Unc13A dynamically stabilizes vesicle priming at synaptic release sites for short-term facilitation and homeostatic potentiation

Jusyte, Meida; Blaum, Natalie; Böhme, Mathias A.; Berns, Manon M.M.; Bonard, Alix E.; Vámosi, Ábel B.; Pushpalatha, Kavya V.; Kobbersmed, Janus R.L.; Walter, Alexander M.

Published in:
Cell Reports

DOI:
10.1016/j.celrep.2023.112541

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Unc13A dynamically stabilizes vesicle priming at synaptic release sites for short-term facilitation and homeostatic potentiation

Highlights
- Neurotransmitter release potentiation from milliseconds to minutes relies on Unc13A
- Unc13A functional domains dynamically regulate release-site occupation
- Phorbol esters or calmodulin binding domain mutation similarly activate Drosophila Unc13A
- STED microscopy reveals nanometer changes in Unc13A at potentiated synapses

Authors
Meida Jusyte, Natalie Blaum, Mathias A. Böhme, ..., Kavya V. Pushpalatha, Janus R.L. Kobbersmed, Alexander M. Walter

Correspondence
awalter@sund.ku.dk

In brief
Jusyte et al. find joint dependence of millisecond and minute presynaptic plasticity on conserved Unc13A. Mutating its Ca$^{2+}$/calmodulin binding domain or phorbol ester treatment blocked plasticity by overactivating Unc13A, possibly by enhancing neurotransmitter release-site occupation through its subsynaptic redistribution. Converging signals on Unc13A dynamically control synaptic output from release sites.
Unc13A dynamically stabilizes vesicle priming at synaptic release sites for short-term facilitation and homeostatic potentiation

Meida Jusyte,1,2 Natalie Blaum,3 Mathias A. Böhme,1,4 Manon M.M. Berns,3 Alix E. Bonard,3 Ábel B. Vámosi,9 Kayva V. Pushpalatha,3 Janus R.L. Kobbersmed,5,6 and Alexander M. Walter1,2,3,7,*

1Molecular and Theoretical Neuroscience, Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin, Germany
2Einstein Center for Neurosciences Berlin, Charité Universitätsmedizin Berlin, Berlin, Germany
3Department of Neuroscience, University of Copenhagen, Copenhagen, Denmark
4Rudolf Schoenheimer Institute of Biochemistry, Division of General Biochemistry, Medical Faculty, Leipzig University, Leipzig, Germany
5Department of Mathematical Sciences, University of Copenhagen, Copenhagen, Denmark
6Center of Functionally Integrative Neuroscience, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
7Lead contact
*Correspondence: awalter@sund.ku.dk
https://doi.org/10.1016/j.celrep.2023.112541

SUMMARY

Presynaptic plasticity adjusts neurotransmitter (NT) liberation. Short-term facilitation (STF) tunes synapses to millisecond repetitive activation, while presynaptic homeostatic potentiation (PHP) of NT release stabilizes transmission over minutes. Despite different timescales of STF and PHP, our analysis of Drosophila neuromuscular junctions reveals functional overlap and shared molecular dependence on the release-site protein Unc13A. Mutating Unc13A’s calmodulin binding domain (CaM-domain) increases baseline transmission while blocking STF and PHP. Mathematical modeling suggests that Ca2+/calmodulin/Unc13A interaction plastically stabilizes vesicle priming at release sites and that CaM-domain mutation causes constitutive stabilization, thereby blocking plasticity. Labeling the functionally essential Unc13A MUN domain reveals higher STED microscopy signals closer to release sites following CaM-domain mutation. Acute phorbol ester treatment similarly enhances NT release and blocks STF/PHP in synapses expressing wild-type Unc13A, while CaM-domain mutation occludes this, indicating common downstream effects. Thus, Unc13A regulatory domains integrate signals across timescales to switch release-site participation for synaptic plasticity.

INTRODUCTION

Stimulated chemical synaptic transmission depends on an action potential (AP)-induced Ca2+ influx triggering the fusion of synaptic vesicles (SVs) with the plasma membrane at release sites of presynaptic active zones (AZs). The released neurotransmitters (NTs) are subsequently detected by postsynaptic receptors.1

Both the pre- and the postsynaptic sides can be involved in synaptic plasticity, the modulation of transmission strength. Plasticity occurs on timescales ranging from milliseconds to a lifetime.2,3

Short-term plasticity (STF), the transient change in synaptic responses upon repetitive stimulation on the millisecond timescale, primarily relies on presynaptic mechanisms and forms a basis for temporal information processing in the nervous system.4-5 Elevation of vesicular release probability (pV) or the number of participating release sites can cause short-term facilitation (STF) in response to repetitive AP stimulation.5-8 Although molecular details are still lacking, several recent studies have highlighted that rapid changes in release-site occupation can mediate STF.9-13

Longer-term synaptic plasticity is essential to stabilize information flow and form memories.2,3,14 Presynaptic homeostatic potentiation (PHP) is an evolutionarily conserved mechanism to enhance AP-induced NT release to ensure transmission in case postsynaptic NT sensitivity is reduced.15 PHP is acutely induced (and observed after minutes) by the pharmacological inhibition of NT receptors at central mammalian synapses and the Drosophila melanogaster neuromuscular junction (NMJ).16,17 Chronic mutation of glutamate receptors of the Drosophila NMJ similarly reduces NT sensitivity, which is offset by a sustained (lifelong) PHP.16,18 While acute and chronic PHP similarly enhances NT release, the underlying mechanisms differ: longer-term PHP depends on cellular transport reactions and coincides with the local enrichment of synaptic components.19-21 In contrast, acute PHP, on the timescale of a few minutes, can be achieved independent of cellular transport, indicating that the initial phase of PHP relies on the available synaptic material, which is “switched” to a state more permissive for NT release.18 As for STF, it is not known how this is achieved molecularly.

The evolutionarily conserved (M)Unc13 proteins (Munc-13 in mammals, Unc13 in other species, like Drosophila and C. elegans) are essential for NT release and mediate the physical...
attachment of SVs to the plasma membrane (SV docking) and their molecular maturation to become responsive to the Ca\(^{2+}\) stimulus (SV priming). Unc13 proteins localize in defined clusters to generate SV release sites across species (Drosophila/mouse/C. elegans). (M)Unc13 proteins contain several evolutionarily conserved domains that influence synaptic transmission. Transmission itself, as well as SV docking and priming, critically depends on the conserved MUN domain, which catalyzes the formation of the neuronal SNARE complex that provides the energy for SV fusion. Furthermore, mutations of the Ca\(^{2+}\)/calmodulin interaction domain (CaM domain) or the Ca\(^{2+}\)/phosphoinositide-binding C2B domain of Unc13-1, ubMunc13-2, and Unc13A alter STP. Moreover, mutation of the protein’s C1 domain or its pharmacological activation with the diacylglycerol (DAG) analog phorbol ester enhances NT release. However, exactly how these manipulations change transmitter release and under what biological circumstances this is relevant remain unclear.

Here, we investigate the relevance of (M)Unc13 regulatory domains in presynaptic plasticity on the timescales of milliseconds and minutes. Electrophysiological recordings of the Drosophila larval NMJ revealed that mutation of the Unc13A CaM domain resulted in increased baseline transmission and loss of STF at and below physiological extracellular Ca\(^{2+}\) concentrations. A previous analysis had shown that this synapse’s STP profile is reproduced by a model assuming a Ca\(^{2+}\)-dependent stabilization of SV priming at release sites, and we here extend this model to describe this by Ca\(^{2+}\)/calmodulin binding to Unc13A. The model not only reproduced the experimentally observed baseline transmission and STP profiles of the NMJ, but also predicted that the Unc13A CaM domain mutation caused an unnatural enhancement of release-site occupation by primed vesicles, thereby limiting the ability to further enhance transmission during STF. Superresolution stimulated emission depletion (STED) microscopy was used to investigate the local AZ topology and revealed changes in the subsynaptic distribution of the Unc13A MUN domain, which was found enriched at the AZ-central plasma membrane in Unc13A CaM domain mutant synapses. We further investigated whether mutation of the Unc13A CaM domain also interfered with presynaptic plasticity on the minute timescale by acutely challenging Unc13A CaM domain mutant synapses with pharmacological inhibition of NT receptors. This demonstrated that the normally observed PHP upon this treatment was lost, indicating that both this PHP and STF depend on plastic Unc13A regulation. Functional synergism between Unc13A regulatory domains was investigated by pharmacologically targeting the Unc13A C1 domain using the phorbol ester phorbol 12-myristate 13-acetate (PMA). This revealed effects similar to those seen upon mutation of the CaM domain (increased baseline transmission and abolished STF at normal physiological extracellular Ca\(^{2+}\) and block of PHP). PMA enhancement was blocked by the Unc13A CaM domain mutation, indicating that both these ways of potentiation induce the same downstream stabilization of SV priming at release sites. Thus, acute regulation of release-site participation is a powerful presynaptic plasticity mechanism for STP and PHP, and Unc13A regulatory domains can regulate this by integrating a variety of intracellular signals.

RESULTS

Mutation of the Unc13A calmodulin binding domain enhances initial synaptic transmission and abolishes STF at low extracellular Ca\(^{2+}\)

STF depends on the accumulation of presynaptic Ca\(^{2+}\) during repetitive AP stimulation with a high Ca\(^{2+}\) sensitivity. The binding of Ca\(^{2+}\)/calmodulin to Munc13 proteins has a similar affinity, and neurons expressing Munc13 mutants with disrupted calmodulin interaction showed altered STF. We sought to elucidate the role of the Unc13A CaM domain in STF using the Drosophila melanogaster third-instar NMJ as a model synapse. For this we used animals expressing either the wild-type Unc13A protein or an Unc13A mutant whose CaM domain was mutated (Unc13A CaMWRWR mutant) by exchanging two tryptophan residues to arginine (Figure 1A) to achieve the same amino acid sequence as in the non-calmodulin-binding Munc13-1 mutant. Synaptic transmission at the Drosophila larval NMJ strongly depends on Unc13A but not on Unc13B, the second Drosophila isoform. The CaM domain is unique to Unc13A and, to avoid any compensatory effects of Unc13B, experiments were performed in its absence (Unc13A and -B null animals were rescued with wild-type or mutant Unc13A; see STAR Methods for exact genotypes). Confocal analysis confirmed similar expression and targeting of Unc13A CaMWRWR mutant and wild-type proteins to NMJ AZs, and simultaneous analysis of the ELKS-family AZ scaffolding protein Bruchpilot (BRP), which localizes Unc13A to AZs, revealed no obvious changes in NMJ morphology, AZ numbers, or protein levels (fluorescence intensity) at this resolution (Figures 1B–1E).

