Munc13-1 is a Ca\textsuperscript{2+}-phospholipid-dependent vesicle priming hub that shapes synaptic short-term plasticity and enables sustained neurotransmission

Graphical abstract

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In brief
Using novel knockin mouse models, Lipstein et al. show that Ca\textsuperscript{2+}-phospholipid binding activates the presynaptic protein Munc13-1 to fine-tune the rate of synaptic vesicle replenishment according to synaptic activity. This process determines short-term synaptic plasticity and the temporal fidelity of synaptic transmission in the auditory brainstem and the hippocampus.

Highlights
- The Munc13-1 C\textsubscript{2}B domain controls synaptic vesicle replenishment rates
- Blocking Ca\textsuperscript{2+}-phospholipid-C\textsubscript{2}B signaling attenuates vesicle replenishment
- Enhancing Ca\textsuperscript{2+}-phospholipid-C\textsubscript{2}B signaling accelerates vesicle replenishment
- This process determines short-term plasticity and fidelity of synaptic transmission
Article

Munc13-1 is a Ca$^{2+}$-phospholipid-dependent vesicle priming hub that shapes synaptic short-term plasticity and enables sustained neurotransmission

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SUMMARY

During ongoing presynaptic action potential (AP) firing, transmitter release is limited by the availability of release-ready synaptic vesicles (SVs). The rate of SV recruitment (SVR) to release sites is strongly upregulated at high AP frequencies to balance SV consumption. We show that Munc13-1—an essential SV priming protein—regulates SVR via a Ca$^{2+}$-phospholipid-dependent mechanism. Using knockin mouse lines with point mutations in the Ca$^{2+}$-phospholipid-binding C2B domain of Munc13-1, we demonstrate that abolishing Ca$^{2+}$-phospholipid binding increases synaptic depression, slows recovery of synaptic strength after SV pool depletion, and reduces temporal fidelity of synaptic transmission, while increased Ca$^{2+}$-phospholipid binding has the opposite effects. Thus, Ca$^{2+}$-phospholipid binding to the Munc13-1-C2B domain accelerates SVR, reduces short-term synaptic depression, and increases the endurance and temporal fidelity of neurotransmission, demonstrating that Munc13-1 is a core vesicle priming hub that adjusts SV re-supply to demand.

INTRODUCTION

Neuronal signaling at chemical synapses requires transmitter release by synaptic vesicle (SV) fusion. A fraction of SVs residing at molecularly defined release sites constitutes the pool of readily releasable SVs (RRP) (Kaeser and Regehr, 2017), which are in a molecularly mature—“primed”—state to allow rapid membrane fusion upon an action potential (AP)-induced increase in cytosolic Ca$^{2+}$ ([Ca$^{2+}$]). To maintain synaptic transmission during continuous activity, empty release sites must be re-populated with fusion-competent SVs (SV recruitment [SVR]).

SVR is generally slow, with a time constant of several seconds (Fuhrmann et al., 2004; Pyott and Rosenmund, 2002; Stevens and Tsujimoto, 1995; von Gersdorff et al., 1997; Wesseling and Lo, 2002), but can be transiently accelerated by an order of magnitude following bouts of synaptic activity (Sakaba and Neher, 2001a; Stevens and Wesseling, 1998), most likely due to the buildup of presynaptic [Ca$^{2+}$], (Dittman and Regehr, 1998; Sakaba and Neher, 2001a). This enables synapses to maintain high levels of transmitter release during presynaptic AP firing, resulting in reduced synaptic short-term depression (STD), and to speed up the recovery of synaptic strength following activity while [Ca$^{2+}$] decays back to resting levels (Dittman et al., 2000; Fuhrmann et al., 2004; Wang and Kaczmarek, 1998). By counteracting SV pool depletion, the SVR speed is a key determinant of synaptic strength and short-term plasticity (STP) and affects multiple complex brain processes (Zucker and Regehr, 2002).

Because [Ca$^{2+}$], controls several steps of the SV cycle and regulates numerous Ca$^{2+}$-binding proteins (for review, see Alabi and Tsien, 2012 and Neher and Sakaba, 2008), the identity of proteins that mediate activity-dependent SVR acceleration has remained enigmatic. Moreover, beyond their [Ca$^{2+}$], sensitivity, SVR-regulating pathways also respond to lipid second messengers. During activity, the presynaptic membrane lipid composition changes as a consequence of SV fusion and the activity of lipid-modifying enzymes, some of which are Ca$^{2+}$ regulated. In neuroendocrine cells, for example, vesicle fusion occurs at membrane sites rich in PIP$_2$ (phosphatidylinositol-4,5-bisphosphate) and PIP$_2$ augments RRP replenishment (Kabachinski et al., 2014; Milosevic et al., 2005; Walter et al., 2017). PIP$_2$ conversion to diacylglycerol (DAG) regulates transmitter release in cultured neurons (Rhee et al., 2002; Wierda et al., 2007) and in situ (Lou et al., 2008; Malenka et al., 1987).
The present study was designed to demonstrate a Ca\(^{2+}\)- and phospholipid-controlled step in SVR and STP regulation. We focused on the SV priming protein Munc13-1, a presynaptic active zone (AZ) component that is essential for transmitter release (Augustin et al., 1999b; Varoqueaux et al., 2002). Munc13-1 operates by regulating the conformation of the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) fusion protein syntaxin, thus promoting partial SNARE complex formation and close SV-plasma membrane contact (Imig et al., 2014; Lai et al., 2017; Ma et al., 2011). The Munc13-1 MUN domain, which mediates SV priming, is located downstream of three regulatory domains: a Ca\(^{2+}\)-calmodulin (Ca\(^{2+}\)-CaM) binding motif (Junge et al., 2004; Lipstein et al., 2012a, 2013; Piotrowski et al., 2020) a DAG-binding C\(_{2}\) domain (Betz, 1998), and a C\(_{2}\) domain (C\(_{2B}\)) that binds phospholipids in a Ca\(^{2+}\)-dependent manner (Michelassi et al., 2017; Shin et al., 2010). These regulatory domains profoundly affect Munc13-1 activity in vitro (Junge et al., 2004; Rhee et al., 2002; Shin et al., 2010). However, a block of CaM binding to Munc13-1 has only subtle effects on SV priming during and after AP trains in intact circuits (Lipstein et al., 2012a; Lipstein et al., 2013). These regulatory domains profoundly affect Munc13-1 activity in vitro (Junge et al., 2004; Rhee et al., 2002; Shin et al., 2010). However, a block of CaM binding to Munc13-1 has only subtle effects on SV priming during and after AP trains in intact circuits (Lipstein et al., 2012a; Lipstein et al., 2013).

