An active zone state switch concentrates and immobilizes voltage-gated Ca^{2+} channels to promote long-term plasticity

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Abstract

A molecularly diverse spectrum of plasticity mechanisms orchestrates brain information processing and storage via positive (“Hebbian”) and negative (“homeostatic”) feedbacks, which, however, mechanistically converge and functionally interact *in vivo*. The presynaptic scaffold proteins that orchestrate active zone (AZ) function undergo plastic remodeling to regulate release potentiation. Here, voltage-gated Ca^{2+} channel function and their exact AZ nanoscale distribution steer release, although how they are involved in AZ remodeling remains unknown. We here establish intravital, dynamic, single-molecule imaging of endogenously tagged Ca^{2+} channel Cacophony (Cac) at *Drosophila* AZs triggered towards homeostatic potentiation. At potentiating AZs, Cac channel numbers increased, and their mobility decreased, while their overall distribution compacted. Mechanistically, RIM-1 and RimBP proteins and their conserved bindings sites, within the Cac channel's C-terminus, were dispensable for Cac immobilization and compaction. Conversely, the absence of ELKS-family homolog Bruchpilot precluded Cac immobilization and compaction. We show that AZs can undergo a state switch, likely via the ELKS scaffold to concentrate and immobilize Ca^{2+} channels and thus boost release.

Introduction

Synapses are key sites of information processing and storage in the brain. The synaptic transmission strength is not hardwired but adapts during synaptic plasticity to provide adequate input-output relationships, to maintain or restore transmission when compromised and to store information^{1–7}. Mechanisms of long-term (more than seconds) postsynaptic plasticity have been extensively studied, and processes targeting postsynaptic neurotransmitter receptors have been convincingly connected to learning and memory processes^{6,7}. Presynaptic long-term plasticity, however, is also prominent, not only at hippocampal mossy fiber bouton synapses, but also at many other synapse types⁸. However, as we still lack a coherent understanding of how long-term presynaptic plasticity manifests itself at the molecular level, analyzing its computational and behavioral role remains challenging. Ca^{2+} channels at the presynaptic membrane are the fundamental and conserved triggers of evoked synaptic vesicle (SV) release and modulate SV release probability through the precise nanoscale “coupling” between Ca^{2+} channels (within 10–200 nm around the active zone (AZ) center) and release-ready SVs. Indeed, AZ scaffold proteins can cluster Ca^{2+} channels with nanoscale precision to release sites within the membrane.

A remarkable and adaptive form of presynaptic plasticity is homeostatic plasticity. Homeostatic plasticity is observed from invertebrates to humans, and actively stabilizes synaptic transmission in response to neural activity perturbation. Notably, homeostatic mechanisms control both presynaptic and postsynaptic function and likely functionally intersect with Hebbian plasticity mechanisms in stable information encoding such as learning and memory^{9,10}. Meanwhile, elucidating the conserved mechanistic basis of presynaptic homeostatic plasticity at the highly-accessible neuromuscular junction (NMJ) of *Drosophila* larvae has provided important insights¹¹. Here, presynaptic homeostatic potentiation (PHP) can be acutely triggered by blocking postsynaptic glutamate receptors with Philanthotoxin (PhTx). Within minutes, PHP precisely counterbalances the reduced postsynaptic responsiveness through enhanced neurotransmitter release, involving both increased release probability for docked SVs at existing release sites and the addition of functional release sites at AZs. During PHP, Ca^{2+} influx increases, and image analysis based on conventional confocal microscopy suggests that additional Ca^{2+} channels are physically recruited to pre-existing AZs at NMJ synapses. Moreover, confocal imaging studies suggests that Unc13A and BRP levels also increase during PHP, implying that additional release sites might get recruited at pre-existing AZs. Notably, at the AZs of plastically remodeling rodent mossy fiber boutons, addition of release sites and accumulation of additional Ca^{2+} channels were also found to promote functional potentiation. Despite such progress, the exact molecular mechanisms by which AZ scaffolds intersect with Ca^{2+} channels to mediate the complex mechanism of PHP still remain enigmatic. In this study, we explore the *in vivo* dynamics of individual Ca^{2+} channels at AZs undergoing homeostatic remodeling over half an hour. We present evidence that the AZ scaffold, specifically via its BRP/ELKS core component, can trigger the compaction and immobilization of Ca^{2+} channels specifically to drive stable expression of plastic potentiation.

Results

Quantifying the *in vivo* mobility of individual Ca^{2+} channels at *Drosophila* active zones

Cacophony (Cac; Dmca1A), the only *Drosophila* Ca_v2 related voltage-gated Ca^{2+} channel is essential for evoked release, equally at NMJ but also central *Drosophila* synapses, which means it represents the collective function of mammalian N- and P/Q-type Ca^{2+} channels in this model. We, here, investigated the *in vivo* dynamic distribution of individual Cac channels at remodeling NMJ AZs undergoing potentiation using single-molecule imaging techniques.

In a recent study, “on-locus” tagging of Cac with GFP (CacsfGFP) close to its intracellular N-terminus was shown to render Cac fully functional, conferring normal transmission and PHP at NMJs. We started by subjecting the CacsfGFP channel distribution at fixed larval NMJs to STED super-resolution imaging. Under STED, CacsfGFP typically formed a single discrete cluster within a STED resolved ring-like structure of the AZ scaffold protein BRP, which aligns with a postsynaptic glutamate receptor field as expected. The membrane-proximal BRP N-terminus, again as expected directly overlay the Cac clusters, and in turn was surrounded by a STED resolved RIM-1 binding protein (RimBP) ring (Fig. 1A-E). Quantification revealed that AZ Cac channel clusters on average spanned 83.3 ± 3.5 nm and 47.4 ± 2.0 nm along their longest and shortest diameters (Fig. 1E). The STED signal deduced positioning and sizing of CacsfGFP clusters within AZs, deduced from STED imaging, concur well with previous observations^{12–14} and form a frame for our further investigations.

We intended to study the behavior of individual molecules Cac channel molecules within AZs. Thus, to employ live single particle tracking via photoactivation localization microscopy (sptPALM)^{15,16}, we exchanged the GFP for a mEOS4b tag, resulting in CacmEOS4b¹⁷. CacmEOS4b flies were fully viable (note that Cac null mutants are embryonic lethal^{18,19}). We then employed sptPALM to follow functional Cac channels in AZs over time. For this, we modified a preparation described previously²⁰, allowing for the use of the oblique illumination profiles of a TIRF microscope to induce stochastic blinking of individual

CacmEOS4b molecules in a focal plane across several boutons of a given NMJ (Fig. 1F-J, Suppl. Fig. 1A-C). Time windows of observation were both limited by the bleaching of the fluorophore, which was irreversible after 3-5 minutes of continuous illumination.

To identify functional synaptic contacts, we monitored activity at NMJs by employing the Membrane-tethered Ca^{2+} sensor Syn-GCamp6f in the postsynaptic muscle of CacmEOS4b animals (Suppl. Fig. 2). Syn-GCamp6f thus reports Ca^{2+} entry through postsynaptic receptors, activated in response to spontaneous presynaptic transmitter release. Notably, frequencies of postsynaptic Ca^{2+} signals were stable over a period of 30 minutes (Suppl. Fig. 2M). This implies that the quick and persistent loss of CacmEOS4b blinking signals did not result from drastic changes in the synaptic properties but rather reflects a limited population and a low turnover of Cac channel molecules within the AZs. For further analysis, we focused on the fluorescent signals of CacmEOS4b whenever robust characteristic blinking behavior indicated the excitation of individual fluorophores and the stability of the focal plane. At an acquisition frequency of 20 Hz, signal intensities were sufficient to gain an average localization accuracy of ~ 30 nm for individual Cac molecules (Suppl. Fig. 1D).

To gain access to the local distribution and dynamics of individual Cac channels, we first localized individual events and reconnected consecutive detections to create single channel trajectories (see materials and methods for details). We selected trajectories longer than 8 points (400ms), as suitable for direct extraction of both, Cac channel dynamic properties and radius of confinement by calculating of their mean square displacements (MSDs) (Fig. 1L-M).

Here, individual Cac channels were confined but not static. From trajectories that were longer than 8 time points, we calculated a median diffusion coefficient (diff.-coeff.) of $0.0075 \mu\text{m}^2/\text{s}$ and from the mean square displacement (MSD) analysis we observed that on average Cac channels were distributed within an average confinement radius of 120 nm (Fig. 1M). Our data show mobile and immobile Cac channel trajectories within the AZs. Here, the immobile fraction of Cac channels comprised about 25% of the channels while 75% of channels stayed mobile but confined over the observation time window (Fig. 1K).

To further characterize the biophysical milieu in which Cac channels operate at AZs, we applied statistical methods to include all trajectories as short as two time points, increasing the statistical power of the analysis. As a result, we extracted additional parameters, such as the drift field and attraction energy for Cac channels confined within the AZs (Suppl. Fig. 3G). We here observed that the trajectories were mostly located in high-density regions ("energy wells"), which also resemble an elliptic Cac channel cluster geometry (long axis 145 nm, short axis 95 nm) (Suppl. Fig. 3G, K), similar to the results observed by STED-imaging (Fig. 1A-E; N).