At the Drosophila muscle 6 NMJ, baseline AP-evoked synaptic transmission and STP can be analyzed by stimulating motor neuron axons and measuring the AP-evoked excitatory postsynaptic currents (eEPSCs) in two-electrode voltage clamp (TEVC) recordings of the muscle. This model synapse shows robust STF and short-term depression (STD) when exposed to low and high extracellular Ca\(^{2+}\) concentrations, respectively, allowing the characterization of both phenomena in the same cell. TEVC recordings in response to paired AP stimulation (10 ms interval) revealed much larger peak eEPSC\(_{1}\) responses to first APs in flies expressing the Unc13A CaMWRWR mutant compared with controls at low (0.4 and 0.75 mM) and slightly increased amplitudes at standard (1.5 mM) extracellular Ca\(^{2+}\) concentrations (Figures 1F and 1G). Responses in both genotypes were similar under elevated extracellular Ca\(^{2+}\) levels (3 and 6 mM) (Figures 1F and 1G). Alterations in STP characteristics became apparent in the CaM mutant when normalizing eEPSCs to the initial eEPSC\(_{1}\) peak responses (Figure 1H). This was quantified by calculating the cell-wise paired-pulse ratio (PPR; ratio of the second EPSC\(_{2}\) amplitude divided by the first EPSC\(_{1}\) amplitude in each cell) (Figure 1I). This revealed that the typically observed STF (indicated by PPR values greater than 1) at low extracellular Ca\(^{2+}\) concentrations was lost in animals expressing the Unc13A CaMWRWR mutant (Figure 1I). Both genotypes showed similar STDs (PPRs smaller than 1) at elevated extracellular Ca\(^{2+}\) concentrations (Figures 1H and 1I). Our electrophysiological findings align with previous reports of increased STD in cultured mouse neurons expressing corresponding Munc13-1 and ubMunc13-2...
CaM domain mutants. Our data furthermore point to an increased baseline evoked transmission in this condition and that these effects strongly depend on the Ca²⁺ concentration in the external medium.

Ca²⁺/calmodulin binding to Unc13 might stabilize SV priming at release sites for STF

We next investigated a possible mechanism by which Ca²⁺/calmodulin interaction with the Unc13A CaM domain shapes STP using mathematical modeling. STP models have implicated fast vesicle replenishment in counteracting STD or even mediating STF, and several models have suggested fast but reversible SV priming. In these models, STF is observed when unpriming commences similarly fast as (or faster than) forward priming, resulting in incomplete release site occupation with primed SVs, thereby limiting first AP responses. Residual Ca²⁺ following APs may speed SV priming by binding to a release-site-resident molecule, and this might drive STF.
We here used a Drosophila NMJ STP model where SV unpriming is slowed by Ca\textsuperscript{2+} \cite{11} and adapted it to incorporate Ca\textsuperscript{2+}/calmodulin interaction with Unc13A (Table 1). We assumed a stable and an unstable primed state (with slow and fast unpriming, respectively) (Figure 2A) and that the transition between them depended on Ca\textsuperscript{2+}/calmodulin binding to Unc13A. An assumption of this (and the original) model is that increasing the extracellular Ca\textsuperscript{2+} concentration in the recording solution increases the intracellular Ca\textsuperscript{2+} of this (and the original) model is that increasing the extracellular Ca\textsuperscript{2+} concentration corresponds to the half-maximal intracellular Ca\textsuperscript{2+} concentration; Kobbersmed et al. \cite{11}.

The Ca\textsuperscript{2+} current (charge) induced by an AP, the total number of release sites, the number of Unc13A proteins jointly stabilizing SV priming at one release site (m), the Ca\textsuperscript{2+} binding affinity of this reaction, the forward priming rate, and the unpriming rate from the non-stabilized (Ca\textsuperscript{2+} unbound) primed state were free parameters and determined by fitting the model to the experimental data (see STAR Methods for details). The unpriming rate from the stabilized primed state was not fitted, as it can be shown that this depends on the other parameters (see STAR Methods for details). The full model (control condition) achieved good agreement between predicted and experimentally determined eEPSC\textsubscript{1} and PPR values if around two Unc13A proteins per release site stabilized SV priming (m = 2.04) (Figures 2G and 2H). The model predicted a Ca\textsuperscript{2+} dissociation constant for stable priming of ~40 nM, similar to affinity estimates of Ca\textsuperscript{2+}/calmodulin binding to Munc13-1/ubMunc13-2 in vitro (20–100 nM). \cite{57,58} This means that Unc13A is predicted to bind to Ca\textsuperscript{2+}/calmodulin at resting internal Ca\textsuperscript{2+} concentrations higher than 3 mM (Figure 2B). The probability that release sites are occupied with primed SVs (pSite\textsubscript{occ}) (Figure 2C) combined with the vesicular release probability (of a primed SV, p\textsubscript{Vr}, Figure S1C) determines the total probability of NT prelease (p\textsubscript{release} = pSite\textsubscript{occ} * p\textsubscript{Vr}, Figure 2D). \cite{9,13,59,60} After AP stimulation, Ca\textsuperscript{2+} accumulates in the synapse (Figure 2E, top), which slows unpriming, increases the release-site occupation with vesicles (Figure 2E, bottom), and enhances the amplitude of the response to a second AP (Figure 2F). In the model, the strongest facilitation is achieved when the initial release site occupation with primed vesicles is low, and many additional sites are populated between stimuli (Figure 2E, bottom). This is the case when intra-/extracellular Ca\textsuperscript{2+} is low (Figures 2C and 2E).

We also, at the same time, used the model to explore why mutation of the Unc13A domain caused the observed effect in the experiment. Assuming that Unc13A CaM\textsuperscript{WRWR} mutation leads to constitutive (Ca\textsuperscript{2+}-independent) stabilization of SV priming caused release-site occupation to be higher than in the control simulations at low (0.4 and 0.75 mM) and intermediate (1.5 mM) extracellular Ca\textsuperscript{2+} concentrations in the model (Figure 2C). This predicted larger eEPSC\textsubscript{1} amplitudes and reduced PPR values for extracellular Ca\textsuperscript{2+} concentrations below 3 mM, consistent with the experimental data from the Unc13A CaM\textsuperscript{WRWR} mutant NMJs (Figures 2F–2H). Thus, an unexpected prediction of our model is that the consensus mutation of the Unc13A CaM domain that blocked Ca\textsuperscript{2+}/calmodulin interaction with Munc13-1/ubMunc13-2 causes a gain of function in terms of stabilizing SV priming at release sites (see discussion).

### Table 1. Mathematical model parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Explanation and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{Ca}^{2+}]_{\text{ext}})</td>
<td>–</td>
<td>external (\text{Ca}^{2+}) concentration</td>
</tr>
<tr>
<td>(Q)</td>
<td>(\frac{Q_{\text{max}} \cdot [\text{Ca}^{2+}]<em>{\text{last}}}{[\text{Ca}^{2+}]</em>{\text{last}}+K_{\text{M, current}}})</td>
<td>total amount of (\text{Ca}^{2+}) charge flowing into the cell</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_{\text{basal}})</td>
<td>(\frac{[\text{Ca}^{2+}]<em>{\text{max}} \cdot [\text{Ca}^{2+}]</em>{\text{last}}}{K_{\text{M, current}}+[\text{Ca}^{2+}]_{\text{last}}})</td>
<td>resting intracellular (\text{Ca}^{2+}) concentration</td>
</tr>
<tr>
<td>(K_{\text{M, current}})</td>
<td>2.679 mM</td>
<td>extracellular (\text{Ca}^{2+}) concentration corresponding to the half-maximal intracellular (\text{Ca}^{2+}) concentration; Kobbersmed et al. \cite{11}</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_{\text{max}})</td>
<td>190 nM</td>
<td>maximal intracellular (\text{Ca}^{2+}) concentration; Kobbersmed et al. \cite{11}</td>
</tr>
<tr>
<td>(k_{\text{unprime}}(\text{Ca}^{2+} = 0))</td>
<td>(\frac{[\text{Ca}^{2+}]<em>{\text{max}} \cdot m \cdot K</em>{D}}{[\text{Ca}^{2+}]<em>{\text{max}}+[\text{Ca}^{2+}]} \cdot K</em>{D} \cdot k_{\text{unprime}}(\text{Ca}^{2+} = 0))</td>
<td>unpriming rate</td>
</tr>
<tr>
<td>(n)</td>
<td>2</td>
<td>number of (\text{Ca}^{2+}) ions binding to a calmodulin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Q_{\text{max}})</td>
<td>14.04 IC</td>
<td>maximum charge flow</td>
</tr>
<tr>
<td>(k_{\text{sp}})</td>
<td>92.16 s(^{-1})</td>
<td>priming rate constant</td>
</tr>
<tr>
<td>(k_{\text{unprime}}(\text{Ca}^{2+} = 0))</td>
<td>265 s(^{-1})</td>
<td>unpriming rate constant in the absence of (\text{Ca}^{2+})</td>
</tr>
<tr>
<td>(N_{\text{total}})</td>
<td>240</td>
<td>number of release sites</td>
</tr>
<tr>
<td>(m)</td>
<td>2.04</td>
<td>number of Unc13 cooperating in stabilizing SV priming at one release site</td>
</tr>
<tr>
<td>(K_{D})</td>
<td>40 nM</td>
<td>(\text{Ca}^{2+}) affinity Unc13/CaM</td>
</tr>
<tr>
<td>Cost value</td>
<td>8.19</td>
<td>see STAR Methods</td>
</tr>
</tbody>
</table>
Figure 2. Mathematical model indicating that Ca\(^{2+}\)/calmodulin binding to Unc13A might stabilize primed SVs and that Unc13ACaM\(^{WRWR}\) leads to constantly high release-site occupation

(A) Schematic energy landscape of the priming reaction in the model (top) and the corresponding reactions (bottom). (Ca\(^{2+}\))\(_2\)/CaM binding to “m” Unc13A proteins promotes a shift from an unstably primed vesicle (fast unpriming) to a stably primed vesicle (slow unpriming) (indicated by a negative value of the enthalpy difference, \(\Delta G\)).

(B) Percentage of Unc13A molecules bound to (Ca\(^{2+}\))\(_2\)/CaM (blue) and the unpriming rate constant (black, control; pink, mutant) as a function of internal Ca\(^{2+}\). For the relationship between unpriming rate and the extracellular Ca\(^{2+}\) concentration see Figure S1.

(C) Probability of release-site occupation with primed SVs (pSite occ) at rest as a function of extracellular Ca\(^{2+}\) concentration for control (black) and mutant (pink) simulations.

(D) Average total release probability (p\(_{\text{release}}\)) as a function of extracellular Ca\(^{2+}\) concentrations for control (black) and mutant (pink) simulations. p\(_{\text{release}}\) is defined as the number of vesicles released between 0 and 10 ms upon the stimulation depicted in (E), divided by the total number of release sites (N\(_{\text{total}}\) = 240). See Figure S1C for the vesicular release probability p\(_{\text{v, \text{release}}\})

(E) Ca\(^{2+}\) transient (top) and number of primed SVs (pSite occ) at rest as a function of extracellular Ca\(^{2+}\) concentration for control (black) and mutant (pink) simulations. The depicted Ca\(^{2+}\) transient is obtained at 120 nm distance from a Ca\(^{2+}\) source. Control and mutant simulations are in black and pink, respectively.

(F) Simulated average eEPSC\(_1\)s for different extracellular Ca\(^{2+}\) concentrations simulated for control (black) and mutant (pink) settings.