In sum, we generated two novel KI mouse lines with specific point mutations in the Munc13-1 C\(_{2B}\) domain that abolish (C\(_{2BDN}\)) or increase (C\(_{2BKW}\)) Ca\(^{2+}\)-dependent phospholipid binding without altering Munc13-1 expression or localization.

**Basal transmission and presynaptic Ca\(^{2+}\) influx in Munc13-1 C\(_{2BDN}\) and C\(_{2BKW}\) KI synapses**

To assess the role of the Munc13-1 C\(_{2B}\) domain in synaptic transmission and STP, we used the calyx of Held synapse, as it is accessible to pre- and postsynaptic recordings (Borst et al., 1995; Forsythe, 1994). Recordings were obtained from homozygous mut and WT littermates (mut\(_{DN}\) and WT\(_{DN}\) for C\(_{2BDN}\) KIs; mut\(_{KW}\) and WT\(_{KW}\) for C\(_{2BKW}\)) at postnatal day (P) 14–P17, i.e., after hearing onset, when Munc13-1 is functionally dominant (Chen et al., 2013). Only minor differences were observed between WT\(_{DN}\) and WT\(_{KW}\) (Table S1).

To assay synaptic strength and evoked excitatory postsynaptic current (eEPSC) kinetics, we recorded unitary eEPSCs in voltage-clamped MNTB PNs (Figure 2A). Unless stated otherwise, recordings were obtained in the presence of 1 mM kynurenic acid (kyn) (STAR Methods; Figure S2). In C\(_{2BDN}\) mice, we observed larger eEPSCs in mut\(_{DN}\) as compared to WT\(_{DN}\) (Figures 2A–2C; Table S1), whereas in C\(_{2BKW}\) mice, eEPSC amplitudes were indistinguishable between mut\(_{KW}\) and WT\(_{KW}\). eEPSC kinetics were unaltered (Figures 2B and 2C; Table S1). Scatter-plots of eEPSC rise time versus eEPSC half-width revealed a positive correlation (Figure S2), reflecting a developmental eEPSC shortening (Joshi et al., 2004; Koike-Tani et al., 2005; Taschenberger and von Gersdorff, 2000). This correlation was similar in all genotypes, indicating no adverse effects of KI mutations on developmental synapse refinement.

We next measured paired-pulse ratios (PPRs) (PPR = eEPSC\(_{2}\)/eEPSC\(_{1}\)) of two consecutive eEPSCs evoked at interstimulus intervals (ISIs) of 5 ms–2 s (Figures 2D and 2E). In many synapses, including the calyx of Held (Debanne et al., 1996; Dobrunz and Stevens, 1997; Taschenberger et al., 2016), PPRs correlate with initial eEPSCs size, indicating that differences in synaptic strength arise, at least partly, from differences in release probability. Regression lines fitted to log-linear plots of 1 – PPR versus ISI provide estimates for average release probabilities (\(\bar{p}\)) and average SV pool replenishment rate constants (K\(_{r}\)) (Betz, 1970). We observed lower PPRs at all ISIs in mut\(_{DN}\) synapses (Figure 2E1; Table S1) as compared to WT\(_{DN}\) synapses, indicative of elevated \(\bar{p}\) in the former.

Homologous mutant (mut) KIs of both lines were viable and fertile but showed an ~30% body weight reduction at 2 weeks of age (Figure 1E), which did not persist. Neither KI line showed signs of distress or gross behavioral abnormalities. Analyses of expression levels of a selected set of presynaptic proteins did not reveal any genotype-related differences (Figures 1F and S1A–S1C). The localization of the mutant Munc13-1 variants in presynaptic compartments contacting principal neurons (PNs) of the medial nucleus of the trapezoid body (MNTB) was examined by immunostaining (Figure S1D). A similar typical pattern of Munc13-1-positive structures co-localizing with the AZ marker Bassoon and surrounding MNTB PN somata was observed in wild-type (WT) and KI samples (Figures S1D–S1F; Chen et al., 2013; Lipstein et al., 2013).

### RESULTS

**Generation of Munc13-1 C\(_{2BDN}\) and C\(_{2BKW}\) KI mice**

The Ca\(^{2+}\)-binding site in the Munc13-1 C\(_{2B}\) domain is composed of negatively charged residues that chelate Ca\(^{2+}\), thus allowing negatively charged phospholipids to bind. We exchanged two such aspartic acid residues by asparagine (D705N and D711N) to create the Munc13-1 C\(_{2BDN}\) KI line (Shin et al., 2010). This exchange abolishes Ca\(^{2+}\)-binding, slightly increases phospholipid binding to its C\(_{2B}\) domain. This causes distinctly higher SVR rates and thus shapes STP, enables sustained transmission, and enhances the temporal fidelity of synaptic signaling.

To examine the role of the Munc13-1 C\(_{2B}\) domain in Ca\(^{2+}\)- and phospholipid-dependent SVR regulation in situ, we generated knockin (KI) mouse lines that express Munc13-1 variants with either abolished or increased Ca\(^{2+}\) and phospholipid binding to the C\(_{2B}\) domain (Shin et al., 2010) and characterized functional consequences in the calyx of Held synapse. We demonstrate that presynaptic activity activates Munc13-1 by Ca\(^{2+}\)-phospholipid binding to its C\(_{2B}\) domain. This causes distinctly higher SVR rates and thus shapes STP, enables sustained transmission, and enhances the temporal fidelity of synaptic signaling.

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contrast, PPRs were similar in mutKW and WTKW synapses, consistent with unchanged \( p \). However, a steeper slope of the log-linear plot of \( 1/C0_{\text{PPR}} \) versus ISI indicated a faster \( k_+ \) in mutKW synapses (Figure 2 E2).

To test whether enhanced presynaptic Ca\(^{2+}\) influx contributes to the elevated \( p \) in mutDN synapses, we recorded pharmacologically isolated voltage-gated Ca\(^{2+}\) currents (\( I_{\text{Ca(V)}} \)) in voltage-clamped calyx terminals (Figures 2 F and 2G). Peak \( I_{\text{Ca(V)}} \) amplitudes were similar in mut and WT terminals, indicating similar voltage-gated Ca\(^{2+}\) channel (VGCC) densities (Figure 2G; Table S1). To assess VGCC gating kinetics, we quantified the charge of \( I_{\text{Ca(V)}} \) (\( Q_{\text{Ca(V)}} \)) elicited by short AP-like depolarizations, assuming that altered activation or deactivation of \( I_{\text{Ca(V)}} \) will affect its current integral (Figure 2F; Li et al., 2007), and detected no differences (Figure 2G; Table S1).