High- and low-density zones of Ca^{2+} channels in active zones

We find that the Cac channels in wild type synapses are strongly confined within the AZs (Fig. 1). This observation allowed us to further exploit the total population of Cac channel localizations, from the sptPALM imaging, to probe whether the channels would preferentially localize to specific "nanodomains" within AZs, as previously proposed for mammalian synapses^{21,22}. To this end, we employed Dual-Delaunay Triangulation Tessellation, a segmentation method to define local density distributions based on cumulative localizations²³. This method allowed identification of the preferred region within which the majority of Cac channel localizations in an AZ occur, defining a boundary for each AZ. We excluded AZs with diameters larger than 600 nm (Fig. 1J, N). Furthermore, the tessellation process also robustly retrieved a sub-region within the AZ clusters, wherein a significant increase in localization density was determined. Henceforth, we call these AZ-central areas "nanoclusters" (Fig. 1J, N). In addition, we employed further filtration parameters similar to the filtering and segmentation parameters of AZ area limits and localizations (for details see materials and methods), to those previously reported in super resolved localization microscopy of scaffold protein BRP at *Drosophila* NMJ AZs^{13,14}. Employing these strict parameters, we robustly found AZ borders of the best in-focus AZs and found that these AZs typically form a single nanocluster in their center.

The Cac channels localizations formed AZ clusters of about 160 nm diameter with a central nanocluster of about 60 nm diameter (Fig. 1N-O). On average, the Cac nanoclusters harbored about 50% of all Cac localizations found within an AZ (Fig. 1N-O). PALM-based localizations of the CacmEOS4B depicts Cac nanocluster diameters of ~ 60 nm, resembling results of previous STED based quantifications²⁴. In addition, the diameter of Cac nanoclusters derived from tessellation analysis of our sptPALM data also falls within the size range of 50 to 90 nm, obtained from nanobody-based detection by 2D STED microscopy at a lateral resolution of 40 nm (Fig. 1A-E). STED based quantification of Cac channel cluster size thus obviously "emphasizes" the central AZ nanocluster derived from our sptPALM data. In addition, these results concur with previous STED data which determined a Cac enrichment zone size of about ~ 70 nm in the AZ center, which is the nanoscopic spacing between SV release sites marked by Unc13A release clusters and Cac channels, and thus ensures the high release probability at NMJ and also central *Drosophila* synapses^{25,26}. Notably, the Ca^{2+} channel cluster sizes observed here are similar to those previously measured in rodent hippocampal synapses by immunohistochemistry, gSTED (~ 70 -100 nm) and electron microscopy²⁷⁻²⁹.

Ca^{2+} channel numbers increase, mobility decreases and distribution compacts in the course of presynaptic homeostatic plasticity

As mentioned above, *Drosophila* NMJ AZs can be triggered towards PHP within minutes by the application of the glutamate receptor antagonist Philanthotoxin (PhTx)^{11,30}. We triggered PHP in CacmEOS4 larvae by application of 50 μM PhTx for 10 minutes in the bath solution, before transferring the larva into the imaging chamber containing fluorescent horseradish peroxidase (HRP)-antibody in imaging buffer to outline the NMJ for better orientation during imaging. After 5 minutes, the animals were washed with plain imaging buffer to remove unbound label. We began live sptPALM imaging of Cac channel populations at NMJ I_b boutons (muscle 4, 6, and 7) 15 minutes after the start of the PhTx application. Of note in this regard, PhTx treatment was shown previously to irreversibly block postsynaptic GluRIIA receptors^{31,32}. Thus, we here intentionally analyzed the behavior of individual Ca^{2+} channels within the time window of upcoming PHP expression, which means after 20 minutes of the pharmacological glutamate receptor blockage (Fig. 2A-H).

In addition, we also counted the relative Cac channel molecule numbers per AZ, at control as well as at plastically remodeling AZs from the same sptPALM data. Here, we analyzed the bleaching behavior of the CacmEOS4b signals at individual AZs upon steady illumination. Discrete bleach steps were identified, which obviously indicate the bleaching of individual mEOS4b tagged Cac channel fluorophores. Under control conditions, about nine channel molecules were

measured within an AZ (9.2 ± 0.5). In contrast, after PhTx treatment, on average twelve channels per AZ were measured (12.2 ± 0.8) (Fig. 2I-K). This analysis thus provides direct single molecule derived evidence that the numbers of Ca^{2+} channels per AZ increase upon PhTx treatment, consistent with previous interpretations based on confocal imaging and Ca^{2+} imaging^{31–33}. We would like to emphasize that this approach does not depend on the use of antibody labeling necessarily confounded with issues of inhomogeneous labeling or limitation in the accessibility of the relevant epitopes. Still, the Cac channel numbers retrieved might well represent an underestimation of the total channel number, as we extracted these relative channel numbers from the live imaging sptPALM data with limited control over fluorophore conversion.

These sptPALM data were further used to compare the dynamics and distribution of Cac channels of animals undergoing PhTx-triggered plasticity to controls (see details in materials and method). We first analyzed the Cac channel dynamic behavior. Notably, we found that our PhTx treatment reduced Cac channel mobility, evident in a profound decrease of the median diffusion coefficient (PhTx treated channels median diffusion coefficient: $0.0027 \mu\text{m}^2/\text{s}$; untreated controls: $0.0074 \mu\text{m}^2/\text{s}$) (Fig. 2L). In addition, the mean radius of confinement of Cac trajectories was slightly but significantly decreased for PhTx treated compared to the untreated animals (Control- 113 ± 0.6 nm S.E.M, PhTx treated 107 ± 0.5 nm S.E.M) (Fig. 2M). Thus, the PhTx treatment seemingly promotes an immobilization and confines the spread of Cac channels within the AZ membrane during PHP.

We next employed tessellation analysis to calculate the spread and densities of Cac channels within the broader AZ cluster and the central nanocluster from Cac channel PALM localization data obtained from PhTx treated or untreated animals (Fig. 2C-H & N-Q). Upon analysis of these AZs, we found that within AZ cluster boundaries, overall diameters significantly decrease (10%) and their channel localization densities significantly increased (21%) in PhTx treated animals compared to controls (Fig. 2N-O). Similarly, analysis of the localization within nanocluster boundaries of PhTx treated animals also displayed a significant increase (16%) in Cac channel density, while the diameter of nanocluster clusters significant decreased by about 8% (Fig. 2P-Q), when compared to controls. This suggest that the high-density, central nanocluster of Cac channels gets further compacted during PHP.

Thus, taken together the PhTx treatment seemingly promotes an immobilization and “compaction” of Cac channel distributions within minutes at the level of the individual AZ.

RIM-1/RimBP binding is dispensable for Ca^{2+} channel immobilization and compaction

We asked how this in-AZ distribution and compaction of Cac channels could be molecularly orchestrated, and whether AZ scaffold proteins might contribute to this process. Ca_v2 class voltage-gated Ca^{2+} channels (including Cac channels) bind to the AZ scaffold via their conserved binding motifs within their intracellular C-termini bind^{24,34–39}, and form contacts to RIM-1 and RimBP. These two highly conserved multidomain proteins are responsible for orchestrating SV release and organize the functional SV release site architectures across evolution⁴⁰. To our surprise, in both mutants, Cac channels showed significantly lower diffusion coefficients compared to controls, indicating that Cac channel mobility at these AZs is indeed reduced, with effects being more pronounced for *RimBP* mutants (Fig. 3I, Control: $0.0071 \mu\text{m}^2/\text{s}$ vs *RimBP*^{-/-} $0.0037 \mu\text{m}^2/\text{s}$; Suppl. Fig. 4I Control: $0.0068 \mu\text{m}^2/\text{s}$ vs *RIM-1*^{-/-} $0.0044 \mu\text{m}^2/\text{s}$). Thus, neither RimBP nor RIM-1 proteins *per se* seem essential for stable AZ anchoring of Cac channels here. At the single AZ level, the radius of Cac confinement was also significantly reduced at the AZs of both mutants (Fig. 3J, Suppl. Fig. 4J). Finally, nanocluster sizes of both *RIM-1* and *RimBP* mutant AZs were significantly reduced as well, with again loss of RimBP displaying the stronger phenotype (Fig. 3M and Suppl. Fig. 4M). These changes resulted in increased Cac localization densities within their AZs and nanocluster boundaries, again particularly pronounced for *RimBP* mutants (Fig. 3L,N and Suppl. Fig. 4L,N). Similar trends in the distribution of Cac channel localizations within the AZ boundary were also observed in the background of *RIM-1*^{-/-} (Suppl. Fig. 4K-L) and *RimBP*^{-/-} mutant backgrounds (Fig. 3K-L).

Thus, instead of increasing Cac motility, as might be expected, lack of RimBP and RIM-1 surprisingly provoked a “pre-compaction” of Cac distributions “already” in the absence of PhTx application.

