dSTORM measurements. Primary antibodies were used in the following dilutions: monoclonal antibody (mAb) BrpNic(2R) (1:250, provided by E. Buchner) and rabbit-GluRIID (1:2,000, provided by S.J. Sigrist). Alexa Fluor 488-conjugated mouse (Invitrogen) and Cy3-conjugated rabbit (Diana) antibodies were used at 1:250. Images were acquired with a Zeiss LSM5 Pascal confocal system (objective: 63×, numerical aperture 1.25, oil). For each set of experiments, all genotypes were stained in the same vial and imaged in one session. To estimate synapse numbers laser power was adjusted individually for each vial.

Brp punctae and GluRIID clusters (NMJ 6/7, segments A2, A3) were examined using ImageJ software (National Institutes of Health) in principle as previously described20. After background subtraction, a Gaussian blur (0.9 px s.d.) was applied to maximum z-projections of confocal stacks and masks were generated (threshold manually adjusted for 25 for Brp and 30 for GluRIID). After superimposing the masks on the original blurred image, spot detection and segmentation via the ‘Find Maxima’ operation was performed to extract particle numbers.

To estimate the number of release sites (N) per AZ, the modelling prediction of N (average value of both models) was divided by the number of AZs on muscle 6 identified in confocal images, that is, half of NMJ 6/7 (ref. 28).

Super-resolution imaging. The mAb BrpNic(2R) was used at a dilution of 1/2,000 to identify AZs. Goat anti mouse F(ab)2 fragments (A10534, Invitrogen) were labelled with Cy5-NHS (PA15101, GE Healthcare) according to standard coupling protocols given by the supplier. Purification of the conjugates was performed by use of gel filtration columns (Sephadex G-25, GE Healthcare). The degree of labelling was determined by absorption spectroscopy (Jasco) as 1.3 for studies of the CAZ ultrastructure and 1.3–1.5 for dilution experiments. Samples were stored in 0.2% sodium azide in PBS and for the experiments, Cy5-labelled secondary antibody was used at a concentration of 5.2 μg/ml.

For dSTORM imaging with Cy5, the sample was embedded in photoswitching buffer, that is, 100 mM mercaptoethylamine, pH 8.0, enzymatic oxygen scavenger system (5% wt/vol) glucose, 5 U ml−1 glucose oxidase and 100 U ml−1 catalase21 and mounted on an inverted microscope ( Olympus IX-71) equipped with an oil immersion objective (60×, numerical aperture 1.45, Olympus) and a low-illumination stage (IX2-NPS, Olympus)22. For excitation of Cy5, a 641-nm diode laser (Cube 640–100C, Coherent) was used. Telescope lenses and mirror were arranged on a translation stage to allow for switching between wide-field, low-angle/highly inclined thin illumination and total internal reflection fluorescence imaging22,62,63. Fluorescence light from Cy5 was detected by a dichroic mirror (650/700 nm) and a band- and long-pass filter (BrightLine 697/75, RazorEdge 647, Semrock), and imaged on an electron-multiplying CCD camera (EMCCD; Ixon DU989, Andor Technology). Additional lenses were used to generate a final camera pixel size of 107 nm. Fifteen thousand frames were recorded with a frame rate of 100 Hz at an irradiance intensity of ~5 kW/cm2. A 488-nm-narrow bandpass filter (488 LP, Coherent) and a polychromatic dichroic mirror (410/504/582/669, Semrock) were used. Fluorescence light from A488 was reflected by a dichroic mirror (630 DCXR, Chroma) and imaged on a second EMCCD camera equipped with a band-pass filter (HQ525/50, Chroma).

Goat anti mouse IgG labelled with A533 (A11002, Invitrogen) and A700 (A21036, Invitrogen) was used at a concentration of 6.25×10−9 M. The degree of labelling was determined as 2.0 (A700) and 4.5 (A533). Imaging of A532 and A700 by dSTORM was performed in PBS containing 100 mM mercaptoethanolamine, pH 8.3. Using appropriate filter sets (dichroic mirrors: 650 or 545/600; band-pass filter: BrightLine 647 or BrightLine 582/75, Semrock), the sample (650 nm) and a dichroic mirror (410/504/582/669, Semrock) and a band- and long-pass filter (BrightLine 697/75, RazorEdge 647, Semrock), and imaged on an electron-multiplying CCD camera (EMCCD; Ixon DU989, Andor Technology).