These data show that basal synaptic transmission is intact in C2BDN and C2BKW KI synapses. No indications of developmental abnormalities or postsynaptic modifications were observed, and normal eEPSC kinetics indicate unaltered SV fusion kinetics. The C2BDN mutation leads to enhanced synaptic strength, partly due to elevated \( p \), but neither mutation alters VGCC expression or kinetics. Thus, the increased \( p \) in mutDN synapses is either caused by a subtle change in AP waveform not affecting eEPSC kinetics or, more likely, by a mechanism downstream of Ca\(^{2+}\) influx that increases the apparent Ca\(^{2+}\) sensitivity of SVs in mutDN terminals. The C2BKW mutation

**Figure 1. Munc13-1 C2BDN and C2BKW KI mice**

(A) Munc13-1 gene, targeting vector, mutated gene after homologous recombination, and mutated gene after Flp recombination to remove the puromycin cassette. Exon 18 is indicated in red. FRT, flippase recognition target; Puro, puromycin resistance gene; TK, herpes simplex virus thymidine kinase. Puro and TK cassettes are not drawn to scale.

(B–D) Genotyping strategy for the Munc13-1 KI mice of the indicated genotypes (F, fRT site).

(C) Gel electrophoresis of PCR products using mouse tail DNA of the indicated genotypes.

(D) Sequence chromatograms of the mutated region in exon 18 of the indicated genotypes.

(E) Scatter dot plots and bar graphs showing individual and average values of body weights of juvenile (P14–P17) C2BDN (left) and C2BKW (right) mice.

(F) Quantitative western blot analysis showing no difference in the expression levels of Munc13-1, ubMunc13-2, and bMunc13-2.

Data depict mean ± SEM. Differences among mean density values were statistically not significant (\( p > 0.05; \) ANOVA). See also Figure S1.
does not affect eEPSC amplitudes but accelerates the recovery from STD induced by single eEPSCs.

The Munc13-1 C2B domain regulates steady-state rates of quantal release during repetitive synaptic stimulation  
During AP trains, calyx of Held synapses typically show STD at low and intermediate (<50 Hz) stimulus frequencies ($f_{stim}$) and sometimes transient facilitation followed by STD at higher $f_{stim}$ (> 50 Hz; Grande and Wang, 2011; Taschenberger et al., 2016). Quantal release at steady state is limited by the SVR rate. To examine the role of the Munc13-1 C2B domain in determining STD, we recorded eEPSC trains for a range of $f_{stim}$ (0.5–200 Hz, 35 APs; Figures 3A1 and 3A2). Normalized average eEPSC train amplitudes showed stronger STD at high $f_{stim}$ for mutDN as compared to WTDN synapses (Figure 3B1; Table S1), whereas reduced STD was seen for all but the highest $f_{stim}$ in mutKW as compared to WTKW synapses (Figure 3B2; Table S1).

The relationship between steady-state quantal release and $f_{stim}$ is illustrated in Figure 3C by plotting eEPSCs and average steady-state release ($eEPSC_{ss} \times f_{stim} = r_\cdot \cdot \cdot q$, where $r_\cdot \cdot \cdot$ and $q$ are the average release rate and quantal size, respectively) against $f_{stim}$. eEPSC_{ss} was larger in mutDN as compared to WTDN synapses for all $f_{stim} \leq 10$ Hz. This is likely a consequence of only minor SV pool depletion during such low-frequency trains, so that eEPSC_{ss} largely reflects the higher $\bar{\mu}_{mut}$ in mutDN. In contrast, for $f_{stim} \geq 20$ Hz, eEPSC_{ss} and $r_\cdot \cdot \cdot q$ were smaller in mutDN as compared to WTDN synapses, indicating that slower SVR limits the amount of release in the former (Figure 3C). In mutKW synapses, a different picture emerged: for all but the three highest frequencies (50, 100, and 200 Hz), eEPSC_{ss} and $r_\cdot \cdot \cdot q$ were larger in mutKW as compared to WTKW synapses. Because eEPSC_{ss} and $\bar{\mu}$ are similar in mutKW and WT KW synapses (Figure 2), this is consistent with a faster SVR in mutKW at $f_{stim} \leq 20$ Hz (Figure 3C).

As SVR enhancement at high firing rates is thought to be mediated by AP-evoked presynaptic Ca(V)²⁺ influx (Dittman and Regehr, 1998; Wang and Kaczmarek, 1998), and as Munc13-1 mutations were reported to affect VGCC behavior in cultured neurons (Calloway et al., 2015), we tested whether presynaptic Ca(V)²⁺ influx is altered in Munc13-1 C2B KI calyces by examining frequency-dependent modulation of calyceal $I_{Ca(V)}$ during trains of AP-like depolarizations. Neither magnitude nor time course of $I_{Ca(V)}$ or its frequency-dependent facilitation was different between mut and WT terminals (Figures 3D and 3E; Table S1).

These data lead to the conclusion that the C2BDN mutation slows SVR preferentially for higher $f_{stim}$ while the C2BK mutation accelerates SVR preferentially for lower $f_{stim}$. This causes reduced steady-state release during high-frequency trains in mutDN and enhanced steady-state release during low frequency trains in mutKW synapses.

The number of readily releasable SVs is not altered in Munc13-1 C2BDN and C2BK synapses  
Given the key role of Munc13-1 in RRP establishment and maintenance (Augustin et al., 1999b; Varoquaux et al., 2002), we next tested whether the C2BDN and C2BK mutations affect the RRP. Pool size estimates corresponding to the subpool of “fast releasing” SVs (FRP) (Sakaba, 2006) were obtained from cumulative eEPSC amplitudes measured during high-frequency trains and corrected for ongoing SVR (Schneggenburger et al., 1999; Figure 4A). An estimate for $\bar{\mu}_{cor}$ is obtained from the ratio eEPSC_{ss}/FRP. FRP estimates increased and $\bar{\mu}_{cor}$ values decreased with increasing $f_{stim}$ likely because of more complete pool depletion (Figures 4A and 4B). We therefore termed these estimates $FRP^p$ and $\bar{\mu}^p$. When plotting 1/$FRP^p$ and $\bar{\mu}^p$ versus ISI, we observed a roughly linear relationship between these quantities and ISI. Assuming this relationship holds for the entire ISI range, we obtained corrected estimates for pool size ($FRP_{cor}$) and release probability ($\bar{\mu}_{cor}$) by extrapolation to ISI = 0 s. $FRP_{cor}$ values were slightly larger when comparing C2BDN and C2BK mut with littermate WT synapses (Figure 4B; Table S1) but statistically not significantly different, although $\bar{\mu}_{cor}$ was significantly higher in mutDN synapses (Figure 4B; Table S1), consistent with their larger initial eEPSCs and reduced PPR (Figures 2C and 2E1).