Other mechanistic means, rather than the individual binding of RimBP or RIM-1 to Cac channels, might be at play here, particularly when considering the severe release deficits characterizing both mutants. Thus, in the background of *CacmEOS4b*, we specifically disrupted, in the background of *CacmEOS4b*, the conserved RIM-1 and RimBP binding sites within the C-terminal region of the Cac channels, allowing us to directly study the role of both the RimBP and RIM-1 binding sites. To do so, we eliminated the C-terminal last six amino acids (to interfere with binding of the RIM-PDZ domain) and introduced a double point mutation (PPTP to APTA at the 2nd PxxP motif within the Cac C-terminus at amino acid positions 1690-1693) to disturb Cac/RimBP interaction^{39,34,41} (Fig. 3O-T, Suppl Fig. 5A). The on locus engineered Cac channel mutant (“*Cac-APTA-Δlast6aa*”) still established nearly normal baseline transmission and displayed normal quantal content levels (Suppl Fig. 5B-M; 5E). Following suit, the dynamic localization and distribution parameters of individual Cac channels derived from the live sptPALM data obtained at these mutant AZs were essentially identical to controls (Fig. 3O-T). In detail, neither the in-AZ mobility, nor the nanodomain organization were different for the on-locus engineered *Cac-APTA-Δlast6aa* mutant channels. In short, consistent with our results analyzing *RimBP/RIM-1* mutant AZs (Fig. 3 and Suppl. Fig. 4), the discrete “classical” binding motifs connecting Ca_v2 class voltage-gated Ca^{2+} channels with these scaffold proteins are obviously dispensable for AZ tethering and immobilization in this context.

The BRP/ELKS scaffold mediates Ca^{2+} channel immobilization in homeostatic plasticity

We went on by genetically analyzing the compaction process by taking a Cac channel perspective. Recent studies demonstrate that the distal C-terminus interaction surface of Ca^{2+} channel harbors a significant number of channel-scaffold interactions, and some have been shown to mediate Ca^{2+} channel localization in cultured rodent neurons⁴². Thus, we generated an on-locus Cac channel C-terminal deletion mutant that removes the last 160 amino acids of the C-terminus (*Cac-Δlast160aa*) (Fig. 4 and Suppl Fig. 5A). To evaluate the functional consequences of this deletion, we performed TEVC recordings in mutant and control animals. The *Cac-Δlast160aa* mutants, different from cognate controls, suffered from a severe reduction of evoked response, and consequently had significantly lower quantal content levels compared to controls (Suppl Fig. 5B-M; 5E). We then performed live sptPALM imaging on *Cac-*

Δlast160aa mutant NMJs, and analyzed their dynamics, distribution, and relative channel numbers (Fig. 4A-H, I-N and O respectively). Our subsequent analysis of channel dynamics from live sptPALM imaging revealed a significantly higher Cac mobility (Fig. 4I: median diffusion coefficient of $0.0163 \mu\text{m}^2/\text{s}$ for *Cac-Δlast160aa* compared to $0.0062 \mu\text{m}^2/\text{s}$ for controls) and a significantly larger radius of confinement (Fig. 4J: Control: $111.5 \pm 0.9 \text{ nm}$; *Cac-Δlast160aa*: $155.8 \pm 1.7 \text{ nm}$). Tessellation analysis of Cac localization distribution within the AZ cluster and nanocluster boundaries showed that the *Cac-Δlast160aa* channels appear dispersed over a larger area as nanocluster diameters were also significantly larger (Fig. 4K,M), while the localization densities within AZ- and nanoclusters reduced concomitantly (Fig. 4L,N). In addition, bleach curves analysis of *Cac-Δlast160aa* PALM data uncovered a significant reduction in channel numbers in *Cac-Δlast160aa* mutants compared to controls (Fig. 4O).

Collectively, the distal 160 amino acids of the Cac C-terminus are thus obviously critical to effectively tether Cac channels at the AZ presynaptic membrane, and densely pack them into the AZ central nanocluster. The remaining Cac channels at *Cac-Δlast160aa* AZs were fewer in number at mutant AZs and rendered atypically mobile.

At *Drosophila* AZs, BRP is a member of the AZ evolutionarily generic Cast/ELKS protein family and is a major building block of the NMJ AZ ultrastructural scaffold (also known as “T-bar”). Combinations of electron and super-resolution light microscopy have previously shown that a concentrated density of Cac channels clusters beneath the AZ center, i.e., the BRP-based T-bar, and that, additionally, the in-AZ Cac channels levels were reduced in the absence of BRP^{24,43}. Similarly, the reduction of ELKS proteins decreased the presynaptic Ca^{2+} influx at hippocampal synapses⁴⁴ and reduced the clustering of $\text{Ca}_v2.1$ channels at the Calyx of Held.

Previous yeast-two hybrid and pulldown assays revealed that a N-terminal BRP fragment (amino acids 1-320), which is conserved between ELKS family members, interacts with the Cac C-terminus. The BRP N-terminus in STED microscopy localizes considerably closer to the Cac channel accumulation when probed via a C-terminal fused GFP. We thus tested whether the distal 160 amino acids of Cac, which are critical for its AZ immobilization, would be critical for binding the BRP N-terminal region. Thus, we mapped the interaction site using yeast two hybrid (Suppl. Fig. 6). Binding is attenuated by deleting the last 60 amino acids from the Cac C-terminus ($\Delta 81\text{aa}$) and is further weakened upon removal of the last 130 amino acids from the Cac channel C-terminus (Suppl. Fig. 6). Thus, it appeared plausible that binding of the AZ membrane proximal N-terminal of BRP to the C-terminal of Cac is crucial for Cac channel immobilization. To further elaborate on this idea, we analyzed the dynamics of CacmEOS4b channels at *BRP* null mutant AZs by sptPALM (Fig. 5). Notably, individual Cac molecules were atypically mobile at *BRP* null mutant AZs and less confined than at control AZs (Fig. 5A-F, G-H). Cac channel mobility and radius of confinement were higher than in controls and, importantly, remained high even after PhTx treatment (Fig. 5G-H) (median diffusion coefficient: control: $0.0054 \mu\text{m}^2/\text{s}$; *BRP*^{-/-}: $0.0104 \mu\text{m}^2/\text{s}$; *BRP*^{-/-} after PhTx: $0.0104 \mu\text{m}^2/\text{s}$; radius of confinement: control: $113.9 \pm 1 \text{ nm}$ S.E.M.; *BRP*^{-/-}: $136.6 \pm 1 \text{ nm}$ S.E.M.; *BRP*^{-/-} after PhTx: $121.2 \pm 2 \text{ nm}$ S.E.M.). We would like to mention that here we used the same control data for both experimental conditions, *BRP*^{-/-} mutant and *BRP*^{-/-} mutant after PhTx, as these conditions were concurrently imaged in one experiment (Fig. 5A-H).

We also attempted to use the localization data from the sptPALM recording for Tessellation analysis of the *BRP* null mutant AZs. Here however, far fewer AZ and nanoclusters clusters could be retrieved per NMJ terminal area by the standard tessellation parameters (AZ clusters: minus 62% in AZs of *BRP* null mutant and *BRP* null mutant after PhTx treatment; NC clusters: minus 54% in AZs of *BRP* null mutant and 69% *BRP* null mutant after PhTx treatment compared to controls) (Suppl. Fig. 7). Given the exceptionally low numbers of remaining clusters that were quantifiable with the tessellation method, we refrained from this type of analysis in this situation.

We also analyzed CacmEOS4b bleach curves from the sptPALM imaging data to extract a measure for the average numbers of Cac molecules per AZ in *BRP* mutant AZs with and without PhTx treatment. Consistent with our previous work using cDNA derived overexpressed GFP-tagged Cac constructs^{24,35}, the endogenously tagged CacmEOS4B line displayed reduced numbers of Cac channels in *BRP* null mutants compared to controls (Fig. 5I). Moreover, there was no significant increase in Cac channel number upon PhTx treatment (Fig. 5I). Important in this regard, we recently found that BRP is crucial to sustain the expression of homeostatic potentiation beyond the initial induction period, while induction persists undisturbed. Concretely, BRP was fully dispensable for PHP at a 10-minute time interval, while its absence eliminated PHP measured after 30 minutes of PhTx application⁴⁵. Collectively, our data thus imply that the immobilization of Cac channels via BRP might be a critical process to drive the stable expression of PHP. This is interesting considering that BRP is essential for the AZ structural remodeling which sustains the PHP^{30,45} and that BRP “itself” has been shown to undergo PhTx-triggered compaction.

Taken together, our data suggest that within about 20 minutes, PhTx triggered plasticity can still trigger a certain accumulation of Cac channel even at AZs lacking BRP (Fig. 5I,J also see discussion), likely derived from extra-AZ sources. However, these additional channels then fail to stably anchor at the BRP-lacking AZs and were rendered atypically mobile, a state incompatible with PhTx plasticity beyond the initial induction period (Fig. 5G-H). This is interesting considering that BRP is essential for the AZ structural remodeling which sustains the⁴⁵ PHP and that BRP “itself” has been shown to undergo PhTx-triggered compaction.

Our analysis therefore suggests that the BRP scaffold is critical for mediating the Cac channel immobilization and compaction (Fig. 2L-Q), thus likely directly contributing to sustaining release increases to allow for stable PHP expression.