(G and H) (G) Mean eEPSC\(_1\) amplitude and (H) PPR for control (black) and mutant (pink) simulations together with the experimental data (gray for control experiments, purple for Unc13ACaM\(^{WRWR}\) mutant; replotted from Figure 1) for different extracellular Ca\(^{2+}\) concentrations. Mean model predictions were computed over 200 stochastic model repetitions. Error bars show SEMs. See Table 1 for model parameters and Table S2 for source data.
The Unc13A MUN domain is closer to the plasma membrane in Unc13A CaM mutants

Because our model suggested that enhanced SV priming at release sites might underlie the Unc13A CaM<sup>WRWR</sup> mutant phenotypes, we next investigated whether this mutation affected the protein’s subsynaptic distribution using dual-color superresolution STED microscopy. Because the Unc13A CaM<sup>WRWR</sup> mutant phenotypes were most clearly seen when external Ca<sup>2+</sup> concentrations were low (Figures 1F–1I and 2G–2H), larval fillets were treated in Ca<sup>2+</sup>-free medium prior to fixation and stained against BRP and Unc13A (Figure 3). We investigated two combinations of primary antibodies. In both cases, BRP was labeled with an antibody (Nc82) recognizing an epitope near the roof of the pedestal with maximal distance from the AZ plasma membrane<sup>40,42</sup> (Figures 3D and 3H). This was combined with either an Unc13A antibody targeting an N-terminal motive<sup>25</sup> (Figures 3A–3D) or an antibody targeting its MUN domain<sup>30</sup> (Figures 3E and 3H). STED microscopy (lateral resolution of ~25 nm, Figure S2) revealed the typical ring-like structures of BRP and Unc13A for AZs in top view<sup>25,62</sup> (yellow dashed lines in Figures 3A and 3E). Ring diameters were quantified by measuring the peak-to-peak distance of fluorescence line profiles across individual AZs in raw images. The mean fluorescence distribution was found after aligning profiles of BRP and Unc13A channels to the fluorescence minima (i.e., ring centers) in each channel. For both channels, intensity values were normalized to the maximal mean fluorescence observed in control animals to allow combination of several experiments (see STAR Methods). The average BRP ring diameters were ~160 nm, in agreement with previous estimates.<sup>25</sup> In one of the experiments, BRP rings were slightly smaller in Unc13A CaM<sup>WRWR</sup> mutant animals (Figure 3F). The Unc13A ring diameters were similar in both genotypes when analyzed with the antibody recognizing the Unc13A N-terminal epitope (Figure 3B), while Unc13A rings were slightly smaller in AZs expressing the Unc13A CaM<sup>WRWR</sup> mutant when visualized using the antibody recognizing the MUN domain (Figure 3F). A change in the fluorescence distribution between genotypes was seen in the Unc13A channel with both the N- and the C-terminal antibodies (Figures 3B and 3F). Yet different effects were seen regarding peak intensities, which were lower in AZs stained with the N-terminal antibody and higher in AZs stained with the C-terminal antibody in synapses expressing the Unc13A CaM<sup>WRWR</sup> mutant (Figures 3F and S3A–S3C).

Regarding a role in SV priming, the vertical Unc13A distribution profile (from cytosol to plasma membrane) may be more relevant. To investigate whether mutation of the Unc13A CaM domain affected this, we analyzed fluorescence line profiles of AZs observed in side view<sup>61</sup> (Figures 3A and 3E, red dotted lines). At each investigated AZ, the distance between the peak fluorescence in the BRP and Unc13A channel was quantified (Figures 3C and 3G). The mean fluorescence distribution was compared by aligning the signals to the peak fluorescence in the BRP channel and averaging across all analyzed AZs (Figures 3C and 3G). As before, intensity values were normalized in the BRP and Unc13A channel to the maximal average intensities in the control genotype (see STAR Methods). The intensities and shapes of the vertical BRP fluorescence profiles were overall similar in the two genotypes (Figures 3C and 3G), indicating that Unc13A CaM<sup>WRWR</sup> mutation did not grossly affect overall AZ morphology. Comparison of the vertical fluorescence profiles in both genotypes stained with the N-terminal Unc13A antibody revealed similar line profiles and maximal intensities (Figures 3C and S3B). The peak intensities in the Unc13A channel were 34.29 ± 3.811 nm (mean ± SEM, Ctrl) and 40.71 ± 3.002 nm (mean ± SEM, Unc13A CaM<sup>WRWR</sup>) below the BRP peak fluorescence in control and Unc13A CaM<sup>WRWR</sup> mutant animals (Figure 3C), in close agreement with previous estimates.<sup>30</sup> In contrast, the C-terminal Unc13A antibody revealed clear differences between genotypes: the fluorescence line profile profiles were different, and higher signals were detected in AZs expressing the Unc13A CaM<sup>WRWR</sup> mutant (Figures 3G and S3D). Moreover, the distances between peak signals in the Unc13A and BRP channels were larger (i.e., Unc13A peak signals were found closer to the AZ plasma membrane) in AZs expressing the Unc13A CaM<sup>WRWR</sup> mutant (Figure 3G). A similar conclusion was reached in an independent analysis by aligning and averaging many individual AZ images, which indicated that the redistribution of the epitope recognizing the Unc13A MUN domain happened throughout the AZ (Figure S4G). Thus, our data point to a local enrichment or higher epitope availability of the MUN domain of Unc13A CaM<sup>WRWR</sup> mutants at the AZ plasma membrane, which might contribute to enhanced SV priming as predicted by our model.

Unc13A CaM<sup>WRWR</sup> mutation abolishes acute (minutes) presynaptic homeostatic potentiation

Our model predicted that the Unc13A CaM<sup>WRWR</sup> mutation leads to an increased stabilization of primed SVs causing higher (and almost total) release-site occupation (pSite<sub>occ</sub> = 100%) at (sub)physiological extracellular Ca<sup>2+</sup> concentrations (0.4–1.5 mM, Figure 2C). The high release-site occupation at rest removes the ability of synapses to potentiate their release by changing pSite<sub>occ</sub>. We therefore wondered whether the Un13A CaM<sup>WRWR</sup> mutation also interfered with longer-term presynaptic plasticity engaging release sites. More active release sites were shown to mediate transmitter release during PHP.<sup>61,62</sup> This plasticity mechanism counterbalances the loss of postsynaptic NT sensitivity by increasing the amount of AP-induced NT release within minutes.<sup>15</sup> Experimentally, PHP can be acutely induced by applying the glutamate receptor antagonist phanithoxotin (PhTx).<sup>16</sup> Recent analysis revealed that immediate presynaptic plasticity on the minutes timescale can be achieved independent of a structural AZ remodeling needed for long-lasting potentiation, indicating that acute PHP can be accomplished with the available presynaptic material.<sup>19</sup> Thus, we wondered to what extent this acute PHP—similar to STF (Figure 1H)—depended on increasing release-site occupation with primed SVs (pSite<sub>occ</sub>) and therefore investigated whether the Unc13A CaM<sup>WRWR</sup> mutation influenced PHP (as it occluded STF).

At third-instar Drosophila larval NMJs (muscle 6, segments A2 and A3), PHP can be effectively monitored in current clamp recordings. This technique more closely resembles the physiological condition in the animal, as the membrane potential of the muscle is not perturbed by the measurement (when no current is injected). Moreover, to assess PHP, it is necessary
Figure 3. STED microscopy reveals subsynaptic changes in Unc13A CaM\textsuperscript{WwWm} mutants

(A) STED images of muscle 4 NMJs of segment A2–4 from third-instar Drosophila larva of the displayed genotypes labeled with antibodies recognizing BRP (green, left) and Unc13A N-Term (purple, right). Yellow dashed lines indicate top-view AZ. Red dashed lines indicate side-view AZs. Quantification of the resolution of the STED microscope can be found in Figure S2.

(B) Top-view fluorescence line profiles (aligned to BRP and Unc13A ring centers and normalized to the highest average intensities in the control genotype) of BRP (top, left) and Unc13A (top, right) for the displayed genotypes. Bottom: quantification of peak-to-peak distances (ring diameters) of BRP (left) and Unc13A (right) rings for the displayed genotypes. For quantification of the maximum intensities see Figure S3.

(C) Side-view line profiles of BRP signal (aligned to the peak in each AZ and normalized to the highest average BRP intensity in the control genotype) (top, left) and Unc13A signal (aligned to the BRP peak in each AZ and normalized to the highest average Unc13A intensity in the control condition) in laterally viewed AZs (top, right) for the displayed genotypes. Bottom: quantification of the distance between the peak intensities in the Unc13A and BRP channel for the displayed genotypes.
to accurately measure postsynaptic responses to the fusion of single SVs, and current clamp recordings provide a better signal-to-noise ratio to detect such “miniature” events. Prior to recording, larvae were incubated for 10 min either in standard hemolymph-like (HL3) control solution or in HL3 solution containing 20 μM PhTx. First, miniature excitatory postsynaptic potentials (mEPSPs) caused by spontaneous synaptic activity were recorded for 60 s (Figures 4A and 4D). Following this, the efferent motoneural axons innervating the same NMJs were stimulated, and the resulting AP-evoked excitatory postsynaptic potentials (eEPSPs) were measured (Figures 4B and 4D, left). The quantal content, the number of vesicles released per AP, was then calculated by dividing the eEPSP amplitude by the mean mEPSP amplitude in each cell (Figures 4B and 4D). We used a lower than typical extracellular Ca2+ concentration (0.15 mM instead of 0.4 mM15,16.) to reduce the strongly elevated eEPSP amplitudes of Unc13A CaMWRWR mutants, which hamper quantal content estimation (only small eEPSPs are linearly summed by mEPSPs). As expected from its inhibitory action on ionotropic glutamate receptors at the NMJ, application of PhTx potently diminished mEPSP amplitudes in both genotypes (Figure 4A). Despite this reduced postsynaptic NT sensitivity, AP-evoked eEPSPs were not diminished in control synapses measured 10 minutes after PhTx exposure because of a compensatory increase in the quantal content (Figure 4B), indicating intact PHP. In contrast, NMJs expressing Unc13A CaMWRWR mutants failed to undergo PHP under these conditions, as PhTx treatment similarly diminished mEPSP and eEPSP amplitudes, and no potentiation of the quantal content was detected (Figures 4C and 4D). Thus, enhanced transmission observed in the Unc13A CaM domain mutant similarly occluded PHP on a minutes timescale, as it abolished STF on a millisecond timescale (Figure 1H). This indicates that both plasticity phenomena share some commonality despite their different time courses and that both STF and PHP might rely on Unc13A-mediated stabilization of SV priming at release sites that increases pSiteocc.