To exclude the possibility of an overcorrection when estimating $FRP_{cor}$, we performed three controls. (1) Assuming a simple SV pool depletion model, an estimate for release probability can be obtained by plotting eEPSC amplitudes during high-frequency trains versus the cumulative sum of previous eEPSCs (Elmqvist and Questel, 1965; Neher, 2015). Such plots show steeper negative slopes for mutDN as compared to WT DN synapses, consistent
with more rapid SV consumption and therefore higher \( \pi \) in the former (Figure 3A1). In contrast, slopes and, therefore, \( \pi \) estimates were similar in WT KW and mut KW synapses (Figure 3A2). Both findings confirm our earlier conclusions (Figure 4B). (2) For a second set of control experiments, we recorded 200-Hz eEPSC trains in WT synapses before and after bath application of tetraethylammonium (TEA), a blocker of voltage-gated potassium channels that broadens calyceal APs and increases AP-evoked \( \text{Ca}^{2+} \) influx and \( \beta \) (Ishikawa et al., 2003; Figure 3B), and observed an apparent FRP increase of \(-40\%\) in the presence of TEA. This is in accord with the average ratio between FRP \( \text{corr} \) and FRP \( \text{100 Hz} \) in both WT DN and also WT KW synapses (Figure 3C), indicating that our strategy to correct FRP estimates for incomplete pool depletion (Figure 4B) does not result in a substantial overestimate. (3) Finally, we assayed SV fusion by measuring \( \Delta C_m \) in response to depolarizing voltage steps of increasing duration in voltage-clamped calyces. \( \Delta C_m \)-based pool estimates do not rely on postsynaptic responses and thus are not susceptible to AMPA receptor (AMPA) saturation or desensitization. However, long-lasting presynaptic depolarizations—during which presynaptic [\( \text{Ca}^{2+} \)] spatially equilibrates—trigger release from an additional subpool of “slowly releasing” SVs (SHP; Sakaba and Neher, 2001b), while during short and spatially restricted AP-evoked [\( \text{Ca}^{2+} \)] elevations, SVs of the SHP fuse to a much lesser extent (Sakaba, 2006). Increasing \( I_{\text{calv}} \) duration led to larger \( \Delta C_m \) (Figure 4C). The average relationship between quantal release and \( \text{Ca}^{2+} \) influx duration was very similar in WT and mutant C2BDN or mutant C2BKW calyces with respect to both \( \Delta C_m \) amplitudes and release kinetics (Figure 4D; Table S1). Despite the caveat that \( \Delta C_m \) represents the sum of fast and slowly releasing SVs (FRP + SRP), these experiments do not reveal changes in SV pool size in mutDN or mutKW synapses.

These data show that the Munc13-1 C2BDN and C2BKW mutations do not affect the number of release-ready SVs in calyx terminals. Thus, changes in SV pool size are unlikely to contribute to the observed differences in steady-state release rates and STP.

**Munc13-1 C2BDN and C2BKW mutations change the average rate constants of SV recruitment**

Knowing steady-state release rates and the total number of release-ready SVs in resting calyces allowed us to approximate the relationship between \( f_{\text{stim}} \) and the average FRP replenishment rate constant \( \langle \hat{R} \rangle \), which represents the rate of release site refilling per empty site averaged over one inter-stimulus interval. Figure 4E plots \( \langle \hat{R} \rangle \) versus \( f_{\text{stim}} \) for WT and C2B mutant synapses. For WT DN and WT KW synapses, a similar relationship between \( \langle \hat{R} \rangle \) and \( f_{\text{stim}} \) was observed (Figure 4E1). At the highest \( f_{\text{stim}} \) of 200 Hz, \( \langle \hat{R} \rangle \) is \(-10\)-fold above its value estimated for \( f_{\text{stim}} = 0.5 \) Hz. The reciprocal of the maximum \( \langle \hat{R} \rangle \), of \( \sim 4 \) s\(^{-1} \) (\( \tau = 250 \) ms) agrees well with the rapid time constant of FRP replenishment measured following prolonged presynaptic depolarizations (\( \tau_1 = 270 \) ms, Lipstein et al., 2013; \( \tau_1 = 360 \) ms, Sakaba and Neher, 2001a). Figure 4E shows that the C2BDN and C2BKW mutations alter the relationship between \( \langle \hat{R} \rangle \) and \( f_{\text{stim}} \) so that the dynamic range of \( \langle \hat{R} \rangle \) is reduced. However, in mutDN synapses, we observed a decreased \( \langle \hat{R} \rangle \) for all \( f_{\text{stim}} \geq 2 \) Hz as compared to WT values (Figure 4E2). In contrast, higher \( \langle \hat{R} \rangle \) values were estimated for all stimulation frequencies \(< 20 \) Hz in mutKW synapses (Figure 4E2).

These data demonstrate lower SVR rate constants for mutDN synapses mainly at high \( f_{\text{stim}} \) and higher SVR rate constants for mutKW synapses for low and intermediate \( f_{\text{stim}} \) consistent with the changes observed during steady-state depression.

**Abolishing \( \text{Ca}^{2+} \) binding to the Munc13-1 C2B domain eliminates a fast component of eEPSC recovery following high-frequency conditioning**

Having established that Munc13-1 C2B mutations alter \( \langle \hat{R} \rangle \) during ongoing stimulation, we next tested eEPSC recovery after conditioning trains. When calyces are conditioned with low-frequency trains, eEPSCs recover with a slow time course that is well described by a single exponential (\( \tau = 4 \) s; Iwasaki and Takahashi, 2001; von Gersdorff et al., 1997). Upon high-frequency conditioning, an additional fast recovery component is observed at calyces (Wang and Kaczmarek, 1998) and other synapses (Cho et al., 2011; Gomis et al., 1999; Wang and Manis, 2008; Yang and Xu-Friedman, 2008). This fast recovery is presumed to depend on elevated \( [\text{Ca}^{2+}]_i \), and \( \text{Ca}^{2+} \cdot \text{CaM} \) binding to Munc13-1 participates in the molecular signaling pathway that mediates it (Lipstein et al., 2013; Sakaba and Neher, 2001a).