For the investigation of different motorneurons, a total of 963 (type Ib) and 579 (type Ia) AZs were identified via their area (300 px to infinity). For the comparative analysis, unspecific background labels exhibited equal localization numbers, were analysed and data (presented as mean ± s.e.m.) were acquired in two imaging sessions, each containing all three genotypes stained in the same vial.

The release probabilities (pvr for model 1, and k1,r for both models), resulting in three free parameters for each model. The remaining parameters were constrained to values previously estimated at the Drosophila NMJ19 with the facilitation parameter (χ) adjusted to 0.4 μm−1 to reproduce the initial facilitation observed here with an extracellular Ca2+ concentration of 1.5 mM.

Individual experiments including depression during 60 Hz train stimulation and the recovery from depression were fitted with either models 1 or 2 as previously described. The fits were judged by a visual inspection for both individual experiments of the different genotypes as mean ± s.e.m. in Fig. 6b.
threshold 0.08 counts). Within a dSTORM image, only selections with a size between 16 and 100 nm (10 px−1) and an ellipticity ≥0.95 were analyzed. The coordinates of localizations within a single selection were aligned to their centre of mass and a 2D histogram of all localizations (209,537 in total) was generated (binning: 4 nm × 4 nm). A 2D Gaussian function was fitted to this histogram (adjusted $R^2 = 0.995$). The s.d. of the Gaussian function ($\sigma_{Brp} = (\sigma_x + \sigma_y)/2$) was determined as $7.16 \pm 0.02$ nm and is stated as localization precision in this work (Fig. 1a,b). This value is comparable to the localization precision obtained with an alternative method based on nearest neighbour analysis24 (Supplementary Fig. 1).

For the investigation of CAZ-units, those structures were chosen that were not grouped together and were viewed en face, that is, with the AZ membrane perpendicular to the optical axis20. For the manual selection of CAZ-units, the genotypes were blinded.

To calculate the radial distribution (Fig. 4g), Mathematica 9.0 (Wolfram Research) was used to automatically calculate the centre of each chosen CAZ-unit as the centre of mass (that is, the average localization of all pixels of the CAZ-unit weighted with the pixel value). Subsequently, the distance of each pixel to the centre of mass was calculated. These distances were then binned, the pixel values were added to the corresponding bins and the values were normalized by the area of each radial slice. The resulting distributions were averaged across all chosen CAZ-units, resulting in mean and s.e.m. values for the radial distributions of each genotype.

Quantification of Brp protein densities. To estimate the number of Brp molecules per CAZ-unit, a number of parameters had to be considered. First, the mAb BrpNc82 specifically recognizes one epitope per Brp molecule. Second, it is unclear how many Cy5-labelled F(ab)2 fragments can bind to the primary mAb BrpNc82 and how many localizations can be expected per Cy5-labelled secondary antibody. Here, it has to be considered that the number of localizations detected per antibody can be strongly influenced by its nano-environment. Hence, the number of localizations expected for an individual labelled antibody should ideally be derived from measurements performed under identical imaging and buffer conditions in the same cellular environment, that is, within the CAZ. In order to derive quantitative values of Brp molecules from the localization data, we performed antibody titrations with (i) different dilutions of Cy5-labelled secondary antibody and a constant concentration of mAb BrpNc82, and (ii) different dilutions of mAb BrpNc82 and a constant concentration of Cy5-labelled secondary antibody.

To evaluate the localization patterns by a Cy5-labelled F(ab)2 fragment attached to Brp via mAb BrpNc82, the concentration of mAb BrpNc82 was kept constant (1/2,000, that is, experimental concentration) and the secondary antibody was diluted ($1, 1/2, 1/10, 1/100, 1/1,000, 1/10,000$ and $1/100,000$). Preparations were simultaneously stained with A488 goat anti mouse F(ab)2 fragments (A11017, Invitrogen) to warrant an overall constant secondary antibody concentration of $5.2 \times 10^{-8} M$ for epitope saturation (for example, 1: 100% Cy5 to 0% A488 and 1/1000; 0.1% Cy5 to 99.9% A488) and to enable the unequivocal identification of the CAZ at low Cy5 antibody concentrations (Fig. 3b). The epifluorescence signal (A488) was background subtracted, blurred and contrast enhanced to identify Cy5 localizations within the CAZ. NMs (8–10) were evaluated for each dilution and the localizations per CAZ were histogrammed and fit to a Poisson model in order to extract the average number of localizations per CAZ ($L_{CAZ}$) as the mean of the distribution. $L_{CAZ}$ as a function of the Cy5 antibody dilution ($l$) was then approximated with the logistic function