The DAG analog PMA affects baseline transmission and STP similar to mutation of the Unc13A CaM domain

An additional, highly conserved regulatory domain in close proximity to the Unc13A Ca2+/calmodulin binding domain is the Unc13A C1 domain, which binds the signaling lipid DAG and phorbol esters like PMA (unnatural DAG analogs) that potently increase in the quantal content (Figures 4A and 4D), indicating intact PHP. As expected from its inhibitory action on ionotropic glutamate receptors at the NMJ, application of PhTx potently diminished mEPSP amplitudes in both genotypes (Figure 4A). Despite this reduced postsynaptic NT sensitivity, AP-evoked eEPSPs were not diminished in control synapses measured 10 minutes after PhTx exposure because of a compensatory increase in the quantal content (Figure 4B), indicating intact PHP. In contrast, NMJs expressing Unc13A CaMWRWR mutants failed to undergo PHP under these conditions, as PhTx treatment similarly diminished mEPSP and eEPSP amplitudes, and no potentiation of the quantal content was detected (Figures 4C and 4D). Thus, enhanced transmission observed in the Unc13A CaM domain mutant similarly occluded PHP on a minutes timescale, as it abolished STF on a millisecond timescale (Figure 1H). This indicates that both plasticity phenomena share some commonality despite their different time courses and that both STF and PHP might rely on Unc13A-mediated stabilization of SV priming at release sites that increases pSiteocc.

Acute application of the DAG analog PMA occludes acute (minutes) PHP

Due to the functional similarity and non-additivity in the potentiation of STP similar to mutation of the Unc13A CaM domain
case without PMA (Figure 6), incubation with both PMA and PhTx reduced mEPSP amplitudes by 50%, indicating a PMA-induced increase in the quantal content, indicating intact PHP (Figures 6A and 6B). Wild-type flies incubated with PMA but without PhTx showed similar (in tendency slightly smaller) mEPSP amplitudes compared with controls without PMA (Figures 6A and 6C), but eEPSP amplitudes were markedly increased, demonstrating a PMA-induced increase in the quantal content (Figures 6B and 6D). As was the case without PMA (Figure 6A), incubation with both PMA and PhTx reduced mEPSP amplitudes compared with PMA-only treated animals (Figure 6C). Yet unlike without PMA,
PhTx reduced average eEPSP amplitudes (statistically not significantly different at a 5% level), and no elevation of the quantal content was seen compared with NMJs treated with PMA alone (Figure 6D). These findings illustrate that presynaptic potentiation induced by acute PMA treatment occluded PHP to counteract the effect of PhTx, consistent with PMA “maxing out” release-site occupation ($p_{\text{Site occ}} = 100\%$). Together, our findings indicate that acute pharmacological activation of Unc13A by PMA (similar to mutation of its CaM domain) enhances synaptic transmission and that this enhancement impedes both STF and PHP.

**DISCUSSION**

Here, we show commonality and joint molecular dependence between STF and PHP, two presynaptic plasticity mechanisms operating on distinct timescales of milliseconds and minutes. This connection further extends to potentiation induced by the phorbol ester PMA. Our main conclusions are the following:

- Mutation of the Unc13A CaM domain increased baseline synaptic transmission at low extracellular Ca$^{2+}$, blocked STF and PHP, and rendered synapses PMA insensitive.
- A mathematical model implicated the stabilization of SV priming at release sites by Ca$^{2+}$/calmodulin binding to Unc13A for STF.
- The model predicted that Unc13A CaM WRWR mutation results in a constitutive stabilization of priming, thereby blocking presynaptic plasticity.
- STED microscopy revealed an enrichment of the functionally essential MUN domain at the synaptic plasma membrane following Unc13A CaMWRWR mutation, which might facilitate priming at release sites.
- Acute Unc13A activation by PMA mirrored phenotypes of Unc13A CaMWRWR mutation, and no further potentiation was seen for coincident manipulation, suggesting the same downstream effect.
- STF and PHP may thus underlie similar increases in release-site occupation due to Unc13A activation.

Figure 4. Unc13A CaMWRWR mutation abolishes PhTx-induced PHP
(A–D) Analysis of PhTx effects in current clamp recordings (muscle 6 NMJs, 0.15 mM extracellular Ca$^{2+}$) of (A and C) spontaneous and (B and D) AP-evoked synaptic activity in (A and B) control (Ctrl) and (C and D) Unc13A CaMWRWR mutant animals. (A and C) Left: representative example traces of spontaneous mEPSPs from (A) Ctrl and (C) Unc13A CaMWRWR mutant synapses preincubated for 10 min either with (+PhTx, 20 $\mu$M) or without (–PhTx/+H$_2$O) philanthotoxin. Middle and right: quantification of mEPSP amplitudes and frequencies. (B and D) Left: representative AP-evoked eEPSP responses (average of five repetitions in one cell) of NMJs preincubated for 10 min either with (+PhTx, 20 $\mu$M) or without (–PhTx/+H$_2$O) philanthotoxin. Middle and right: quantification of eEPSP amplitude and quantal content. Genotypes (see STAR Methods for details): Unc13A and -B null animals expressing either wild-type Unc13A (Ctrl) or the Unc13A CaMWRWR mutant (Unc13A CaMWRWR). Number of cells (n) and animals (N) investigated: n/N(Ctrl; /PhTx) = 29/17, n/N(Ctrl; +PhTx) = 30/18, n/N(Unc13A CaMWRWR; /PhTx) = 35/17, n/N(Unc13A CaMWRWR; +PhTx) = 30/16. Statistical analysis with Student’s two-tailed t test. Data show the mean values ± SEM; n.s., p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Source data available in Table S4.
Release probability and readily releasable pool sizes in dynamic priming models

The amount of NT release depends on the product of the vesicular release probability with the probability that release sites are occupied and the total number of release sites ($NT_{\text{release}} = p_V \cdot p_{\text{Site occ}} \cdot N_{\text{total}}$). The total number of SVs primed at release sites is also referred to as the readily releasable pool ($RRP = p_{\text{Site occ}} \cdot N_{\text{total}}$). If transitions between unprimed and primed states are fast and can vary on timescales relevant for STP, this can complicate the interpretation of whether certain effects can be attributed to alterations in $p_V$, $RRP/p_{\text{Site occ}}$, or both. For instance, phorbol esters have been assumed to exert potentiation by increasing the vesicular release probability $p_V$, but typical effects (e.g., enhanced baseline responses, increased STD) can also be interpreted in a redistribution between distinct primed states.

One problem can be that established paradigms to quantify the RRP, which rely on strong and extended stimulation (e.g., using high-frequency AP stimulation, extended voltage depolarization, step-like Ca$^{2+}$ uncaging, hypertonic sucrose application), are outpaced by changes in priming and therefore likely sample replenishing SVs in addition to the resting RRP. In such cases a decomposition using mathematical approaches can be helpful. We here show that manipulations of external Ca$^{2+}$ concentration, mutation of the Unc13A CaM domain, and PMA and/or PhTx might be useful to distinguish altered states of release-site occupation experimentally at the larval fly NMJ. Our experiments cannot be used to distinguish whether RRP changes coincide with changes in SV docking at release sites, but experiments monitoring SV attachment at the plasma membrane by TIRF or electron microscopy are likely to provide valuable insights into this question.

Figure 5. Respective occlusion of presynaptic potentiation by activation of Unc13A via CaM mutation or PMA treatment

TEVC experiments from larval M6 NMJs at 0.4 mM (A and C) and 1.5 mM (B and D) extracellular Ca$^{2+}$. (A and B) Analysis of control (Ctrl) animals at extracellular Ca$^{2+}$ concentrations of (A) 0.4 mM and (B) 1.5 mM. Left: example traces of AP-evoked paired-pulse responses preincubated with DMSO (–PMA) or 2 µM PMA in DMSO (+PMA). Middle: quantification of eEPSC$_1$ amplitudes. Right: quantification of PPR ratios (10 ms interstimulus interval [ISI]). (C and D) Same analysis as in (A) and (B) for Unc13ACaM$^{WRWR}$ mutant flies. Genotypes (see STAR Methods for details): Unc13A and -B null animals expressing either wild-type Unc13A (Ctrl) or the Unc13A CaM$^{WRWR}$ mutant (Unc13A CaM$^{WRWR}$). Number of cells (n) and animals (N) investigated: n/N(Ctrl; –PMA; 0.4 mM Ca$^{2+}$) = 9/5, n/N(Ctrl; +PMA; 0.4 mM Ca$^{2+}$) = 8/5, n/N(Unc13A CaM$^{WRWR}$; –PMA; 0.4 mM Ca$^{2+}$) = 9/5, n/N(Unc13A CaM$^{WRWR}$; +PMA; 0.4 mM Ca$^{2+}$) = 8/5, n/N(Ctrl; –PMA; 1.5 mM Ca$^{2+}$) = 17/10, n/N(Ctrl; +PMA; 1.5 mM Ca$^{2+}$) = 14/9, n/N(Unc13A CaM$^{WRWR}$; –PMA; 1.5 mM Ca$^{2+}$) = 18/11, n/N(Unc13A CaM$^{WRWR}$; +PMA; 1.5 mM Ca$^{2+}$) = 12/7. Dots represent individual cells/NMJs, mean and SEM are shown. Statistical analysis with Student’s two-tailed t test; n.s., p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Source data available in Table S5.
and BRP) rings in our STED experiments (Figure 3F), and these changes likely have an impact on the probability of release. On the one hand, closer release-site placement to the AZ center, where the voltage-gated Ca\(^{2+}\) channels are located, profoundly increases pVr because shorter distance increases the probability that Ca\(^{2+}\) influx activates the vesicular fusion machinery.\(^{25,77}\) On the other hand, smaller AZs generally have lower total NT release, as they typically contain fewer Ca\(^{2+}\) channels and Unc13A release sites, which might indicate secondary, compensatory changes to lower the enhanced transmission caused by the Unc13A CaMWRWR mutation.\(^{19,30,78,79}\) Future experiments tracking single AZ activity might help clarify this.

The Unc13A CaM\(^{WRWR}\) mutation causes a gain-of-function phenotype at the Drosophila NMJ

In central mouse neurons, mutation of the conserved CaM domain in Munc13-1 and ubMunc13-2 early on identified a relevance in STP.\(^{41}\) The Drosophila Unc13A CaM domain mutant studied here is based on this mutation, and our data confirm its relevance for STF at the Drosophila NMJ. We also found an unexpected potentiation of initial responses. Both effects were most prominent at low extracellular Ca\(^{2+}\) concentrations (Figures 1F–1I). Our mathematical modeling indicated that under ordinary circumstances, Ca\(^{2+}\)-dependent calmodulin binding to Unc13A may mediate STF by rapid stabilization of SV priming at release sites. Optimization of model parameters predicted a Ca\(^{2+}\) dissociation constant of \(40\) nM for this reaction, which is similar to, but somewhat lower than, in vitro estimates of ubMunc13-2 binding to calmodulin-coated beads (\(100\) nM).\(^{57}\) Differences could be due to some erroneous assumptions in our model (e.g., the relation between external and internal Ca\(^{2+}\) concentrations; Figure S1C) or rely on the slightly different amino acid sequence of the CaM domain (the consensus motif in Drosophila contains two tryptophan residues where the mammalian counterpart has one). On the other hand, our model predictions closely agree with estimates using photoaffinity labeling (\(30\) nM).\(^{58}\) Future experiments tracking single AZ activity might help clarify this.