To examine SVR, we applied 100-Hz (25 stimuli) or 200-Hz (50 stimuli) trains to induce strong FRP depletion and monitored recovery of synaptic strength by measuring eEPSC test amplitudes at different intervals (Figure 5A1). When plotting fractional recovery (eEPSC test - eEPSC control)/(eEPSC control - eEPSC control) versus recovery interval (Figure 5A3; Table S1), we observed for WT DN synapses...
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a biphasic time course requiring a fast component to account for the recovery during the initial 500 ms (Figure 5A3; Lipstein et al., 2013, their Figure 5), which was selectively and completely abolished in mutDN synapses (Figures 5A2 and 5A3). After 16 s, full recovery of eEPSC\textsubscript{test} was seen in both mutDN and WT\textsubscript{DN} synapses. Closer inspection of the time course of fractional recovery revealed values slightly below zero in mutDN synapses for the two shortest intervals (125 and 250 ms; Figure 5A3). This can be explained by acknowledging that release probability increases during conditioning stimulation and that this synaptic facilitation decays faster than FRP recovery proceeds. Such an effect is likely occluded by the fast SVR component in WT synapses but uncovered in mutDN synapses that lack this SVR component.

These data show that a fast SVR component is absent in mutDN synapses with abolished C\textsubscript{2B} domain binding to Ca\textsuperscript{2+} and phospholipids. Because a fast SVR component is observed in WT\textsubscript{DN} synapses only after high-frequency conditioning, we expected the recovery time course after 10- or 20-Hz conditioning to be largely unaltered in mutDN as compared to WT\textsubscript{DN} synapses. This is indeed the case (Figure S4), demonstrating that the slow component of eEPSC recovery is unaffected in mutDN synapses.

**Ca\textsuperscript{2+}-CaM binding to Munc13-1 is independent of Ca\textsuperscript{2+} binding to the C\textsubscript{2B} domain**

The effects of the C\textsubscript{2BDN} mutation on SVR resemble those reported previously for a Ca\textsuperscript{2+}-CaM-binding-deficient Munc13-1 mutant (W464R; CaMWR; WT\textsubscript{WR} and mut\textsubscript{WR} for WT and mutant littermates, respectively; Lipstein et al., 2013). To compare STP at C\textsubscript{2BDN} and CaMWR calyces under identical conditions, we performed recordings similar to those described above also for WT\textsubscript{WR} and mut\textsubscript{WR} synapses and analyzed normalized eEPSC amplitudes during stimulus trains (Figure S5A), FRP, and \( \bar{p} \) estimates (Figures S5B–S5D); the relationship between \( f_{\text{stim}} \) and steady-state release \( (r \cdot q_{0}) \); and the apparent average replenishment rate constant \( \bar{k}_{r} \) (Figures S5E and S5F) for WT\textsubscript{WR} and mut\textsubscript{WR} synapses. While FRP and \( \bar{p} \) estimates were unchanged, we observed lower steady-state release during high-frequency stimulation in mut\textsubscript{WR} synapses, which corresponded to reduced \( \bar{k}_{r} \). Accordingly, the dynamic range for \( \bar{k}_{r} \) was reduced as seen for mut\textsubscript{DN} synapses (Figure 4E2). As reported (Lipstein et al., 2013), we observed slower recovery after depleting stimulus trains in mut\textsubscript{WR} synapses (Figures S5G1 and S5G2). However, contrary to the C\textsubscript{2BDN} mutation and consistent with Lipstein et al. (2013), differences in the eEPSC recovery time course were less pronounced after 200- as compared to 100-Hz conditioning. The presence of residual fast SV replenishment after 200-Hz conditioning in mut\textsubscript{WR} calyx synapses suggests that the C\textsubscript{2B} pathway operates also in the absence of the Ca\textsuperscript{2+}-CaM pathway and partially compensates for its loss.

Because of the similarity of the functional deficits between mut\textsubscript{DN} versus mut\textsubscript{WR} synapses and the spatial proximity of the C\textsubscript{2B} domain and the Ca\textsuperscript{2+}-CaM binding site, we tested whether blocked Ca\textsuperscript{2+} binding to the C\textsubscript{2B} domain perturbs Ca\textsuperscript{2+}-CaM binding. Co-immunoprecipitation experiments showed unaltered Ca\textsuperscript{2+}-CaM binding of the C\textsubscript{2BDN} mutant Munc13-1 (Figure 5B).

These data indicate that the Munc13-1 regulation via the C\textsubscript{2B} domain is likely downstream of or independent from the regulation by Ca\textsuperscript{2+}-CaM and that Ca\textsuperscript{2+} binding to the C\textsubscript{2B} domain is not a pre-requisite for Ca\textsuperscript{2+}-CaM binding. Whether these pathways operate independently or synergistically to regulate Munc13-1 function and SVR remains to be studied.

**The Munc13-1 C\textsubscript{2BK}W mutation accelerates eEPSC recovery following conditioning trains**

We next analyzed SVR in C\textsubscript{2BK}W synapses. The recovery time course in mut\textsubscript{KW} synapses was profoundly accelerated as compared to WT\textsubscript{KW} (Figure 6A) and well described by a single fast exponential time constant. Little differences in fractional recovery were observed between mut\textsubscript{KW} and WT\textsubscript{KW} synapses for the two shortest recovery intervals (125 and 250 ms), while already 2 s after conditioning, eEPSC\textsubscript{test} had either nearly completely (100 Hz) or completely (200 Hz) recovered in mut\textsubscript{KW} synapses (Figures 6A2 and 6A3; Table S1). For 200-Hz conditioning, we noticed average amplitudes of eEPSC\textsubscript{test} slightly larger than those of eEPSC\textsubscript{1}.

To exclude that accelerated eEPSC\textsubscript{test} recovery in mut\textsubscript{KW} synapses reflects \( p \) augmentation rather than faster SVR, we recorded eEPSC\textsubscript{test} pairs to monitor PPRs. If \( p \) were indeed higher in mut\textsubscript{KW} synapses at 2- or 4-s recovery intervals as compared to 16 s, we would expect different PPRs at these time points. PPRs

![Figure 4. Total number of releasable SVs and average initial release probability in C\textsubscript{2BDN} and C\textsubscript{2BK}W calyx synapses](image). Estimates for the readily releasable SV pool were derived from high-frequency eEPSC trains (FRP; A and B) and presynaptic \( J_{c_{m}} \) measurements (FRP + SRP; C and D).