Discussion

Exact numbers, density, and nanoscale positioning of voltage-gated Ca^{2+} channels (VGCCs), within the AZ at the presynaptic membrane, are central for sensitively tuning AZ release function. Ca^{2+} channels seemingly get confined to specific positions within the presynaptic membrane^{28,29,46}, and AZ scaffold proteins have been shown to dynamically change on the minute timescale²⁹. To better understand presynaptic plasticity processes at the level of VGCC dynamics, we here exploited the *Drosophila* NMJs as a particularly well-suited model to establish quasi intravital imaging of individual Ca^{2+} channels at

remodeling AZs. A fundamental finding here is that the lateral dynamics of individual Ca^{2+} channels declines, in the context of homeostatic potentiation, accompanied by a compaction of the Ca^{2+} channels within the AZs, which coincides and most likely directly contributes to the sustained increase in presynaptic SV release. We also suggest BRP/ELKS as *the* critical scaffold protein mediating these changes in Ca^{2+} channel number, immobilization, and compaction, to promote these sustained release increases (stable PHP). We suggest this based on the following findings:

1. BRP is essential for Cac channel immobilization and compaction, regardless of PhTx application (Fig. 5);
2. BRP is essential for stable expression but not induction of PHP (Fig. 5);
3. the distal Cac C-terminus including the BRP binding region is essential for immobilization and compaction (Fig. 4 and 5)^{30,45};
4. BRP undergoes PhTx triggered compaction based on dSTORM analysis of fixed NMJs¹⁴.

In detail, we found Ca^{2+} channels to significantly slow their mobility at AZs when undergoing homeostatic remodeling (Fig. 2L). At the same time, their confinement area became “compacted”, evident in a decreased size of both AZ cluster and the AZ-central nanocluster (Fig. 2M, N, P). Consequently, the density of individual Ca^{2+} channels per unit area increased strongly, particularly in the center of the AZ (Fig. 2N-Q). In addition, here, the (relative) numbers of Ca^{2+} channels per AZ, estimated via bleach curves from *in vivo* sptPALM movies increased significantly (Fig. 2I-K). As mentioned above, during PHP, Ca^{2+} influx is increased, and previous confocal microscopic analyses have shown Cac channel increases during PHP. Biophysical analysis has suggested that SV release dominantly operates in a distance less than 100 nm from the AZ center, i.e., the nanocluster center^{12,47}. It thus appears most plausible that the increased Ca^{2+} channel density accounts for the PhTx-triggered increase of Ca^{2+} influx and consequently directly contributes to PHP expression. An increase in the total number of channels has been proposed as a first order mechanism^{11,33} assuming that Ca^{2+} channels will behave independently in respect to their voltage dependent activation. However, it has also been shown that Ca^{2+} channels can act cooperatively, depending on their proximity to each other and on their activation history^{48,49}. It is thus conceivable that the AZ and nanocluster compaction of Ca^{2+} channels within the AZ probably serves to promote channel-scaffold interactions in favor of more reliable Ca^{2+} signaling, particularly within the identified nanocluster of each AZ. The condensation of Cac channels within just a single nanocluster per AZ supports the idea that beyond the increased channel number, a change in channel density entails a cooperative modulation of Ca^{2+} channel activity with an impact on the PHP related increase of SV release. The compaction of Cac channels may further add to the isolation of Ca^{2+} influx dedicated to SV release from peri-AZ Ca^{2+} influx related to SV endocytosis⁵⁰. Due to the supra-linear dependence of the SV release on intracellular calcium concentration as shown for many synapses, even already the nanoscale change in Cac-channel organization can be very effective and work as fast feedback for acute activity changes. Several mechanisms can be envisioned to support and maintain the compaction of the central Ca^{2+} channel cluster over time, for example differences in local molecular crowding within the membrane prolonging the dwell time of channels in particular membrane domains. Also, specific interactions with particular intracellular scaffold proteins^{36,39,41,51} or through interactions with scaffold protein complexes by liquid-liquid phase separation are possible^{52–54} (see below).

An interesting result of our analysis is also that the absence of RimBP and RIM-1 rendered the AZs in a pre-compacted state of high Cac channel density and immobility. This is likely explained by the fact that these mutant AZs operate under conditions of chronically inefficient SV release and reduced Cac levels that likely renders the channel distribution into a compact state despite the absence of a PhTx treatment (Fig. 3). This result is now to be kept in mind when interpreting mutants interfering with PHP expression but also from evoked base line release deficits. BRP mutants, despite suffering from reduced evoked release as well (although not as severely as in RimBP mutants), are obviously unable to pre-compact given the role of BRP/ELKS as a “master scaffold for compaction”.

Our analysis showed that RIM-1 and RimBP with their structurally well defined, “punctate” Ca^{2+} channel C-term interaction motifs were dispensable for PhTx triggered Ca^{2+} channel immobilization measured via live sptPALM imaging. In contrast, the ELKS homologue BRP clearly is essential to serve as an AZ anchor and immobilize the Ca^{2+} channels, while additional Ca^{2+} channels are seemingly still trafficked to the presynaptic plasma membrane upon PhTx (Fig. 5I). However, PhTx treatment failed to trigger an in-AZ immobilization of Ca^{2+} channels when BRP was missing (Fig. 5A-H, J). Given that, BRP is essential for stable PHP expression (but not induction), these results further suggest that Ca^{2+} channels need to be stably anchored, at least to display their full impact in triggering SV release. The Ca^{2+} channel association with RIM-1 and RimBP in our intravital system is not crucial for stable *in vivo* Ca^{2+} channel anchorage at AZs, which is obviously essential for proper SV priming as shown for rodent synapses^{40,42,55}.

How then might BRP mediate this Cac channel compaction? Notably, a recent study using STORM analysis of fixed NMJs showed that the BRP AZ scaffold seemingly also undergoes a compaction-like process in response to PhTx, as suggested by measuring an increased nanoscale density of the C-terminal BRP epitope evaluated in this study¹⁴. The biophysical nature of BRP scaffold and its ability to undergo compaction, like Ca^{2+} channels, might contribute to a consequent connection to Ca^{2+} channels, which likely drives their distribution and compaction. Here, liquid-liquid phase separation (LLPS)-like processes are prime candidates in our eyes for the interaction of Ca^{2+} channels with the BRP/ELKS C-terminus. Recently, ELKS-type AZ scaffold proteins were found to undergo liquid-liquid-phase separation (LLPS), with “liquid” states promoting assembly but “rigid” states promoting release function^{52,54,56,57}. The relevance of such collective states changes for functional plasticity remained unexplored yet. Notably, extended regions of AZ proteins RIM-1, RimBP, ELKS/BRP, as well as the Ca^{2+} channel intracellular C-terminus were shown *in vitro* to form LLPS-like condensates^{52,54,56,57}. Given the apparently “collective nature” of this new form of AZ plasticity, relating its mechanisms to other state-switches, particularly LLPS like processes, should be a subject warranting future research.

Materials And Methods

Experimental Model and subject details

Fly husbandry, stocks, and handling

Fly strains were reared under standard laboratory conditions and raised at 25 °C on semi-defined medium (Bloomington recipe). For RNAi experiments flies and larvae were kept at 29 °C. For experiments male third instar larvae were used. The following genotypes generated in the wild-type background of CacmEOS4b were created as follows:

RimBP^{Null}: *RimBP*^{Stop1}/*RimBP*^{S2.01}; *BRP*^{Null}: *brp*^{Δ6.1}/*brp*⁶⁹; *RIM*^{Null}: *rim*^{ex1.103}/Df(3R)ED5785

Stocks were obtained from: *RimBP*^{Stop1}, *RimBP*^{S2.01}, *rim*^{ex1.103}; *brp*^{Δ6.1.24}, *brp*⁶⁹, SynapGCaMP6f (MHC-CD8-GCaMP6f-Sh)⁵⁹, lines were provided by the Bloomington *Drosophila* Stock Center.

***Drosophila* genetics and genome engineering:**

Endogenous expression of calcium channels and on-locus deletion mutants:

Fly strains were reared under standard laboratory conditions at 25°C and 65–70% humidity in incubators. Wandering third instar male larvae were used for analysis in all experiments, except when indicated differently. The endogenously tagged fly lines CacsGFP and CacmEOS4b were generated in the O'Connor Giles laboratory as previously described in Gratz et al., 2019³³. Briefly, N-terminally tagged Cac was generated using a scarless CRISPR/piggyBac-based approach (flyCRISPR.molbio.wisc.edu).

The following generated transgene animals based on CRISPR mediated mutagenesis were performed by Well Genetics (Taipei City, Taiwan) Inc. using modified methods of Kondo and Ueda (2013). The following transgene animals have been generated using the CRISPR/Cas9 mediated genome editing approach by homology dependent repair (HDR) using 1 guide RNA and a dsDNA plasmid donor:

CacmEOS4b-Δlast160AA:

We generated a deletion allele of the *Cac* gene by truncating its distal C-terminal sequence of the last 160 AA.

In brief, the gRNA sequence GACGGTTTGCTGGGAGTCGG[AGG] (same gRNA used for CacmEOS4b-APTA) and AGGAGGATTGGTGCTAGCAA[AGG] were cloned into U6 promoter plasmids. Cassette PBacDsRed containing two PBac terminals 3xP3 DsRed and two homology arms were cloned into pUC57 Kan as a donor template for repair. Cac/CG43368 targeting gRNAs and hs-Cas9 were supplied in DNA plasmids, together with a donor plasmid for microinjection into embryos of control strain CacmEOS4B/FM7a. F1 flies carrying the selection marker of 3xP3 DsRed were further validated by genomic PCR and sequencing. CRISPR generates a 734 bp deletion allele of Cac/CG43368, deleting the last 160 AA of Cac/CG43368 and is replaced by cassette PBacDsRed.