Figure 6. Acute potentiation with PMA blocks PhTx-induced PHP

(A–D) Analysis of PhTx effects in current clamp recordings (muscle 6 NMJs, 0.2 mM extracellular Ca\(^{2+}\)) of (A and C) spontaneous and (B and D) AP-evoked synaptic activity in wild-type (w\(^{1118}\)) animals (A and B) without or (C and D) with prior PMA treatment. (A and C) Left: representative example traces of spontaneous mEPSPs from w\(^{1118}\) animals preincubated for 10 min with (A) control solution (–PMA) or (C) 2 \(\mu\)M PMA (+PMA) followed by 10 min incubation either with 20 \(\mu\)M PhTx (+PhTx) or without (–PhTx). Middle and right: quantification of mEPSP amplitudes and frequencies. (B and D) Left: representative AP-evoked eEPSP responses (average of five repetitions in one cell) of NMJs preincubated for 10 min (B) with control solution (–PMA) or (D) with 2 \(\mu\)M PMA (+PMA) followed by 10 min incubation either with (+PhTx, 20 \(\mu\)M) or without (–PhTx) philanthotoxin. Middle and right: quantification of eEPSP amplitude and quantal content. Number of cells (n) and animals (N) investigated: n/N (–PMA; –PhTx) = 15/8, n/N (–PMA; +PhTx) = 17/9, n/N (+PMA; –PhTx) = 14/7, n/N (+PMA; +PhTx) = 17/9. Genotype (see STAR Methods for details): w\(^{1118}\) wild type. Statistical analysis with Mann-Whitney U test. Mean values ± SEM are indicated in plots; n.s., p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Source data available in Table S6.
domain disruption aligns with autoinhibitory functions described for other (M)Unc13 domains (C2A, C1, and C2B), yet why a mutation that inhibits calmodulin interaction (at least in the mammalian orthologs) should cause this is not clear. One possibility is that under ordinary circumstances this interaction causes local buffering of Ca\(^{2+}\) directly at the release sites and that loss of this local buffering allows other Ca\(^{2+}\)-binding molecules relevant during SV priming and fusion to bind Ca\(^{2+}\) (see below). Another possibility is that the mutation itself renders Unc13A hyperactive by, e.g., inducing a conformational/functional change that bypasses the need for Ca\(^{2+}\)/calmodulin binding.

**Subsynaptic changes in Unc13A to dynamically regulate release site priming?**

While similar protein localizations and levels were seen in confocal microscopy (Figures 1B–1E), dual-color STED imaging resolved changes in the subsynaptic distribution of antibodies recognizing different Unc13A epitopes (Figure 3). Stronger signals at the AZ center were revealed by an antibody recognizing the C-terminal Unc13A MUN domain in synapses expressing Unc13A CaM\(^{WRWR}\) mutant proteins (Figures 3F, 3G, and S3). This could be consistent with a local enrichment of the protein or higher epitope availability. Another consideration is that the emission depletion in STED non-linearly depends on the laser intensity, and therefore, signals of surrounding pixels can influence this, meaning that intensity changes could be secondary to differences in signal localization. Consistent with this, we observed a shift in the locations of signal maxima from antibody labeling of the Unc13A MUN domain in Unc13A CaM\(^{WRWR}\) mutants closer to the AZ center and the plasma membrane (Figures 3F and S3). Conformational Unc13A changes could affect both local levels and epitope availability and such changes have been proposed to regulate NT release and STP. Our data are thus consistent with both propositions that closer membrane localization increases NT release and STP. The CaM\(^{WRWR}\) mutation might “lock” Unc13A in this high activity state (Figures 3D and 3H). While potentially beneficial to SV priming, a disadvantage of constitutive enrichment of the Unc13A C terminal at the plasma membrane could relate to SV replenishment under sustained stimulation, because “capturing” newly incoming vesicles might require their interaction with the C2C domain of (M)Unc13 in its extended conformation. This might explain why inhibiting (M)Unc13-calcmodulin interaction impairs RRP replenishment after strong stimulation.

**Different phases of PHP by cooperating mechanisms**

Classical experiments demonstrated robust clamp PHP in current recording preparations performed at low extracellular Ca\(^{2+}\). In these experiments, low Ca\(^{2+}\) was chosen for the technical reason that the quantal content can be assessed only when APs induce small eEPSPs, as otherwise these do not sum linearly from the mEPSPs recorded at rest. Moreover, our STP model predicts high capacity for potentiation by enhanced release-site priming at low extracellular Ca\(^{2+}\) due to a low steady-state release-site occupation (pSite\(_{occ}\)) (Figure 2C). As increased pSite\(_{occ}\) appears possible during millisecond STF (Figure 2E), PHP might be much faster than previously thought, and this mechanism could correspond to the “rapid, low-gain” homeostatic response on a timescale of seconds.

Following this immediate “ultrafast PHP,” further compensatory reactions appear necessary to reset the functional state of the synapse. Remarkably, when assayed 10 min after PhTx treatment, potentiated synapses have the same baseline STP profile as non-potentiated ones, and TEVC recordings at this time demonstrated PHP even at an extracellular Ca\(^{2+}\) concentration of 15 mM. This is not consistent with a mere increase in pSite\(_{occ}\) (which according to our model should change STP and should not be possible at high external Ca\(^{2+}\); Figure 2E). Instead, under these conditions the local enrichment of AZ components (BRP, RIM-BP, Unc13A) already seen on this timescale (which is essential for long-term stabilization of PHP) may be relevant. Thus, while it was demonstrated that PHP at 10 min could be maintained even when such enrichment was blocked, it is currently unknown whether under intact conditions an exchange of PHP mechanisms already occurs at this timescale. Thus, an important question future studies should address is how long-term PHP achieves a stable reset of the physiological properties from the “ultra-rapid” PHP at low external Ca\(^{2+}\) we describe here.

**(M)Unc13 proteins as integration hubs for release-site-based plasticity**

Our data indicate that STF, PHP, and PMA-induced plasticity phenomena at the Drosophila NMJ jointly converge on Unc13A. All types of potentiation were blocked by mutation of the Unc13A CaM domain (Figures 1A, 4, and 5). This is different from analyses of central Munc13-1/2 double-knockout mouse neurons rescued with Munc13-1, ubMunc13-2, or Munc13-3 CaM domain mutants, whose synapses showed potentiation of NT release following phorbol ester treatment. Also, PHP was shown to be intact in another study of the Drosophila NMJ where the sequence of phorbol ester and PhTx treatment was inverted (i.e., PhTx applied before the phorbol ester PdBu). The reason for these differences is unknown, but could relate to species-specific differences, genetic background, or different treatment conditions (PMA vs. PdBu, duration of treatment, extracellular Ca\(^{2+}\) concentration). Apart from a direct action on the (M)Unc13 C1 domains, phorbol esters also recruit and activate protein kinase C, which regulates synaptic targets, and we have not explored to what extent this signaling is relevant here. Another yet unexplored possibility is that differences in the local lipid environment (e.g., DAG/PI(4,5)P\(_2\) levels) between different synapse types cause altered sensitivities to these compounds.

Even though our data show that STF and PHP are blocked by activating the Unc13A CaM domain or PMA treatment, our experiments do not allow us to identify which signals are natively responsible for these plasticity phenomena. For STF, additional Ca\(^{2+}\)-dependent interactions, like Ca\(^{2+}\) binding to the (M)Unc13As C2B domain, seem relevant. Moreover, loss of the Ca\(^{2+}\)-sensing protein synaptotagmin-7, which also binds calcmodulin and functions in STF, SV replenishment, and vesicle (re)Docking, similarly increased baseline transmission
and abolished STF at low Ca^{2+}, as we observe here^{95,100} (Figure 1). This could indicate a cooperative function with Unc13A for STF. In addition, the Ca^{2+}-sensing protein Doc2 interacts with Munc13-1 and affects its subcellular localization, which could affect release-site occupation on distinct timescales and contribute to Doc2’s role in asynchronous/spontaneous NT release or synaptic augmentation.^{101–104} Thus, a picture is emerging in which Unc13A proteins are highly relevant plasticity targets that respond to a multitude of signals (Ca^{2+}, Ca^{2+}/calmodulin, DAG, and P(4,5)P_2/Ca^{2+}) to regulate release-site participation. The broad range of signals integrated likely relates to different forms of synaptic plasticity (e.g., facilitation/augmentation/potentiation) across extended timescales that all converge on Unc13A proteins for release-site-based plasticity.

**Limitations of the study**

Modeling studies can provide explanations of features of the data but can never prove that a certain theory is right. In this regard, while our model shows that our experimental data are consistent with the Unc13A CaM^{WRWR} causing a stabilization of priming, thereby blocking presynaptic plasticity, our analysis does not prove this. We have explored a limited set of conditions, and alternative options cannot be ruled out. For instance, we here found that changes in release-site occupation (pSite_{occ}) are sufficient to account for Unc13A CaM^{WRWR} mutant phenotypes but did not explore possible pV, changes (see above). Thus, while models are useful to conceptualize findings and explore hypotheses, they remain a stark simplification of reality.

Our STED analysis was limited to the situation of genetic/chronic mutation of the Unc13A CaM domain, and we therefore do not know whether such changes may also be observed acutely (e.g., upon PMA or PhTx treatment). However, a recent single-molecule imaging study reported a shift in Unc13A signals closer to the AZ center following PhTx treatment, which aligns the smaller Unc13A rings we observe in Unc13A CaM^{WRWR} mutants (Figure 3F).^{105} On the other hand, as these analyses relied on immunolabeling with antibodies, uncertainties in the exact protein positions are added by the dimensions of the label. Analysis of endogenously labeled proteins using single-molecule imaging, as recently established for the fly NMJ, combined with pharmacological treatment could be used to complement the analysis provided here.^{106,107}

PHP experiments were performed only at low external Ca^{2+} concentrations, which was necessary to accurately estimate quantal content in current clamp experiments (to ensure near-linear summation of mEPSPs to eEPSPs). However, how these phenomena are affected by the Unc13A CaM^{WRWR} mutation or PMA treatment under physiological conditions (1.5 mM extracellular Ca^{2+}) was not investigated. Doing so in the future (e.g., by use of TEVC recordings) will help clarify how different phases of PHP intersect to reestablish STP profiles and to ensure potentiation when the demand cannot be offset by an increase in pSite_{occ} alone (e.g., at extracellular Ca^{2+} concentrations above 3 mM, where our model predicts full site occupation under standard conditions).

Finally, whether the observed effects of PMA treatment are due to a direct activation of the Unc13A C1 domain or an indirect effect on Unc13A via PKC activation is not known.^{94} Future experiments using a mutant of the C1 domain that prevents DAG/PMA interaction can clarify this.^{44,46}

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **Key Resources Table**
- **Resource Availability**
  - Lead contact
  - Materials availability
  - Data and code availability
- **Experimental Model and Subject Details**
  - Fly Husbandry, stocks, and Handling
- **Method Details**
  - Mathematical modelling and simulation
  - Electrophysiology: Setup and data acquisition
  - Baseline transmission and STP in Unc13ACaM^{WRWR} mutants
  - Rapid PHP in Unc13A CaM^{WRWR} mutants
  - Assessment of phorbol ester induced presynaptic potentiation in Unc13-CaM mutants
  - Phorbol ester induced presynaptic potentiation in rapid PHP
  - Immunostaining, confocal/STED microscopy
  - Analysis of STED images
- **Quantification and Statistical Analysis**

**Supplemental Information**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112541.

**Acknowledgments**

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to A.M.W. (Emmy Noether Programme, project 261020751, and the TRR 186, project 278001972) and the Novo Nordisk Foundation (Young Investigator Award NNF19OC0056047 to A.M.W.). M.J. was supported by the Einstein Foundation Berlin (grant EZ-2014-226). We thank Dr. Stephan Sigrist for helpful comments and sharing antibodies.