- **(A)** Mean cumulative eEPSC amplitudes measured in response to stimulation with 50-, 100-, and 200-Hz trains (55 APs) in C\textsubscript{2BDN} (A1) and C\textsubscript{2BK}W (A2) synapses of WT (left) and mut (right) littermates. Solid, broken, and dotted lines represent regression lines correcting for ongoing SVR for 200-, 100-, and 50-Hz trains, respectively, assuming a constant average release \( \bar{p} \). Intersections of these lines with the abscissa represent apparent pool size estimates (FRP).
- **(B)** 1/FRP and apparent average release probability for eEPSC, \( (\bar{p} = \text{eEPSC}_{1}/\text{FRP}) \) (insets) plotted versus inter-stimulus interval for C\textsubscript{2BDN} (B1) and C\textsubscript{2BK}W (B2) calyx synapses. Gray and colored symbols represent mean values for WT and mut synapses, respectively. Solid lines represent linear regressions to the scatterplots. Intersections of the line fits with the abscissa at ISI = 0 ms represent corrected estimates for 1/FRP and \( \bar{p} \).
- **(C)** Traces of presynaptic \( f_{\text{app}} \) (left) and \( J_{c_{m}} \) (right) elicited by step depolarizations of 1, 2, 5, 10, and 20 ms duration in voltage-clamped C\textsubscript{2BDN} (C1) and C\textsubscript{2BK}W (C2) calyx terminals of WT (top row) and mut (bottom row) littermates.
- **(D)** Average \( J_{c_{m}} \) values plotted versus step duration for C\textsubscript{2BDN} (D1) and C\textsubscript{2BK}W (D2) calyx terminals. The numbers of SVs, obtained by assuming a single SV capacitance of ~80 aF (Sakaba, 2006), are shown on the right axis.

![Figure S4. Changes in release probability and fractional recovery of eEPSCs](image). **(A)** Relationship between synaptic release probability \( p \) and fractional recovery of eEPSC \( (\text{FRP}) \). For WT\textsubscript{DN}, both \( p \) and FRP were reduced as compared to WT\textsubscript{WR}. For mut\textsubscript{DN}, we observed lower steady-state release during high-frequency stimulation in mut\textsubscript{DN} synapses, which corresponded to reduced \( \bar{k}_{r} \). Accordingly, the dynamic range for \( \bar{k}_{r} \) was reduced as seen for mut\textsubscript{DN} synapses (Figure 4E2). As reported (Lipstein et al., 2013), we observed slower recovery after depleting stimulus trains in mut\textsubscript{WR} synapses (Figures S5G1 and S5G2). However, contrary to the C\textsubscript{2BDN} mutation and consistent with Lipstein et al. (2013), differences in the eEPSC recovery time course were less pronounced after 200- as compared to 100-Hz conditioning. The presence of residual fast SV replenishment after 200-Hz conditioning in mut\textsubscript{WR} calyx synapses indicates that the C\textsubscript{2B} pathway operates also in the absence of the Ca\textsuperscript{2+}-CaM pathway and partially compensates for its loss.

Data depict mean ± SEM; n values and statistical significance are summarized in Table S1. See also Figures S3 and S6.
measured after 2- and 4-s recovery in mutKW synapses were indistinguishable from those measured at 16 s in either mutKW or WT KW synapses, indicating similar values at the respective recovery intervals (Figure 6B).

To further corroborate that faster recovery from STD in mutKW synapses primarily stems from faster SVR, we assayed SV pool recovery directly by presynaptic DCm recordings. The duration of presynaptic depolarizations was limited to 3 ms to limit fusion of slowly releasing SVs (Sakaba and Neher, 2001b). Recovery of DCm was noticeably faster in mutKW as compared to WT KW calyx terminals (Figure 6C) and resembled the accelerated eEPSC recovery time course (Figure 6A).

We then tested whether accelerated recovery from STD in mutKW synapses is only observed after high-frequency conditioning (100 and 200 Hz), leading to substantial temporal summation of AP-evoked presynaptic global [Ca2+]i transients, or if it is also seen following low-frequency conditioning (10 and 20 Hz), during which individual AP-evoked [Ca2+]i transients decay nearly completely (Müller et al., 2007 and see below).

We found that lowering the number of conditioning stimuli together with their frequency considerably slows down eEPSC recovery (Figures S4A3 and S4B3). However, at any tested conditioning frequency, recovery from STD occurred faster in mutKW as compared to WT KW synapses (Figures 6A and S4C). A detailed analysis of the relationship between the estimated steady-state K, during stimulus trains versus the fractional recovery at different intervals after a conditioning train for all three mutants at the level of individual synapses is presented in Figure S6.

These data show that, in the Munc13-1 C2BKW mutant with enhanced Ca2+-dependent phospholipid binding to the C2B domain, SVR is accelerated after low- and high-frequency conditioning. Faster recovery of eEPSC amplitudes is not driven by an elevated release probability during the recovery period.

**Augmenting presynaptic AP-evoked Ca2+ influx does not mimic the acceleration of SV recruitment caused by the C2BKW mutation**

The accelerated eEPSC recovery after high-frequency trains in mutKW synapses is reminiscent of faster recovery from STD upon increased presynaptic Ca2+ influx in calyx synapses (Wang and Kaczmarek, 1998). We tested whether widening of calyceal APs by TEA, thus augmenting presynaptic Ca2+ influx, induces fast SVR in WT synapses similar to that seen in mutant C2BKW synapses. Bath application of TEA in the presence of kyn enhanced eEPSCs 1-4-fold and strongly increased STD (Figure 6D1). Unexpectedly, though, we observed only a moderate acceleration of the eEPSC recovery time course (Figure 6D2).

To resolve the apparent discrepancy between our results and those of Wang and Kaczmarek (1998), we repeated our experiments in the presence of the high-affinity, slowly dissociating AMPAR antagonist NBQX instead of the low-affinity, fast-dissociating antagonist kyn. With 100 nM NBQX, adequate voltage-clamp is ensured by reducing eEPSCs to a size comparable to that observed with kyn in the bath, while eEPSCs are still subject to AMPAR saturation and desensitization (Wadiche and Jahr, 2001). Augmentation of eEPSCs following TEA application was considerably smaller (<3-fold; Figures S7A and S7B) in the presence of NBQX instead of kyn, indicative of postsynaptic AMPAR saturation (Taschenberger et al., 2002, their Figure 8). eEPSC recovery after conditioning 100- or 200-Hz trains was profoundly accelerated by TEA (Figure S7A2), while eEPSCs were similar to control conditions (100-Hz conditioning) or even reduced...
(200-Hz conditioning; Figure S7A1), which is unexpected if SVR during steady state was strongly enhanced by TEA.

These data show that, under conditions that limit AMPAR saturation and desensitization, SVR acceleration by enhanced Ca\textsuperscript{2+} influx is substantially smaller than that seen in mut\textsubscript{KW} synapses. We conclude that the C\textsubscript{2}BK-W-mutation-induced SVR enhancement is not mimicked solely by augmenting AP-evoked Ca\textsuperscript{2+} influx. Rather, it reflects a combined regulatory effect of Ca\textsuperscript{2+} and phospholipid binding on Munc13-1 activity, whose magnitude is unique.