CacmEOS4b-APTA-ΔLast6AA:

In brief, the gRNA sequence AGGAGGATTGGTGCTAGCAA[AGG] was cloned into U6 promoter plasmids. Cassette PBacDsRed containing two PBac terminals and 3xP3 DsRed and two homology arms were cloned into pUC57 Kan as donor template for repair. cac/CG43368 targeting gRNAs and hs-Cas9 were supplied in DNA plasmids, together with donor plasmid for microinjection into embryos of control strain Cac mEos APTA CRISPR / FM7a. F1 flies carrying selection marker of 3xP3 DsRed were further validated by genomic PCR and sequencing. CRISPR generates a 21 bp deletion allele of cac/CG43368, deleting the last AA of cac/CG43368 and is replaced by cassette PBacDsRed.

Translation of CacmEOS4b-APTAΔ-last 6aa after excision of screening cassette:

The following 6 AA are deleted: DEEDWC

Nucleotides:

ACTGGAATATATAGCACCGCTGTCGTTTGAACAAGCGCTGGCTATGGGCCGAACAGGTCGTGTACTGCCATCTCCAGTTCTAAACGGTTTTAAGCCAAAGAGTGGTCTG/

Protein:

LEYIAPLSFEQALAMetGRTGRVLPSPVLNGFKPKSGLNPRHSDS Stop

Yeast-2 Hybrid Assay

In principle all experiments were conducted according to the yeast two-hybrid protocols of Clontech using the strain AH109 with minor adjustments meeting the requirements of the interaction-domain mapping experiments. Co-transformation of the yeast strain AH109 with both prey and bait constructs (Clontech) was conducted using the following protocol: The yeast was plated on an YPDA (yeast, peptone, dextrose, adenine) medium agar plate and cultured for two days at 30° C. An appropriate amount of YPDA medium was inoculated with a single clone and cultured at 30° C overnight while shaking at approximately 200 rpm. After dilution to OD600 0.2 (10 mL culture volume per transformation) the culture was grown until OD600 reached 0.6. The cells were pelleted, washed in 1xTE (Tris-EDTA)- medium at pH 8.0, re-pelleted and resuspended in TE/LiAc (100 µl per 10 mL culture volume; LiAc: Lithium acetate). 100 µl resuspended yeast were mixed with the transformation mix which was composed as follows, 500 ng of each of the two plasmids and 10 µl (= 100 µg) Herring Testes carrier DNA (Clontech). After adding 600 µl PEG (Polyethylene glycol)/LiAc, the mix was vortexed and left to incubate at 30°C for 30 minutes in a shaker. Then 70 µl DMSO (10 % final concentration) were added, and the cells were heat shocked for 15 minutes at 42°C. After chilling on ice, the yeast was

carefully sedimented, the pellet suspended in 100 µl 1xTE-medium pH 8.0, and the transformation plated on minimal SD (synthetic defined) /-Leu/-Trp medium plates, to ensure the presence of both plasmids. After growing for 2 - 3 days at least 10 clones each were analyzed on SD/ -Ade/ -His/ -Leu/ -Trp/ X-α-gal medium plates to select for positive interaction. If > 70% of the clones plated on SD/ -Ade/ -His/ -Leu/ -Trp/ X-α-gal medium plates grew, this was regarded as weak positive interaction (+), > 80% as Intermediate interaction (++) and > 90% and blue clones as strong interaction (+++). Negative controls consisted both of laminin as bait and the prey to be tested and the corresponding bait and the empty prey vector. In the positive control pGBKT7 p53 was transformed with pGADT7 containing the SV40 large T antigen.

The BRP D1 and Cac fragments depicted in Suppl 4 were cloned into pGADT7 using the following primer pairs:

Cac C-term_Fwd: 5' GATGCCATGG CG TTA TTC GCT TTG ATT CGT GAG 3'

Cac C-term_Rev: 5' GTCTCTCGAGTTA TCA GCA CCA ATC CTC CTC ATC 3'

CacΔ304 aa_Fwd: 5' GATGCCATGG CG TTA TTC GCT TTG ATT CGT GAG 3'

CacΔ304 aa_Rev: 5' CAAGCTCGAGTTATCA CAAGCTAGGCGATCGCCGATG 3'

CacΔ130 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ130 aa_Rev: 5' GTCTCTCGAGTTATCAGTGTGTATGTGTGGGACTGG 3'

CacΔ81 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ81 aa_Rev: 5' GTCTCTCGAGTTATCACAGGCGATCGCGATCCCTAT 3'

CacΔ60 aa_Fwd: 5' GATGCCATGG CG TTA TTC GCT TTG ATT CGT GAG 3'

CacΔ60 aa_Rev: 5' CAAGCTCGAGTTATCA TTCGCGTTCGTACAACTCTCGT 3'

CacΔ31 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ31 aa_Rev: 5' GTCTCTCGAGTTATCAACCTGTTGCGCCCATAGCCA 3'

BRP D1_Fwd: 5' GTCTATCGGAATTCATGGGCAGTCCATACTAC 3'

BRP D1_Rev: 5' GGAATTCATCGATGAGCTCGTCCTCTAGGTAC 3'

The BRP D1 and Cac fragments depicted in Suppl 4 were cloned into pGBKT7 using the following primer pairs:

Cac C-term_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

Cac C-term_Rev: 5' GTCTGTGCACTTATCAGCACCAATCCTCCTCATCCGAA 3'

CacΔ304 aa_Fwd: 5' GATGCCATGG CG TTA TTC GCT TTG ATT CGT GAG 3'

CacΔ304 aa_Rev: 5' GTCTGTGCACTAAGCTAGGCGATCGCCGATG 3'

CacΔ130 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ130 aa_Rev: 5' GTCTGGATCCTTATCAGTGTGTATGTGTGGGACTGG 3'

CacΔ81 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ60 aa_Fwd: 5' GATGCCATGG CG TTA TTC GCT TTG ATT CGT GAG 3'

CacΔ60 aa_Rev: 5' GTCTGTGCACTTATCA TTCGCGTTCGTACAACTCTCGT 3'

CacΔ31 aa_Fwd: 5' GATGCCATGGCGTTATTCGCTTTGATTCGTGAG 3'

CacΔ31 aa_Rev: 5' GTCTGGATCCTTATCAACCTGTTGCGCCCATAGCCA 3'

BRP D1_Fwd: 5' GTCTATCGGAATTCATGGGCAGTCCATACTAC 3'

BRP D1_Rev: 5' GGAATTCGTCGACGAGCTCGTCCTCTAGGTAC 3'

Immunofluorescence cytochemistry of larval NMJs

Dissections were performed following standard protocols⁶⁰ and are described here: Third instar larvae were opened in HL3 by opening dorsally along the midline and removing the innards. Filets were fixated with ice-cold MeOH for 10 min for all experiments to quench autofluorescence of sfGFP/mEOS4b to subsequently boost them with an antibody or nanobody booster, after testing all antibodies function optimally in MeOH. After fixation, the filets were washed with PBS plus 0.05% Triton X-100 and blocked for 60 min in 5% normal goat serum. The larvae were incubated with primary antibodies at 4°C overnight and subsequently washed in a PBS plus 0.05% Triton X-100 solution for 2 h at room temperature for immunostaining. Larvae were then incubated for 4h with secondary antibodies at room temperature followed by the same washing procedures. Immunocytochemistry was done the same way for both conventional confocal and STED microscopy except for the mounting medium. Larvae were finally mounted either in Mowiol (Sigma-Aldrich) for confocal microscopy or Prolong Gold (ThermoFisher-Scientific) for STED. The following antibodies were used at the indicated concentrations: mouse anti-Bruchpilot (Brp) Nc82/ BRP^{C-term} (DSHB, catalog #nc82; RRID:AB_2314866) and BRP^{N-term} (1:500⁶¹), rabbit RIMBP^{C-term} (1:500⁶²

); mEOS4B Nanobody coupled to atto580 or GFP nanobody coupled to STAR635P at 1:300 (Nanotag, Catalog N0304 or N3102), Rabbit or Guinea pig anti-GluRIIA (generated in house), and anti-HRP conjugated to AlexaFluor 405 at 1:200–1:500 (Jackson ImmunoResearch). Species-specific Alexa Fluor 488, atto490LS and Alexa Fluor 594 secondary antibodies (Invitrogen, Jackson ImmunoResearch) were used at 1:300.

Acute PHP/PhTx assay:

Third instar control and mutant larvae in CacsGFP/CacmEOS4b backgrounds were treated with a 10-minute PHP assay using Philantotoxin-433 drug (PhTx) (AOBIOs). Larvae were carefully pinned down at mouth and tail while avoiding any stretching of body wall muscles and minimally dissected in 50µl solution containing 50 µM PhTx in Ca²⁺ and Mg²⁺-free HL3 or No PhTx-HL3. Animal's hemolymph was mixed with the treatment solution by regularly mixing of the solutions during the 10-minute treatment window undertaken with care to not stretch or pull the larvae muscles. For live-sptPALM assays treated animals were moved into the imaging solution containing HL3 with 4mM Mg²⁺ (to reduce muscle contractions) and basal 1.5mM Ca²⁺.

STED imaging and analysis:

gSTED microscopy was performed using an Abberior Instruments Expert Line STED setup equipped with an inverted IX83 microscope (Olympus), two pulsed STED lasers for depletion at 775 nm (0.98-ns pulse duration, up to 80-MHz repetition rate) and at 595 nm (0.52-ns pulse duration, 40-MHz repetition rate) and pulsed excitation lasers (at 488 nm, 561 nm, and 640 nm).