**Author Contributions**


**Declaration of Interests**

M.J. is currently an employee of PPD Germany GmbH & Co. KG (Hansastrasse 32, 80686 Munich, Germany). M.A.B. is currently an employee of Lilly Deutschland GmbH (Werner-Reimers-Straße 2-4, 61352 Bad Homburg, Germany).

**Inclusion and Diversity**

We support inclusive, diverse, and equitable conduct of research.
REFERENCES


STAR METHODS

KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-guinea pig Alexa 488</td>
<td>Life Technologies</td>
<td>Cat# A11073, RRID: AB_2534117</td>
</tr>
<tr>
<td>Goat anti-mouse-Cy3</td>
<td>Jackson ImmunoResearch</td>
<td>Cat# 115-165-146, RRID: AB_2338690</td>
</tr>
<tr>
<td>Guinea-pig Unc13-N-term</td>
<td>Böhme, et al.</td>
<td>N/A</td>
</tr>
<tr>
<td>Rabbit Unc13-C-term</td>
<td>Reddy-Alla, et al.</td>
<td>N/A</td>
</tr>
<tr>
<td>Mouse monoclonal NC82</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>Cat# nc82, RRID: AB_2314865</td>
</tr>
<tr>
<td>Goat anti-rabbit StarRed</td>
<td>Abberior</td>
<td>STRED-1002-500UG</td>
</tr>
<tr>
<td>Goat anti-mouse StarOrange</td>
<td>Abberior</td>
<td>STORANGE-1001-500UG</td>
</tr>
<tr>
<td>Goat anti-guinea pig StarRed</td>
<td>Abberior</td>
<td>STRED-1006-500UG</td>
</tr>
<tr>
<td>Anti HRP-647</td>
<td>Jackson ImmunoResearch</td>
<td>123-605-021, AB_2338967</td>
</tr>
<tr>
<td>D. melanogaster: Wild type: w1118</td>
<td>Lab stock</td>
<td>N/A</td>
</tr>
<tr>
<td>D. melanogaster: unc13Bnull/unc13Bnull, P84200/P84200</td>
<td>Böhme, et al.</td>
<td>N/A</td>
</tr>
<tr>
<td>Software and algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matlab scripts for alignment STED images</td>
<td>This paper, accessible in Zenondo</td>
<td><a href="https://doi.org/10.5281/zenodo.7852141">https://doi.org/10.5281/zenodo.7852141</a></td>
</tr>
<tr>
<td>Matlab scripts unpriming model</td>
<td>This paper, accessible in Zenondo</td>
<td><a href="https://doi.org/10.5281/zenodo.7852141">https://doi.org/10.5281/zenodo.7852141</a></td>
</tr>
<tr>
<td>Clampfit software</td>
<td>Molecular Devices</td>
<td>V10.6.2.2</td>
</tr>
<tr>
<td>ImageJ</td>
<td>NIH</td>
<td>Version 1.53t</td>
</tr>
<tr>
<td>Matlab</td>
<td>MathWorks</td>
<td>R2020a</td>
</tr>
<tr>
<td>Calc</td>
<td>Jan, et al.</td>
<td>Version 6.8.6</td>
</tr>
<tr>
<td>GraphPad Prism</td>
<td>GraphPad Software</td>
<td>Version 9.4.1 (681)</td>
</tr>
<tr>
<td>Inspector Software</td>
<td>Max Planck Innovation</td>
<td>Version 16.3.16118-w2224; 16.3.14267-w2129</td>
</tr>
<tr>
<td>Adobe illustrator</td>
<td></td>
<td>Version 2022</td>
</tr>
</tbody>
</table>

RESOURCE AVAILABILITY

Lead contact
Further information and request for resources should be directed to and will be fulfilled by the lead contact, Alexander Mathias Walter (awalter@sund.ku.dk)

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Source data for each figure is available in the Tables S1–S7.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly Husbandry, stocks, and Handling
Fly strains were reared under standard laboratory conditions and raised at room temperature (Bloomington recipe). For experiments both male and female third instar larvae (larval stage 3) were used. The following genotypes were used: Wild-type: +/+
Mathematical modelling and simulation

We performed model simulations to investigate the possible mechanisms underlying the experimentally observed effects in Unc13CaMWRRR mutants. For this purpose, we used a published model of synaptic release describing the priming, unpriming and fusion of vesicles as a function of Ca\(^{2+}\). \(^{11}\) In brief the model works as follows:

First, for various extracellular Ca\(^{2+}\) concentrations (0.4mM, 0.75mM, 1.5mM, 3mM, 6mM) and for different locations from the Ca\(^{2+}\) sources (location 120 nm was used to plot Figure 2E), Ca\(^{2+}\) transients induced by two APs (10 ms interstimulus interval) were determined. This was done by deterministic simulation using the Calcium Calculator program, version 6.8.6 (CalC 6.8.6) created by Victor Matveev. \(^{110}\) The total amount of Ca\(^{2+}\)-charge flowing into the cell followed:

\[
Q = \frac{Q_{\text{max}} \cdot [Ca^{2+}]_{\text{ext}}}{[Ca^{2+}]_{\text{ext}} + K_{\text{M, current}}}
\]  
\[\text{(Equation 1)}\]

Where \(Q_{\text{max}}\) is the maximum charge flow, which was fitted (see below). \(K_{\text{M, current}}\) was set to 2.679 mM as in \(^{11}\). The resting intracellular calcium concentration was also assumed to be dependent on the extracellular Ca\(^{2+}\) concentration and followed:

\[
[Ca^{2+}]_{\text{basal}} = \frac{[Ca^{2+}]_{\text{max}} \cdot [Ca^{2+}]_{\text{ext}}}{K_{\text{M, current}} + [Ca^{2+}]_{\text{ext}}}
\]  
\[\text{(Equation 2)}\]

In this equation, \([Ca^{2+}]_{\text{basal}}\) is the resting intracellular Ca\(^{2+}\) concentration, \([Ca^{2+}]_{\text{max}}\) is the maximal intracellular Ca\(^{2+}\) concentration (\([Ca^{2+}]_{\text{max}} = 190\text{nM}\), \([Ca^{2+}]_{\text{ext}}\) is the extracellular Ca\(^{2+}\) concentration and \(K_{\text{M, current}}\) is the extracellular Ca\(^{2+}\) concentration corresponding to the half of maximal intracellular Ca\(^{2+}\) concentration (see Table 1).

Next, \(N\) release sites were placed at heterogeneously located positions from the Ca\(^{2+}\) source. The Ca\(^{2+}\) transient corresponding to each release site was read out and used as input for exocytosis simulation. This simulation stochastically computes vesicle priming, unpriming and fusion of primed vesicles. Synaptic vesicle fusion was modelled following the single-sensor model designed by, \(^{68}\) and \(\text{rederived the function for } k_{\text{unprime}} \text{ based on the following considerations:}\)

The rate of unpriming from the non-stabilized primed state (without any Ca\(^{2+}\)-dependent stabilization) depends on the energy difference between this state and the transition state of the reaction according to the Eyring/Arrhenius equation:

\[
k_{\text{unprime}}(Ca^{2+} = 0) = A \cdot e^{-\frac{E^*}{k_B T}}
\]  
\[\text{(Equation 3)}\]

Where \(E^*\) signifies the energy of the transition state, \(k_B\) is the Boltzmann constant and \(T\) the absolute temperature in Kelvin. Maximal stabilization of the primed state (e.g., when Ca\(^{2+}\) exerts its maximal effect) decreases the free energy of the primed state for each (m) Unc13A protein that stabilizes it. That increases the energy difference to the transition state and therefore slows the unpriming rate:

\[
k_{\text{unprime}}(Ca^{2+} = \text{max}) = A \cdot e^{-\frac{E^* - m \Delta_rG(Ca_n - CaM - Unc13A)}{k_B T}}
\]  
\[\text{(Equation 4)}\]

Where \(\Delta_r\text{G}(Ca_n - CaM - Unc13A)\) is the enthalpy provided for each Ca\(^{2+}\)/Calmodulin/Unc13A interaction and “n” is the number of Ca\(^{2+}\) ions bound per complex. Above equation simplifies to:

\[
k_{\text{unprime}}(Ca^{2+} = \text{max}) = A \cdot e^{-\frac{m \Delta_rG(Ca_n - CaM - Unc13A)}{k_B T}} + e^{-\frac{m \Delta_rG(Ca_n - CaM - Unc13A)}{k_B T}}
\]  
\[\text{(Equation 5)}\]

\[
k_{\text{unprime}}(Ca^{2+} = \text{max}) = k_{\text{unprime}}(Ca^{2+} = 0) \cdot e^{-\frac{m \Delta_rG(Ca_n - CaM - Unc13A)}{k_B T}}
\]  
\[\text{(Equation 6)}\]

The affinity of Ca\(^{2+}\)/Calmodulin/Unc13A interaction can be described by the dissociation constant of the complex:

\[
K_0 = \frac{[Ca^{2+}]^n \cdot [CaM - Unc13A]}{[Ca_n - CaM - Unc13A]}
\]  
\[\text{(Equation 7)}\]
Where \([\text{Ca}^{2+}]\) is the intracellular \(\text{Ca}^{2+}\) concentration. The Gibbs free energy of \(\text{Ca}^{2+}/\text{Calmodulin}/\text{Unc13A}\) binding relates to the steady state affinity as following:

\[
\Delta_n G(Ca_n - \text{CaM} - \text{Unc13A}) = -k_B T \times \ln \left(\frac{1}{K_D^n}\right)
\]

(Equation 8)

Or

\[
\Delta_n G(Ca_2 - \text{CaM} - \text{Unc13A}) = k_B T \times \ln(K_D^n)
\]

(Equation 9)

Combining above equations gives:

\[
k_{\text{unprime}}(\text{Ca}^{2+} = \text{max}) = k_{\text{unprime}}(\text{Ca}^{2+} = 0) \times e^{\frac{-m k_B T \ln(K_D^n)}{R T}}
\]

(Equation 10)

Which simplifies to

\[
k_{\text{unprime}}(\text{Ca}^{2+} = \text{max}) = k_{\text{unprime}}(\text{Ca}^{2+} = 0) \times K_D^{-m}
\]

(Equation 11)

Thus, the unpriming rate from the stabilized primed state is directly related to the affinity of \(\text{Ca}^{2+}\) binding to Unc13A/Calmodulin.