**AP-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients in calyx terminals are unaltered in C\textsubscript{2}BDN and C\textsubscript{2}BK\textsubscript{W} synapses**

To rule out changes in the dynamics of presynaptic [Ca\textsuperscript{2+}]\textsubscript{i} due to differences in Ca\textsuperscript{2+} buffering or clearance contribute to the altered eEPSC recovery kinetics in mut\textsubscript{DN} and mut\textsubscript{KW} synapses, we measured [Ca\textsuperscript{2+}]\textsubscript{i} transients in response to afferent-fiber stimulation in nearly unperturbed calyx terminals preloaded with the low-affinity Ca\textsuperscript{2+} indicator dye Cal520FF (~1 min; Figures 7A and 7B; Habets and Borst, 2005; Müller et al., 2007). Maximum [Ca\textsuperscript{2+}]\textsubscript{i} amplitudes during trains were on average ~7 (100 Hz) and 16–18 (200 Hz) times larger than those of single AP-evoked transients. As described (Müller et al., 2007), calyceal [Ca\textsuperscript{2+}]\textsubscript{i} transients decayed bi-exponentially with a slow τ in the hundreds of millisecond range, which became more prominent after repetitive AP firing. When superimposing [Ca\textsuperscript{2+}]\textsubscript{i} transients recorded in either wt or WT C\textsubscript{2}BDN or C\textsubscript{2}BK\textsubscript{W} terminals, their waveforms were indistinguishable (Figure 7C; Table S1).

Having established the average time course of global [Ca\textsuperscript{2+}]\textsubscript{i} transients induced by conditioning trains, we modeled the recovery from SV pool depletion in C\textsubscript{2}BDN and C\textsubscript{2}BK\textsubscript{W} synapses by considering a SVR process that obeys a first-order rate law (Hosoi et al., 2007) and by using the previously established relationship between $f_{\text{stim}}$ and $\bar{R}_\text{m}$ (Figure 3E). We assumed that the latter also predicts the relationship between global [Ca\textsuperscript{2+}]\textsubscript{i} and $\bar{R}_\text{m}$, i.e., we postulated a linear relationship between $f_{\text{stim}}$ and [Ca\textsuperscript{2+}]\textsubscript{i} at steady state. Numerical simulations (Figure 7D) capture the essential features of the C\textsubscript{2}B-mutation-induced changes, indicating that the altered dynamic regulation of $\bar{R}_\text{m}$ allows us to predict changes in pool recovery time course that correspond to the experimental observations. On the other hand, the simulations do not reproduce the initial “negative” or the “overshooting” eEPSC recovery observed in mut\textsubscript{KW} (Figure 5A3) and mut\textsubscript{KW} (Figure 6A3) synapses, respectively, indicating that a more detailed model, covering the heterogeneity in $p$ as well as changes in $p$ during and after conditioning stimulation, is required to more faithfully reconstruct the eEPSC recovery time course.

The data above—along with the facts that $I_{\text{Ca}\textsubscript{v}W}$ amplitudes, VGCC gating kinetics, and $I_{\text{Ca}\textsubscript{v}W}$ facilitation time course during AP-like stimulus trains are unaltered—indicate that changes in presynaptic Ca\textsuperscript{2+} signaling do not contribute to altered SVR kinetics in C\textsubscript{2}BDN and C\textsubscript{2}BK\textsubscript{W} synapses. Numerical simulations based on measured parameters predict the major features of C2B-mutation-induced changes in the pool recovery time course.

**Temporal precision of information processing at calyx synapses deteriorates in the absence of fast SV recruitment**

Calyx of Held synapses operate with high temporal precision, even at high transmission rates, leading to reliable AP firing of MNTB PNs (Guinan and Li, 1990; Kopp-Scheinpflug et al., 2008; Lorteije et al., 2009). In addition to other features (Borst and Soria van Hoeve, 2012; Schneggenburger and Forsythe, 2006; von Gersdoff and Borst, 2002), an activity-dependent SVR upregulation may critically contribute to sustained and temporally precise transmission. We thus tested whether the Munc13-1 mutations affect information processing at calyx synapses by analyzing postsynaptic AP timing following afferent fiber stimulation.

Recordings from MNTB PNs in cell-attached configuration represent a minimally invasive form of monitoring postsynaptic Na\textsuperscript{+} spikes with high signal-to-noise ratio, allowing analyses of AP timing with microsecond precision (Figures 6A and 6B; Lorteije et al., 2009). Extracellularly recorded APs measured during the recovery period following conditioning stimulation are shown superimposed in Figures 8A2 and 8B2. In WT synapses, a clear right shift in the AP timing relative to stimulus onset is seen for short recovery intervals, showing that small evoked excitatory postsynaptic potentials (eEPSPs), generated while the FRP is...
Figure 7. AP-evoked global volume-averaged $[\text{Ca}^{2+}]_i$ transients in calyx terminals

(A) Fluorescence images of a calyx terminal pre-loaded with the low-affinity Ca$^{2+}$ indicator dye Cal520FF ($K_D = 9.8 \, \mu M$) during a brief whole-cell episode (~1 min; pipette concentration 400 $\mu M$; final cytosolic concentration ~200 $\mu M$) at rest (top) and during 200-Hz stimulation (50 APs, center). The difference image is shown in the bottom panel.

(B) Presynaptic $[\text{Ca}^{2+}]_i$ transients ($\Delta F/F_0$) elicited by a single AP (top) or trains of 25 (100 Hz, center) or 50 APs (200 Hz, bottom), corresponding to stimulus trains used for Figures 6A, 6A, and 6D. Each trace represents the average of 6 (single AP) or 4 (AP trains) repetitions, and the gray areas represent ±SEM. AP discharge pattern recorded in response to afferent fiber stimulation under current-clamp conditions during the preloading period is illustrated in the top right insets. Red traces represent double-exponential fits to the decay of the $[\text{Ca}^{2+}]_i$ transients. Fast- and slow-decay time constants are given next to the $[\text{Ca}^{2+}]_i$ transients. The single AP response is shown superimposed to the train responses in the center and bottom panels for comparison.

(C) Normalized average presynaptic $[\text{Ca}^{2+}]_i$ transients recorded in WT (back) and mut (red and blue) C$_2$BDN (C1) and C$_2$BKW (C2) terminals in response to a single AP (left), a 100-Hz AP train (25 APs, center), or a 200-Hz AP train (50 APs, right).