Multi-channel 2D confocal and gSTED images were acquired with a 100× oil-immersion objective lens (UPLSAP0100XO, Olympus, NA = 1.4), with a pixel dwell time of 2 µsec, with 10x and 30x line accumulation, respectively, at 16-bit sampling and a field of view of 10 µm x 10 µm. Lateral pixel size was set to 20 nm. The dyes STAR 635P, Alexa Fluor 594 and ATTO490LS were depleted at 775 nm. Alexa Fluor 488 was depleted at 595 nm. Time gating was set at 750 ps with a width of 8 ns. Fluorescence signals were detected sequentially by line by avalanche photodiode detectors at appropriate spectral regions (STAR 635P and ATTO490LS: 680 nm–765 nm, Alexa Fluor 594: 584 nm–630 nm, Alexa Fluor 488: 500 nm–551 nm). Alexa Fluor 488 confocal and gSTED images were acquired following acquisition of the other channels. These procedures were operated by the software Inspector (version 16.3.13367, Abberior Instruments, Germany). Raw gSTED images were processed for Richardson-Lucy deconvolution with default settings using the Inspector software (version 16.3.13367, Abberior Instruments, Germany). The point spread function was automatically computed with a 2D Lorentzian function having a full-width half-maximum of 40 nm, based on measurements with 40 nm crimson beads. gSTED analysis to determine CacsGFP spot size (Feret's diameter) was performed by particle segmentation above the threshold signal with the function "Find maxima" in ImageJ (ImageJ-1.52g, NIH).

Electrophysiology

Two-electrode voltage clamp (TEVC) recordings were performed at room temperature on muscle 6 of 3rd instar larval NMJs in the abdominal segments A2 and A3. Male third instar larvae were dissected in modified Ca²⁺-free hemolymph-like saline (HL3; in mM: NaCl 70, KCl 5, NaHCO₃ 10, MgCl₂ 20, Sucrose 115, Trehalose 5, HEPES 5) Recordings were obtained with a bath solution of HL3 with 1.5 mM CaCl₂. Recordings were made from cells with an initial V_m between -50 and -80 mV, and input resistances of ≥ 4 MΩ, using intracellular electrodes with resistances of 30-50MΩ, filled with 3 M KCl. Glass electrodes were pulled using a Flaming Brown Model P-97 micropipette puller (Sutter Instrument, CA, USA). Recordings were made using an Axoclamp 2B amplifier with HS-2A x0.1 head stage (Molecular Devices, CA, USA) on a BX51WI Olympus microscope with a 40X LUMPlanFL/IR water immersion objective (Olympus Corporation, Shinjuku, Tokyo, Japan). mEJCs were recorded for 90 seconds with the voltage clamped at -80 mV, all other recordings were performed while clamping the voltage at -60 mV. eEJCs were recorded after stimulating the appropriate motor neuron bundle with 5 V, 300 µs at 0.2 Hz using an S48 Stimulator (Grass Instruments, Astro-Med, Inc., RI, USA). Signals were digitized at 10 kHz using an Axon Digidata 1322A digitizer (Molecular Devices, CA, USA) and low pass filtered at 1 kHz using an LPBF-48DG output filter (NPI Electronic, Tamm, Germany). The recordings were analyzed with pClamp 10 (Molecular Devices, Sunnyvale, CA, USA), GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Stimulation artifacts of eEJCs were removed for clarity. mEJCs were further filtered with a 500 Hz Gaussian low-pass filter. Using a single template for all cells, mEJCs were identified and averaged, generating a mean mEJC trace for each cell. An average trace was generated from 20 eEJC traces per cell for 0.2 Hz stimulation and 10 ms ISI paired pulse recordings and from 10 traces for

30 ms ISI paired pulse recordings. Rise time was calculated from the average trace of the 0.2 Hz stimulation recording as the time from 10% to 90% of the total amplitude before the peak. Decay constant τ was calculated by fitting a first order decay function to the average trace of the 0.2 Hz stimulation recording starting from 60% of the total amplitude after the peak until the baseline was reached. The amplitude of the average eEJC trace from the 0.2 Hz stimulation recording was divided by the amplitude of the averaged mEJC, for each respective cell, to determine the quantal content. 10 ms and 30 ms ISI paired pulse ratios were calculated by dividing the amplitude after the second pulse by the amplitude after the first pulse. The baseline for the second amplitude was set at the last point before stimulation artifact onset.

Single-particle tracking PALM

All live sptPALM experiments were conducted on male third instar larvae. Larval body walls designated for single particle tracking were prepared according to Ramachandran and Budnik 2010 and Marter et al., 2019^{20,63} and imaging experiments were performed using an inverted total internal reflection fluorescence (TIRF) setup. The microscope (Nikon Eclipse Ti) was equipped with a 100x NA 1.49 Apo TIRF oil objective (Nikon). Up to 10,000 images were captured using a EMCCD camera (iXon+ 897, Andor Technology) controlled by NIS-Elements (Nikon) at a frame rate of 20 Hz. The TIRF set-up is based upon an inverted microscope within a whole microscope incubator at 25°C. (eclipse Ti microscope, Nikon GmbH) was equipped with a 100 x Apo TIRF oil objective (1.49 NA; Nikon). CaeMOS4b containing larvae were imaged at the live HRP-488 stained Z-plane. Briefly, we used laser diodes to photoconvert and excite the mEOS4b fluorophore by continuous illumination of the probe with a 405 nm laser (2–5 % of 100 mW) and a 561 nm laser (25% to 40% of 100 mW), and to further improve the separation of the mEOS signal from auto fluorescence and background signals, an emission bandpass filter (ET620/60 nm; *AHF analysentechnik*) was used. Fluorescence was excited by oblique illumination of the probe with a combined laser system (Coherent; MPB communications Inc.) and image sequences (up to 10000 frames) were captured by an EMCCD camera (iXon+ 897, Andor Technology) controlled by NIS-Elements Advanced Research acquisition software (Nikon). Images were recorded at a frame rate of 20 Hz, controlled by MetaMorph imaging software (*Universal Imaging*). We used a 1.6 magnification lens to reduce the pixel size to 107×107 nm. The N-terminal expressed mEOS4b on the Cacophony channel was excited by continuous illumination with 405/561 nm light using 2-5% of the initial laser power of the 405 nm laser line (100 mW) and 25-40 % of the 561 nm laser line (100 mW) for up to 10000 frames at 50 ms acquisition rate.

Dissection chambers were assembled by gluing a magnetic foil (7.4 cm length, 5.4 cm width, 2 mm thickness) to an equally sized glass plate, both (rather than just the foil) with a central hole of 2 cm diameter. The hole was closed by a cover slip of 3.2 cm diameter and 0.17 mm thickness, glued underneath the glass plate, and the inner edge was sealed with silicon. Larvae were placed dorsal side up into the chamber and clips consisting of stainless pins with tin-made holders on the magnetic foil were used to clamp the animals at either end. Dissections were then carried out as described in ^{20,63} Ca²⁺ and Mg²⁺ free HL3.1 saline (in mM: NaCl 70, KCl 5, NaHCO₃ 10, Sucrose 115, Trehalose 5, HEPES 5). Subsequently, the larvae were washed briefly in HL3.1 imaging buffer containing 4 mM Mg²⁺ and 1.5 mM Ca²⁺ for all control situations and/or 0.2 mM for the experiment in Suppl. Fig. 2, to remove debris of innards. Next, the larvae were live stained in a non-stretched position in the imaging buffer solution containing Alexa 488-conjugated anti-horseradish peroxidase antibody (1:1000), serving as a surface marker to visualize the outline of NMJs during subsequent imaging, for 5 minutes. The larvae were briefly washed in the HRP-free imaging buffer to remove excess HRP label. Motor nerves were cut close to the ventral nerve cord and the brains were removed to ensure reduced muscle contractions during live imaging. Clamped at the tip, body walls were turned around such that the muscles were facing the cover slip of the imaging chamber. Samples were straightened carefully using six clips and subjected to TIRF illuminated PALM imaging. An optimal imaging focal plane was chosen, guided by the HRP signal, within which the highest number of boutons of one type 1b NMJ (from segments A2-A4 on Muscle 4 or 6/7) could be captured within the focal plane.

Male Mutant and control larvae were subjected to 10-minute PhTx or HL3 incubation, followed by a live HRP-488 stain were imaged at the HRP Z-plane of type 1b NMJs (from segments A2-A4 on Muscle 4 or 6/7). Localization maps were generated from the acquired data using PalmTracer (MetaMorph, provided by J.-B. Siberita, Bordeaux). Prior to localization detection the movies were drift corrected and cropped to exclude movement artefact by using the ImageJ plugin NanoJ ⁶⁴ or Thunderstorm ⁶⁵. The subsequently generated trajectories (PalmTracer) were further analyzed by calculating the mean square displacement (MSD). Analysis of the local channel density within confined regions were performed by cluster analysis based on Voronoi tessellation constructed from localized channels using the software package SR-Tessler ²³.