We further assumed that the proportion of stably and unstably primed vesicles is in equilibrium and that this depends on the \(\text{Ca}^{2+}\) binding to Unc13A/Calmodulin. The equilibrium between stabilized release sites \(N_{\text{stable}}\) and non-stabilized release sites \(N_{\text{unstable}}\) depends on the number (\(m\)) of Unc13A proteins contributing to stabilization and takes the following form:

\[
\frac{[\text{Ca}^{2+}]^{n-m} + N_{\text{unstable}}}{N_{\text{stable}}} = K_D^{-m}
\]

(Equation 12)

The unpriming rate was decomposed to a combination of unpriming reactions from stably and non-stably primed vesicles:

\[
k_{\text{unprime}}(\text{Ca}^{2+}) = \frac{N_{\text{unstable}}}{N_{\text{total}}} \times k_{\text{unprime}}(\text{Ca}^{2+} = 0) + \frac{N_{\text{stable}}}{N_{\text{total}}} \times k_{\text{unprime}}(\text{Ca}^{2+} = \text{max})
\]

(Equation 13)

\[
k_{\text{unprime}}(\text{Ca}^{2+}) = \frac{N_{\text{unstable}}}{N_{\text{total}}} \times k_{\text{unprime}}(\text{Ca}^{2+} = 0) + \frac{N_{\text{stable}}}{N_{\text{total}}} \times k_{\text{unprime}}(\text{Ca}^{2+} = 0) \times K_D^{-m}
\]

(Equation 14)

Where \(N_{\text{total}}\) is the total number of release sites. Assuming mass conservation of sites:

\[
N_{\text{total}} = N_{\text{stable}} + N_{\text{unstable}}
\]

(Equation 15)

One can express the population of sites in the two states as:

\[
N_{\text{stable}} = \frac{[\text{Ca}^{2+}]^{n-m} \times N_{\text{total}}}{K_D^{-m} + [\text{Ca}^{2+}]^{n-m}}
\]

(Equation 16)

And

\[
N_{\text{unstable}} = \frac{K_D^{-m} \times N_{\text{total}}}{K_D^{-m} + [\text{Ca}^{2+}]^{n-m}}
\]

(Equation 17)

Combining above equations simplifies the expression to:

\[
k_{\text{unprime}}(\text{Ca}^{2+}) = \frac{[\text{Ca}^{2+}]^{n-m} + 1}{K_D^{-m} + [\text{Ca}^{2+}]^{n-m}} \times K_D^{-m} \times k_{\text{unprime}}(\text{Ca}^{2+} = 0)
\]

(Equation 18)

For the model the number of \(\text{Ca}^{2+}\) ions bound per Calmodulin complex was set to \(n=2\). For mutant simulations, \(k_{\text{unprime}}\) was set to be \((\text{Ca}^{2+}\)–independently)

\[
k_{\text{unprime}}(\text{Unc13A CaM}^{\text{WRWR mutant}}) = k_{\text{unprime}}(\text{Ca}^{2+} = 0) \times K_D^{-m}
\]

(Equation 19)

for all \(\text{Ca}^{2+}\) concentrations.
Baseline transmission and STP in Unc13ACaMWRWR mutants

Q_{\text{max}}, K_{D}, k_{\text{unprime(Ca}^{2+} = 0)}, N_{\text{total}}, m, and the priming rate constant were fitted to the eEPSC_{1} amplitudes and PPRs obtained in the control and Unc13CaMWRWR experimental conditions (Figure 1). Other parameters were set to the same values as in the original model\(^\text{11}\) (see Table 1). Fitting was performed using fminsearch in Matlab, which minimized the following cost function:

\[
\text{cost}\left(\text{eEJC}_{1,\text{sim,ctrl}}, \text{eEJC}_{2,\text{sim,ctrl}}, \text{eEJC}_{1,\text{sim,Unc13CaMWRWR}}^{\text{mm}}, \text{eEJC}_{2,\text{sim,Unc13CaMWRWR}}^{\text{mm}}\right) = \sum_{k=1}^{5} \left( \frac{\text{eEJC}_{1,\text{sim,ctrl}} - \text{eEJC}_{1,\text{exp,ctrl}}}{\text{eEJC}_{1,\text{exp,ctrl}}} \right)^{5} + \left( \frac{\text{eEJC}_{2,\text{sim,ctrl}} - \text{PPR}_{\text{exp,ctrl}} * \text{eEJC}_{1,\text{exp,ctrl}}}{\text{PPR}_{\text{exp,ctrl}} * \text{eEJC}_{1,\text{exp,ctrl}}} \right) + \sum_{k=1}^{5} \left( \frac{\text{eEJC}_{1,\text{sim,Unc13CaMWRWR}}^{\text{mm}} - \text{eEJC}_{1,\text{exp,Unc13CaMWRWR}}^{\text{mm}}}{\text{eEJC}_{1,\text{exp,Unc13CaMWRWR}}^{\text{mm}}} \right)^{5} + \left( \frac{\text{eEJC}_{2,\text{sim,Unc13CaMWRWR}}^{\text{mm}} - \text{PPR}_{\text{exp,Unc13CaMWRWR}}^{\text{mm}} * \text{eEJC}_{1,\text{exp,Unc13CaMWRWR}}^{\text{mm}}}{\text{PPR}_{\text{exp,Unc13CaMWRWR}}^{\text{mm}} * \text{eEJC}_{1,\text{exp,Unc13CaMWRWR}}^{\text{mm}}} \right) \right)
\]

(Equation 20)

In this function, k indicates the Ca\(^{2+}\) concentration tested (k(1:5) = 0.4, 0.75, 1.5, 3, 6 mM). Simulated eEJC amplitudes (eEJC\(_{1/2,\text{sim}}\)) were obtained via deterministic model simulations. The initial values for the fitting were: Q_{\text{max}} = 13.77 fC, priming rate constant = 135 s\(^{-1}\), k_{\text{unprime(Ca}^{2+} = 0)} = 236 s\(^{-1}\), m = 2.5, K_{D} = 100 nM. Figures were generated by running the stochastic version of the model 200 times using the fitted parameters Q_{\text{max}} = 14.04 fC, priming rate constant = 92.16 s\(^{-1}\), k_{\text{unprime(Ca}^{2+} = 0)} = 265 s\(^{-1}\), m = 2.04, K_{D} = 40 nM, N = 240. To reduce the number of data points in the figure simulated number of primed vesicles over time and the eEPSCs were downsamples to every 100th data point. All simulations were performed on MATLAB (version R2020a).

**Electrophysiology: Setup and data acquisition**

For all electrophysiological experiments, third instar larvae were dissected using standard procedures\(^\text{108,111}\). In brief, crawling third instar larvae were pinned dorsal side up onto a piece of Sylgard (184, Dow Corning, Midland, MI, USA) with two pins at the anterior and posterior end. A drop of either Ca\(^{2+}\)-free HL3\(^\text{112}\); 70 mM NaCl, 5 mM KCl, 20 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM trehalose, 115 mM sucrose, 5 mM HEPES (pH adjusted to 7.2), or modified low Mg\(^{2+}\) HL3\(^\text{113}\); 70 mM NaCl, 5 mM KCl, 5 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 10 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM Trehalose, 115 mM D-Saccharose, 5 mM HEPES (pH adjusted to 7.2) covering the animal was added. The larva was opened by cutting the dorsal side along its entire length. The larva was opened and the fillet pinned down with four additional pins. The Sylgard piece was transferred to the recording chamber containing HL3 solution with Ca\(^{2+}\). Electrophysiological current clamp and TEVC recordings were performed at 21°C from muscle 6 of abdominal segments A2 and A3 using sharp glass electrodes (borosilicate glass with filament, 0.86 x 1.5 x 80 nm, Science products, Hofheim, Germany) pulled with a P97 pipette puller (Sutter Instrument, CA, USA).

Signals were recorded using a 5 KHz lowpass filter at a sampling frequency of 20 kHz using the Digitax 1440A digitizer (Molecular devices, Sunnyvale, CA, USA) with Clampex (v10.6) software and an Axoclamp 900A amplifier (Axon instruments, Union City, CA, USA) with Axoclamp software. Cells with resting membrane potentials higher than -49 mV and resistances below 4 MΩ prior to measurements were excluded from the datasets. For all TEVC recordings, the postsynaptic muscle cell was clamped at -70 mV and only cells with absolute leak currents below 10 nA throughout individual experiments were included into the analysis. Obtained data was analyzed using Clampfit software (v10.6.2.2).

**Rapid PHP in Unc13CaMWRWR mutants**

For experiments shown in Figures 4 and 6, current clamp experiments were performed using low Mg\(^{2+}\) HL3\(^\text{113}\); 70 mM NaCl, 5 mM KCl, 10 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM Trehalose, 115 mM D-Saccharose, 5 mM HEPES). Rapid PHP was induced using a final concentration of 20 µM of the glutamate receptor blocker Philantotoxin433 (Phtx433, SigmaAldrich, MO, USA, stored at 20C as 4 mM stock in double distilled water (ddH\(_2\)O)). The same volume of ddH\(_2\)O was used in control...
Assessment of phorbol ester induced presynaptic potentiation in Unc13-CaM mutants

For experiments shown in Figure 5, TEVC experiments were performed using normal HL3 (pH adjusted to 7.2). Prior to dissection, animals were pre-incubated for 10 minutes in HL3 (0 mM Ca\(^{2+}\)) containing 2 \(\mu\)M of phorbol ester Phorbol-12-myristat-13-acetate (PMA, Sigma Aldrich, Germany), stored at -20°C as 10 mM stock in dimethyl sulfoxide (DMSO), or the same volume of DMSO for control experiments. For this, a vertical incision to the larval body wall muscles was made and 30 \(\mu\)l of the incubation solution was pipetted into the abdominal larval cavity. During the last 2 minutes of incubation, larvae were dissected, rinsed 3 times with Ca\(^{2+}\)-free HL3 once incubation was completed and transferred into the recording chamber containing HL3 (containing 0.4 mM or 1.5 mM Ca\(^{2+}\)). The postsynaptic muscle cell was clamped at -70 mV in TEVC mode and only cells with absolute leak currents below 10 nA throughout recordings were included in the analysis. Cells were stimulated 10 times by giving two paired stimulating pulses to the innervating motoneuron (300 \(\mu\)s pulses of 8 V, paired pulse stimulation with 10 -ms- inter-stimulus interval repeated at 0.1 Hz). Analysis of AP-evoked responses was performed using pClamp software (Molecular devices, Sunnyvale, CA, USA) by estimating the amplitudes for eEPSC\(_1\) and eEPSC\(_2\) for each stimulation and averaging these to generate cell averages. Paired pulse ratios (PPRs) were calculated by dividing the amplitude of eEPSC\(_2\) by eEPSC\(_1\) and averaging these values for 10 stimulations in each cell.