$$L_{CAZ} = L_0 + (L_1 - L_0)/(1 + (d/C_0)^{-p})^{-1}$$

where the lowest localization value ($L_0$) is equivalent to the number of localizations corresponding to a single F(ab)2 fragment attached to Brp via mAb BrpNc82 ($L_1$ is the maximum localization value, $p$ is the Hill coefficient, which was fixed to 1; Fig. 3b). Such titrations are not equivalent to the number of localizations corresponding to a single F(ab)2 fragment

$$L_{CAZ} \approx L_1 (1 - e^{-d/C_0})^{1/p}$$

$$L_{CAZ} \approx L_1 (1 + d/C_0)^{-1/p}$$

$$L_{CAZ} \approx L_1 (1 - d/C_0)^{-1/p}$$

This gives a measure of the number of Brp molecules expected per CAZ-unit.

Cluster analysis. For the analysis, we used a home-written density-based algorithm. The base of the algorithm comes from the known algorithm, density-based spatial clustering of applications with noise: this algorithm simply looks for localizations that reside within the middle of a circle of radius $Eps$ and enclose at least $k$ other localizations25,26. Since our data contains a large number of localizations, in between putative clusters, we added more constraints on cluster detection. The algorithm starts with finding local maxima of density. The density is defined as the number of localizations within an Eps radius circle around a localization (Eps-environment). Each local maximum that has a density that is more than $k$ will be defined as a cluster centre. For each cluster centre, the localizations contained within its Eps-environment are examined for holding the condition of $k$ localizations. When the condition is held, the current localization along with all the other localizations within the Eps-environment will become members of this cluster. The algorithm then moves on to another localization that was found within the circle of radius $Eps$ from the cluster centre and examines if it holds the conditions. If not, this localization will be a boundary point for the cluster and the expansion will end. If it does, this localization will be considered a core-object of the same cluster, and the cluster will keep expanding until it reaches a boundary point. In addition to the above conditions, each localization added to the cluster should have a lower density than the localization that discovered it. If the algorithm detects an increase in density, this localization will not be part of the previous cluster. This separates adjacent clusters and prevents creating saddle points between them.

We chose a search Eps of 20 nm that roughly corresponds to the estimated radius of an EM filament with the antibody complex attached to it. For a chosen Eps, the density is calculated as the number of localizations within the Eps-environment of the current localization. The parameter $k$ was chosen based on the density distribution that had a peak ~12–18, and $k$ was large enough to separate from noise but not too large as to find a sparse number of clusters ($k = 16$).

After defining the clusters and the non-clustered localizations, we set to examine a set of different parameters that characterize the clusters. We analysed the following cluster properties: (a) the number of localizations belonging to each cluster, and (b) cluster shape and area. We found that most clusters did not show an exact circular shape but were more elliptic. Therefore, a 2D ellipse was tightly fitted to each cluster. From this ellipse, the parameters: shape (minor radius divided by major radius), minor and major axes are calculated. Clusters were defined as comprising a CAZ-unit if the density was at least 4 clusters within a circle of radius 200 nm.

Statistics. Statistical tests were used as indicated. In the figures, the level of significance is denoted by asterisks: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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Author contributions
A.D., M.H., M.S. and R.J.K. designed; and D.L., N.E., S.v.d.L. T.H. and X.Z.K. performed the experiments. A.A., A.R., M.S., N.E., S.H., R.J.K., S.v.d.L., T.H., U.A. and X.Z.K. evaluated the data. R.J.K. and M.S. supervised the project and wrote the manuscript with N.E. and contributions from A.A., A.D., A.R., M.H., S.H., S.v.d.L. and U.A.

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