Localization and trajectory reconnection of mEOS signals was carried out using wavelet-based algorithm and a simulated annealing algorithm as previously described, which considers molecule localization and total intensity. It has been reported that mEOS4b molecules can show blinking-like behavior ⁶⁶. To avoid false reconnections between trajectories, all sub-trajectories were analyzed as individual trajectories. Diffusion coefficients (D) were calculated by linear fitting of the first four points of the MSD plots. MSD plots of immobilized molecules (on fixed samples) revealed that, under our imaging conditions, molecules with $D \geq 0.002 \mu\text{m}^2/\text{s}$ can be considered as mobile. The offset of MSD curves from immobile molecules was taken as empirical measure for the localization accuracy under the live imaging conditions (31.3 nm IQR 25.9/36.6 nm, 247 trajectories, suppl. Fig. 1 C). Calculation of the localization accuracy and number of collected photons per individual fluorophores (suppl. Fig. 1 A,B) were calculated by the use of the Thunderstorm plugin in ImageJ, matching the value obtained from the MSD curves of immobile molecules.

Calcium imaging

Spontaneous synaptic activity was monitored by imaging of postsynaptic calcium transients reflecting the stochastic release of synaptic vesicles. We used the genetically encoded membrane tethered GCaMP6f reporter. Spontaneous calcium transients were recorded in imaging buffer containing 4mM Mg^{2+} and 1.5mM Ca^{2+} for all control situations or 0.2 mM Ca^{2+} to decrease the frequency of spontaneous release events. Images were acquired with a frame rate of 20 Hz. SynapGCaMP6f was excited with a 488 nm laser and transient fluorescent emission changes were detected at emission wavelengths between 520-550 nm (Suppl. Fig. 2). The analysis of calcium transient maxima ($\Delta F/F_0$) was performed on background-corrected maximum projections using a custom written routine in ImageJ software (*N/H*) and involved detection of synaptic puncta with SynapGCaMP6f fluorescence signal exceeding a threshold of 2x the standard deviation (calculated 30 frames prior to stimulus) in response to the stimulus.

Cac channel number analysis by Bleach curves:

The blinking of mEOS4b labelled cacophony channels was used as an approximate measure for the number of channels within individual active zones (AZ), due to the one-to-one stoichiometry between the pore forming subunit and the genetic encoded fluorophore. Only image stacks were analyzed where individual blinking events could be identified. The localization of the channel population within individual AZs was defined within average projections of image stacks. Before further analysis, images were processed by background subtraction and drift correction using the Fiji plugin NanoJ⁶⁴. The confined localization of the channels within the AZ allowed for the counting of fluorescent units within regions of 5x5 pixels, which was sufficient to cover the localization of the fluorescent signal. Individual symmetric AZ within single boutons were selected manually for analysis of the bleach curve. The fluorescent signal was completely bleached within less than 5000 frames (20 Hz acquisition rate), which allowed identification of single fluorophores as one step photobleaching events. The quotient of the maximal fluorescent value and the averaged single fluorophore response was taken as a rough estimate for labelled channel molecules within the AZ. Due to the acquisition rate, as well as the shortcomings of live imaging parameters, and the varying positions of AZs within individual boutons there are likely many missed bleaching steps recorded and thus the calculated channel numbers are most likely an underestimate of the actual number of molecules. Comparative Cac channel numbers under control conditions and after the PHP assay as well as for the BRP and Cac-mutants (CacmEOS4B- Δ 160AA mutants) were analyzed (Fig 2, Fig. 4 and 5).

Well analysis

A. We scanned through all possible local grid bin sizes between 50 nm and 100 nm with a step size of 5 nm, for each bin size dx. For each value of dx, we performed the following iterative procedure starting at iteration :

- i. First, we restricted the analysis to the trajectory points falling in a square region of size centered at the high-density bin center (if , the square is centered at the center of the ellipse found in the previous iteration instead). We obtained the well boundary by fitting the 95% confidence-ellipse to the spatial distribution of these points.
- ii. Inside this ellipse, we computed the depth of the well based on a circular estimator computed on the drift map based on a local grid centered at the well center with bin sizes dx while the diffusion coefficient was obtained by considering the well as a single bin.
- iii. The iteration was scored using the well parabolic score.
- iv. We go back to step i. increasing by 1 and stopped iterating when nm and kept the iteration with the minimum score where the well possessed more than 20 trajectory points and 5 drift bins with at least 7 trajectory displacements per bin.

B. The selected well for a given high-density region was chosen as the well with the minimum parabolic score over all local bin grid sizes.

Finally, to obtain the statistics, only the wells with a parabolic score < 0.5 and an energy < 10 kT were kept.

The ring ellipses in Supplemental Figure 3D,E,F were obtained by manually selecting wells forming an elliptic region and fitting an ellipse around their centers using a minimum volume ellipsoid algorithm with a tolerance of 0.01.

The residence time of a molecule inside a well reported in Supplemental Figure 3I,J was computed for each well as: .

43,89

Tessellation Analysis of Cac localizations within AZ cluster and Nanocluster distributions:

SR-Tessler software, ThunderSTORM, and the ImageJ NanoJ-SRRF were used to process and segment each Cac localization recorded by Live sptPALM of all mutant and control images. Tessellation was performed on drift corrected image stacks. The first 300 frames were rejected to avoid the detection of multiple molecules fluorescent at the same time. Individual molecules could be detected within the following frames and used to localize individual molecules.

Tessellation settings: AZ boundary settings: Voronoi object: 30 density factor, min Area: 2000, Min Localization: 5/10 NC boundary settings: Voronoi Nanocluster object: 3 density factor, min Area: 50, Min Localization: 5/10. List of AZ clusters and their nanoclusters: size, area, number of localizations, density, and number of clusters were exported and filtered to remove all AZ clusters lacking a nanocluster and were also within 30-600nm AZ and Nanoclusters limits. In addition, we applied further limitations to Cac localization number=AZ boundary: 3000 NC boundary: 500, diameter size= AZ boundary: 600-30nm. NC boundary: 300-30nm, Area: AZ boundary: 50000 nm². NC boundary: 7000 nm² and density=AZ & NC boundary: 0.072 localizations/nm² to define well the nanodomain of Cac channels at the *Drosophila* NMJ at the presynaptic membrane. We took into consideration the parameters set for BRP localizations from recent PALM and QPAINT imaging studies and our average diameter size and imaging of average Cac spot size (Fig 1A-H) ^{13,14}. Of note here, the controls data were not independent for *RIM-1*^{-/-} and *RimBP*^{-/-} mutant experiments (Suppl. Fig. 4 and Fig 3A-N). The data shown in Fig. 5 for *BRP*^{-/-}; *BRP*^{-/-}+PhTx, and controls was done in one concomitant experiment.

Software and Online resources

RESOURCE	SOURCE	IDENTIFIER
Fiji	NIH	https://fiji.sc/
FitMaster	HEKA Elektronik	http://www.heka.com/downloads/downloads_main.html#down_fitmaster
GraphPad PRISM 6 and 7	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
ImageJ	NIH	https://imagej.nih.gov/ij/
Imspector	Abberior Instruments	https://imspector.abberior-instruments.com/
Microsoft Office Excel, Word, Powerpoint 2010	Microsoft	https://www.office.com/?omkt=de-de
MetaMorph Microscopy Automation and Image Analysis Software	Molecular Devices	https://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software
NanoJ-SRRF (ImageJ/Fiji plugin)	Gustafsson et al., 2016	http://sites.imagej.net/NanoJ/ ; http://sites.imagej.net/NanoJ-SRRF/
SR-Tessler	Levet et al. 2015	https://github.com/flevet/SR-Tesseler/releases/tag/v1.0

Quantification and Statistical Analysis

The statistical analysis was carried out using Prism software (GraphPad). Normality of distribution was verified by D’Agostino & Pearson omnibus normality test. To test the statistical significance of differences between two conditions Dunn’s test and a Kolmogorov-Smirnov test was used for diffusion coefficient, radius of confinement data and tessellation data. For the comparison of multiple datasets, non-parametric Kruskal-Wallis one-way ANOVA was used as indicated in the corresponding figure legend. All statistical tests were performed as two-tailed tests. Effects and differences were considered as significant at p < 0.05. Data are presented throughout the text as mean ± standard error of the mean (SEM) or the median with the inter-quartile range (IQR) as indicated. sptPALM data analysis for mobility, tesslation, well analysis and channel numbers are shown either as median and an interquartile range, or as mean±SEM. Significance levels are given as *, p<0.05; **, p<0.005; ***, p<0.0005; ****, p<0.0001; and n.s., nonsignificant.

Data and Software Availability

Fly lines are available through the corresponding author.

Declarations

Author Contributions

Conceptualization: T.G, M.H. and S.J.S.; Investigation, T.G., M.E, K.E., J.L.; Well and PALM analyses, P.P, and M.H.; Methodology, U.T., M.M.; Resources, U.T., SG, S.R. and K.O.C.G; Writing Original Draft: T.G, M.H. and S.J.S.; Funding Acquisition, A.W. S.J.S.; Supervision, M.H. and S.J.S.

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Graphical Abstract

The graphical abstract is available in the supplementary files section.