Phorbol ester induced presynaptic potentiation in rapid PHP

For data presented in Figure 6, current clamp experiments were performed using low Mg\(^{2+}\) HL3 (pH adjusted to 7.2: 70 mM NaCl, 5 mM KCl, 10 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM Trethylalose, 115 mM D-Saccharose, 5 mM HEPES). Prior to dissection, animals were first pre-incubated for 10 minutes in low Mg\(^{2+}\) HL3 (10 mM Mg\(^{2+}\), 0 mM Ca\(^{2+}\)) containing 2 \(\mu\)M of phorbol ester Phorbol-12-myristat-13-acetate (PMA, Sigma Aldrich, Germany), stored at -20°C as 10 mM stock in DMSO) or the same volume of DMSO for control experiments. For this, a vertical incision to the larval body wall muscles was made and 30 \(\mu\)l of the incubation solution was pipetted into the abdominal larval cavity. After 10 minutes, the first incubation solution was removed exchanged by 30 \(\mu\)l of low Mg\(^{2+}\) HL3 (0 mM Ca\(^{2+}\), 10 mM Mg\(^{2+}\)) containing PhTx (20 \(\mu\)M) or the same volume of ddH\(_2\)O in control treatments and incubated for further 10 minutes. After preparation, the larvae were rinsed 3 times with Ca\(^{2+}\)-free low Mg\(^{2+}\) HL3 (10 mM Mg\(^{2+}\)) transferred into the recording chamber containing 0.2 mM Ca\(^{2+}\). This concentration of extracellular Ca\(^{2+}\) was chosen based on a baseline experiment where eEPSP responses after PMA treatment were probed (not shown in this paper), so that a ceiling effect of evoked responses could be avoided and the QC could be determined accurately. \(^{113}\) mEPSPs were recorded for 60 s prior to evoking eEPSP. eEPSP responses were elicited by giving 5 short stimulation pulses (300 \(\mu\)s, 8 V, 0.1 Hz) to the respective nerve. For analysis, mEPSP traces were post hoc filtered with a 500 Hz Gaussian low pass software filter and mEPSP templates were generated for each cell. Using this template, events were identified throughout the 60 s trace and used for analysis. eEPSP amplitudes were calculated by averaging all 5 stimulated responses and quantal contents were calculated by dividing average eEPSP by the average mEPSP- amplitude in each cell.

Immunostaining, confocal/STED microscopy

Third-instar larvae were placed onto a dissection plate with their dorsal sides facing up. Larvae were fixed by placing fine pins in the head and tail (Austerlitz INSECT PINS; 0.10 mm). Afterwards 50 \(\mu\)l ice-cold modified hemolymph-like solution 112 (HL3, pH adjusted to 7.2: 70 mM NaCl, 5 mM KCl, 20 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM Trethylalose, 115 mM D-Saccharose, 5 mM HEPES) was pipetted onto the larvae. Sharp dissection scissors were used to make a small incision in the dorsal, posterior midline of each larva. From that cut larvae were completely opened along the dorsal midline to their anterior end. Following this, the epidermis was pinned down twice on each side and slightly stretched. The internal organs and tissues were removed carefully with forceps. Subsequently, the dissected larvae were washed 3x with 50 \(\mu\)l ice-cold HL3 on the pad and then transferred into a 5% native goat serum (NGS; Sigma-Aldrich, MO, USA, S2007) diluted in phosphate buffered saline (Carl Roth, Germany) with 0.05% Triton-X100 (PBT). The sample was blocked for 1h at room temperature and then incubated with primary antibodies mouse Nc82 = anti-BRP\(^{C-term}\) 61 (1:200 for confocal (Figures 1B, 1C, and 1E) and 1:500 (Figures 3E–3G, S3C, S3D and S4) or 1:100 (Figures 3A–3C, S3A and S3B) for STED microscopy, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; AB Registry ID: AB_2314865) and guinea-pig Unc13A 25 (1:500 for confocal (Figures 1B and 1D) and 1:200 (Figures 3A–3C) for STED microscopy) or rabbit Unc13-C-Term 30 (Figures 3E–3G, 1:100) diluted in 5% NGS in PBT overnight at 4 degrees. The next day, the sample was briefly rinsed 5x with PBT and then washed again 4x in PBTP for 20 minutes. Afterwards, the larvae were incubated for 4h with fluorescence labeled secondary antibodies. For confocal imaging: goat anti-guinea pig Alexa-Fluor 488 (1:500, Life Technologies A11073, CA, USA), goat anti-mouse-Cy3 (1:500, Jackson ImmunoResearch 115-165-146) and goat anti-HPR-647 (1:500, Jackson ImmunoResearch 123-605-021, PA, USA). For STED microscopy: goat anti-rabbit Star Red (1:100, Abberior STED-1001-500UG), goat anti-guinea pig Star Red (1:100, Abberior STED-10006-500UG) and goat anti-mouse Star Orange (1:100, Abberior STORANGE-1002-500UG). All secondary antibodies were diluted in 5% NGS in PBT. Afterwards, the samples were briefly...
washed 3x with PBT and then washed again 4x in PBT for 15 minutes. For confocal imaging larvae were mounted in Vectashield (Vector labs, CA, USA) and for STED imaging in ProLong™ Gold Antifade Mountant (Thermo Fisher P36930) or Prolong™ Glass Antifade Mountant (Thermo Fisher P36980) on a microscope slide (Carl Roth, Germany; H868) and sealed with a coverslip (Carl Roth, Germany, H 875).

Confocal microscopy was performed with a Leica SP8 microscope (Leica Microsystems, Germany). Images of fixed and live samples were acquired at room temperature. Confocal imaging of NMJs was done using a z-step of 0.25 μm. The following objective was used: 63 x 1.4 NA oil immersion for NMJ confocal imaging. All confocal images were acquired using the LAS X software (Leica Microsystems, Germany). Images from fixed samples were taken from muscle 4 of 3rd instar larval 1b NMJs (segments A2-A5). Confocal stacks were processed with ImageJ software (http://rsbweb.nih.gov/ij/).

AZ protein levels were quantified using the BRP signal as a reference. Briefly, the signal of the HRP-647 antibody was used as template for a mask, restricting the quantified area to the shape of the NMJ. To determine the synaptic protein levels, ImageJ was used to detect local intensity maxima in the BRP channel, thus identifying individual AZs. These locations were used to generate equally sized regions of interest (ROIs, size: 5 pixel, pixel size: 100 nm) from which pixel intensities (integrated density) were read out in both the BRP and Unc13A channel. Images for figures were processed with ImageJ software to enhance brightness using the brightness/contrast function.

An Abberior Infinity Line confocal and 3D STED super-resolution microscope was used to record two-color STED images. The microscope combines four pairs of excitation laser beams of 640 nm, 561 nm, 485 nm and 405 nm wavelength with one STED fiber laser beam at 775nm. For this analysis, the excitation lasers 640 nm and 561 nm were used together with the STED laser. 2D STED was performed on a single confocal section using an Olympus 60X NA1.4 (Oil) [UPLXAP060XO] objective. The pixel size was set to 10 nm (Figures 3A–3C, S3A and S3B) or 20 nm (Figures 3E–3G, S3C, S3D and S4) and the power of the excitation and STED laser chosen such that we gained the highest resolution with least background noise and kept for all NMJs imaged on one day. Ispector Software (16.3.14287-w2129 or 16.3.16118-w2224, Max Planck Innovation, Germany) was used to acquire all STED images. For figures the images were processed with ImageJ software 1.53c to adjust brightness/contrast.

To calculate the STED microscope’s effective lateral point spread function, we used 20-nm CRIMSON beads (Abberior; excitation and emission maxima at 625/645 nm). STED imaging of nanobeads was performed using the same objective, 640 nm excitation laser and 775 nm STED fiber laser beam. The pixel size was set to 10 nm. Line profiles were generated along X and Y axes of 6 smallest nanobeads using ImageJ (version 1.53t). These line profiles were fitted with a gaussian distribution using Graphpad Prism (version 9). FWHM (full width at half maximum) amplitude of the Gaussian was calculated from the standard deviation (SD) using the following relationship:

\[
FWMH = 2.355 \times SD
\]

Analysis of STED Images

Intensity/line profiles (Figure 3) were generated from co-stained STED images (BRP stained together with Unc13A C-term or Unc13A N-term). For AZ in side view, we manually drew line ROIs through the center of elongated BRP structures. ROIs were always drawn from plasma membrane towards cytoplasm. The same ROIs were then used in the Unc13A channel. We then plotted the line profile using ImageJ (version 1.53t). Only AZs with clear discernible intensity peaks in the BRP and Unc13A channel were included. Next, we identified the peak in the BRP intensity values. This location was set as zero. Intensity values from 120 nm from this point towards the plasma membrane to 120 nm towards the cytoplasm were then selected. The same ROIs were then used in the Unc13A channel. We then plotted the line profile out in both channels (size 25x25 pixel, pixel size 20 nm). Then, we manually drew line ROIs through the center of the elongated BRP structure in all individual images (first channel). The lines were placed such that they followed the longitudinal section of the BRP signal/structure. The lines were saved in a third channel. To indicate the location of the plasma membrane lines were added facing towards the cytoplasm and saved as a fourth channel. A custom-written MATLAB (2022a) script was used to align all AZs to the middle and rotated such that all AZs face towards the plasma membrane. In this script, we first determined the midpoint of the line ROIs (third channel) by taking the average of the coordinates of the start and end points of the line. The computed midpoint was rounded to whole integer values. The images in the BRP and Unc13 channel, as well as the line ROI itself and the direction line in

Cell Reports 42, 112541, June 27, 2023 23
the fourth channel, were translated by the difference between the computed midpoint of the line and the desired midpoint of the line (coordinate (13,13)). Subsequently, we rotated the images in the four channels such that the line ROIs would be orientated horizontally. For this we first computed the current angle of the line ROI with the horizontal midline using:

$$\alpha = \tan^{-1}\left(\frac{\text{Perpendicular}}{\text{Base}}\right)$$

(Equation 22)

The perpendicular and base line were computed by taking the difference between y-coordinates and x-coordinates, respectively, of the start and end points of the translated line ROI. All four channels were rotated with this angle using the MATLAB function imrotate. As a final step in the rotation of the images, we rotated all channels by 180 degrees when the direction line ROI in the fourth channel was below the horizontal midline in the image. This generates images in which the plasma membrane is facing downwards. Individual images were normalized such that the total value of all elements in the 25x25 image summed to 1. Afterwards, average images were computed. To generate the line profiles shown in Figure S4, we extracted the intensity values in the BRP and Unc13 channels of the vertical line through the midpoint of the image. For each individual AZ, we scaled these intensity values such that the area under the curve would sum to 1. Bootstrapping of the lines profiles was performed 100x and for each bootstrap repetition the average of the scaled intensity lines was taken. To generate the average images and line profiles for AZs viewed from the top, we followed a similar procedure. In this case, we, however, drew circles on the top view BRP images and aligned images from both the BRP and the Unc13 channel to the midpoint of this circle (no rotation was performed).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data were analyzed using Prism (GraphPad Software, CA, USA). To compare two groups, two tailed t tests or Mann-Whitney U tests were used for all datasets. For the comparison of fluorescence profiles depicted in Figure 3, a repeated measures two-way ANOVA analysis was used. p values and N values are given in each figure legend and in Tables S1–S7. Means are annotated ± SEM. Asterisks are used to denote significance: *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s. (not significant), p > 0.05.