Figures

Figure 1

Live *in vivo* mobility and distribution of individual voltage-gated Ca²⁺ channels at Drosophila NMJ synapses. (A-D) Representative four channel confocal (A) and gSTED (B-D) images of NMJs (muscle 4) of 3rd instar larvae. Differential labeling of AZs with CacsfGFP (red), BRP N-term (green), RBP C-term (blue) and of postsynaptic GluRIIA (gray) is evident on side and top views (C and D). (E) Sizing of Cac spots from N=24 images with a total of 651 synapses, measured along their Ferret's longest (a) and shortest (b) diameters. N=24 images from which 651 synapses were analyzed. n=2 independent sets. G(F-J) Live Cac channel dynamics based on sptPALM of CacmEOS4b in 1.5 mM Ca²⁺ extracellular imaging buffer. Localization, trajectory, and tessellation maps (G-I) of the

boxed bouton in (F) illustrate stepwise analysis leading to the identification of Cac nanoclusters (NC) inside AZs (I, J). (K-O) Quantification of live dynamics of Cac sptPALM imaging, N=70 images, 8 sets, 14013 MSD and radius of confinement values. For a total of 1166 synapses, 14013 MSD and radius of confinement values were analyzed (K-M). Tessellation analysis revealed the diameters of AZs and NCs (N) and the density of Cac channels localizations within AZ and NC boundaries (O). Data distribution is statically tested by Kolmogorov-Smirnov test. Statistical significance is denoted as asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Scale bars: 5 μ m in A and F, 0.5 μ m in B, F, 200 nm in C, D.

Figure 2

Live *in vivo* Imaging of Cac channels at remodeling AZs.

(A-H) Live sptPALM imaging of CacmEOS4b at muscle 4 or 6/7 NMJs, performed in 1.5mM Ca^{2+} 5 min after a 10 min incubation in HL3 with PhTx or in plain HL3 (Control). Images show representative PALM recordings (A and E), trajectory maps (B and F) and tessellation analysis representations of CacmEOS4B (C and D, G and H). Scale Bar= 2 μ m (A-C, E-G) and 200 nm (D and H). (I-J). Recording of stepwise photobleaching as a reference for counting the relative number of CacmEOS4b molecules within AZs of control and PhTx treated NMJs during a period of continuous illumination (405 nm / 561 nm). (K) Quantification of channel numbers from bleaching curves (I and J), derived from the same sptPALM recordings used in L-Q. 43 AZs (control) and 49 AZs (PhTx) were analyzed based on 12-16 images/NMJ from 3-4 animals for each condition, p<0.0003 (unpaired t-test). (L-M) Quantification of diffusion coefficients and radii of confinement from live Cac channel sptPALM imaging. Control NMJs: N=54 images; 8013 radius of confinement values; PhTx treated NMJs: N=58 images; 9139 values. n=5 Sets with concurrent controls. (N-Q) Tessellation analysis from the same sptPALM data set as visualized and analyzed in A-K. Diameters and densities of Cac channels localizations within AZ and NC cluster boundaries. 644 AZs (control) and 892 AZs (PhTx) included in N=54/58 images for either condition was analyzed. n=5 sets with concurrent controls. Statistical significance is denoted as asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Data distribution is statistically tested with a Kolmogorov-Smirnov test. Statistical significance is denoted as asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 3

Cac channels at *CacmEOS4b;RimBP*^{-/-} null mutant AZs and *CacmEOS4b-APTA-Δ6aa* mutants visualized for their live mobility and distribution at AZs. (A-H) Live sptPALM imaging of *CacmEOS4b* and *CacmEOS4b;RimBP*^{-/-} mutants imaged in 1.5mM Ca²⁺ extracellular imaging buffer. Representative boutons displaying PALM images(A,E), trajectory maps (B-F) and representation of tessellation analysis (C-D,G-H) of individual Cac channels in a bouton of *CacmEOS4B* (control) and *CacmEOS4B;RimBP*^{-/-} mutant animals. Scale bar: A-C,E-G- 1μm; D,H:200nm. (I-J), Diffusion coefficient and confinement quantification of live sptPALM data of *CacmEOS4B* (Control a.k.a Con) and *CacmEOS4B;RBP*^{-/-} NMJs, N=28 (Con) and 26 (*RBP*^{-/-}) images from which 2212 (Con) and 1753 (*RBP*^{-/-}) radius of confinement values were analyzed. (K-N) Tessellation analysis of the diameter and density of Cac channels localizations within AZs (K-L) and Nanoclusters (NC) (M-N), N=28(Con) or 26 (*RBP*^{-/-}) images from which 229 (Con) and 264 (*RBP*^{-/-}) AZs were analyzed. n=3 *RimBP*^{-/-} experiments were conducted with concurrent controls. (O-P) Live sptPALM imaging analysis of *CacmEOS4b* (Control or Con) and *CacmEOS4b-APTA-Δ6aa* mutants (*APTA-Δ6aa*) imaged in 1.5mM Ca²⁺ extracellular imaging buffer. Diffusion coefficients (O) and, radius of confinement quantifications (P) based on N=28 (Con) and 17 images from which 2320 (Con) and 1890 (*APTA-Δ6aa*) confinement values were analyzed. (Q-T) Tessellation analysis of the diameter and density of Cac channels localizations within AZ (Q-R) and NC (S-T) cluster boundaries, N=28(Con) or 17 (*APTA-Δ6aa*) images from which 441 (Con) and 276 (*APTA-Δ6aa*) AZs were analyzed. n=2 independent sptPALM experiments with concurrent controls. PALM Data distribution is statistically tested with Kolmogorov-Smirnov test. Statistical significance is denoted as asterisks: ns (not significant) p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 4

Live *in vivo* imaging of Cac channels in *CacmEOS4B-Δ160aa* mutants. A-H; Representative Live sptPALM imaging of CacmEOS4B (Control or Con) and *CacmEOS4B-Δ160aa* ($\Delta 160aa$) mutants imaged in 1.5mM Ca^{2+} extracellular imaging buffer displaying the PALM localization (A-B), trajectory map (C-D), and a visualization of the tessellation analysis (E-H), in CacmEOS4B/ Control and $\Delta 160aa$ boutons (C) and individual AZs (D). Analysis of this data (I-O). I, live sptPALM Diffusion coefficient, J, radius of confinement quantification of CacmEOS4B and *CacmEOS4B; $\Delta 160aa$* NMJs, N=23 (Control) and 15 ($\Delta 160aa$) images, 3 sets, 2996 (Control) and 2436 ($\Delta 160aa$) radius of confinement values analyzed. K-N tessellation analysis of the diameter and density of Cac channels localizations within AZ (K-L) and NC (M-N) cluster boundaries, N=23 (Control) or 15 ($\Delta 160aa$) images, 3 sets, 303 (Control) and 371 ($\Delta 160aa$) AZs analyzed. PALM Data distribution is statistically tested by Mann-Whitney-U test followed by Kolmogorov-Smirnov test. Statistical significance is denoted as asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns (not significant) $p > 0.05$. O) For comparison quantification of Cac channel numbers extracted from the same live sptPALM data is further characterized within control conditions (for CacmEOS4b (Control a.k.a Con) and *CacmEOS4b $\Delta 160aa$* ($\Delta 160aa$) mutants. The data are from 3-4 NMJ out of 3-4 larvae for each condition from 43 AZ (control) and 49 AZ (PhTx), $p < 0.0003$ (unpaired t-test). N= 12-16 NMJs from 4 larvae for each condition from which 15 AZ (Control) and 35 AZ ($\Delta 160aa$), $p < 0.0003$ (unpaired t-test).

Figure 5

Live *in vivo* imaging of Cac channels in BRP mutant AZs. A-C; Live sptPALM imaging of CacmEOS4B (Control a.k.a Con) (A, B) and *CacmEOS4B; BRP^{-/-}* (*BRP^{-/-}*) mutants (C-F), pre-incubated in 10-minute PhTx in HL3 (E-F) or plain HL3 (C-D) and imaged in 1.5mM Ca²⁺ extracellular imaging buffer visualized in A,C,E PALM images and B,D,F trajectory maps. G-H, Live sptPALM diffusion coefficient and confinement quantification of CacmEOS4B, *CacmEOS4B; BRP^{-/-}* and PhTx treated *CacmEOS4B; BRP^{-/-}* NMJs, N=17 (Con) and 13 (*BRP^{-/-}*) or 12 (*BRP^{-/-} + PhTx*) images, 3 sets, 2192 (Con) and 1644 (*BRP^{-/-}*), radius of confinement values. N=30(Con), N=8(*BRP^{-/-}*) and N=12(*BRP^{-/-} + PhTx*) images. n=3 individual *BRP^{-/-}* experiments conducted with concurrent controls. PALM Data distribution is statistically tested by a Kolmogorov-Smirnov test. Statistical significance is denoted as asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. I) Quantification of Cac channel numbers extracted from live sptPALM data further characterized in A-F within control conditions (for CacmEOS4b (Control a.k.a Con) and *CacmEOS4b; BRP^{-/-}*) and after induction of homeostatic plasticity with a 10-minute incubation of the larvae in Philanthotoxin (PhTx) in extracellular imaging solution. The data are from 12-16 NMJs/images from 3-4 animals for each condition from 72 AZ (control), 80 AZ (*BRP^{-/-}*), and 26 AZ (*BRP^{-/-} + PhTx*), p<0.0003 (unpaired t-test). J; A schematic representation of Ca²⁺ channel distribution and mobility upon 20 minutes PHP and in the background of *BRP^{-/-}* and *Cac 160aa* mutants.

Supplementary Files

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- [CacmEOSGcamp0.2mM CaSupplFig2CGhelani210122.avi](#)
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