(D) Numerical simulations of SV pool recovery after 100-Hz (left) and 200-Hz (right) trains assuming first-order kinetics and a $[\text{Ca}^{2+}]_i$-dependent $R_f$. For simplicity, we approximated the relationship between $R_f$ and $[\text{Ca}^{2+}]_i$ by assuming a linear relationship between $f_{\text{slow}}$ and $[\text{Ca}^{2+}]_i$ at steady state. The decay of $R_f$ immediately after conditioning trains (corresponding to $t = 0$ s in D) was modeled with a double-exponential function using decay time constants established by fitting the average $[\text{Ca}^{2+}]_i$ transients shown in (C).
largely depleted, mostly trigger postsynaptic APs with longer latencies. Strikingly, mutDN synapses, which lack the fast component of eEPSC recovery, tended to show even longer latencies between stimulus and postsynaptic spike for short recovery intervals.

Temporal jitter and timing of postsynaptic APs was further analyzed in whole-cell configuration to obtain stable long-term recordings and to collect more repetitions of a given protocol (Figures 8C and 8D). In rare cases, individual aberrant postsynaptic APs were triggered during conditioning, presumably by asynchronous release events generating supra-threshold eEPSPs, so that the number of postsynaptic APs exceeded the number of stimuli in some trials (Figure 8C1). As in cell-attached recordings, we observed longer AP latencies at short recovery intervals in mutDN as compared to WTDN synapses. In addition, we found that the temporal jitter increased (Figure 8D2). Conversely, mutKW synapses showed improved temporal precision as compared to WT synapses, so that AP latencies were shorter and spike latency jitter was reduced for a range of short recovery intervals (0.5–4 s; Figure 8D3). Figures 8E and 8F plot the average increase in spike latencies relative to those measured for the longest recovery interval (16 s) and the mean of the standard deviation of the timing of AP peaks as a measure of spike timing variability. In both WT and mutKW synapses, AP latencies were ~200 μs longer for the shortest recovery interval (0.125 s) than at those after 16 s recovery in mutDN synapses. In mutDN synapses, the spike latency increase for short recovery intervals was nearly twice as large and remained larger for all but the 4-s and 8-s intervals. In mutKW synapses, on the other hand, AP latencies declined much faster with increasing recovery intervals. For 2–16 s recovery, no differences in spike timing were measurable, consistent with the fact that, for recovery intervals, ≥2 s eEPSCs had nearly fully recovered after conditioning 100-Hz stimulation (Figure 6A3). A similar picture emerged for the temporal jitter of AP latencies. For intervals ≤1 s, the jitter was larger in mutDN but generally smaller in mutKW synapses as compared to WT synapses (Figure 8F).

These data show that the modulation of SVR speed mediated by Ca2+-phospholipid binding to the Munc13-1 C2B domain influences synaptic fidelity. This phenomenon is expected to alter synaptic computation at the level of single synapses and circuits.

**Faster rebound from synaptic depression at hippocampal synapses upon acceleration of SV recruitment**

To show that the rate of Munc13-1-dependent SVR also determines STP at synapses that are less prone to rapid SV pool exhaustion at their typical firing rates (Mizuseki and Buzsáki, 2013), we analyzed transmission at P16–P21 hippocampal Schaffer collateral/commissural synapses with CA1 neurons (SC/C-CA1), focusing on C2BKW mice (Figure 8G). We conditioned SC/C-CA1 synapses with 10-Hz trains. This stimulus frequency was chosen to limit the contribution of synaptic augmentation during recovery from STD and to avoid post-tetanic potentiation. We observed a slight initial paired-pulse facilitation (1.09 ± 0.05 and 1.05 ± 0.03 in WTKW and mutKW synapses, respectively), followed by depression, during which steady-state eEPSC amplitudes were reduced to ~55% as compared to the maximum eEPSC size measured during trains, indicating an RRP depletion of ≥45%. Following conditioning, eEPSCs recovered within 5–10 s to an amplitude that was ~20% larger than eEPSCs of the conditioning train (Garcia-Perez and Wesseling, 2008). While levels and decay time constants (~9 s) of synaptic augmentation were similar in WTKW and mutKW synapses, the rebound from STD was accelerated in mutKW synapses, particularly at 1- to 2-s recovery intervals (Figures 8H1 and 8H2). To corroborate a more complete SV pool recovery after 2 s recovery from STD, we approximated in a subset of synapses the relative RRP occupancy by estimating the ratio of RRP2 / RRP16 s, assuming that pool recovery has completed after 16 s recovery. The corresponding ratio was larger in mutKW (96% ± 3%) as compared to WTKW (82% ± 5%) SC/C-CA1 synapses (Figure 8H3). These data show that SVR acceleration by Munc13-1 affects short-term plasticity also at strongly augmenting synapses, indicating a fascinating interplay between different short-term plastic processes that shapes the distinct features of different synapse types.

**DISCUSSION**

Munc13s are essential SV priming proteins, in whose absence no fusion-ready SVs are formed and synaptic transmission ceases (Sigler et al., 2017; Varoqueaux et al., 2002). Studies with cultured neurons showed that Munc13 activity can be regulated by Ca2+-CaM binding to an amphipathic helix motif (Junge et al., 2004; Lipstein et al., 2012), by DAG binding to a central C1 domain (Betz et al., 1998; Rhee et al., 2002), and by Ca2+-phospholipid binding to an adjacent C2B domain (Shin et al., 2010). What has remained unknown is how these regulatory processes affect synapse function downstream of SV priming and whether they are used purposively in intact networks to shape computational synapse properties. We show here that Munc13-1 is regulated by convergent Ca2+- and phospholipid-dependent signaling to adjust SV priming rates, enable sustained transmission, and ensure temporal fidelity of synaptic signaling in intact circuits.

To assess the role of Ca2+- and phospholipid-dependent regulation of Munc13-1 function in intact circuits, we generated KI mice that express Munc13-1 variants with abolished (Munc13-1<sup>D706S/711H</sup>) or increased Ca2+-dependent phospholipid binding to the C2B domain (Munc13-1<sup>K706W</sup>; Shin et al., 2010) and characterized functional consequences in the calyx of Held synapse. Our data demonstrate an activity-dependent activation of Munc13-1 via its C2B domain. In the absence of Ca2+-regulated phospholipid binding to the Munc13-1 C2B domain, fast SVR during and immediately after high-frequency AP trains is strongly attenuated, resulting in reduced transmitter release and perturbed temporal precision of synaptic transmission following bursts of activity. Conversely, increasing the Ca2+-dependent PIP<sub>2</sub> affinity of the Munc13-1 C2B domain enhances SVR, augments synaptic strength during and following presynaptic AP trains, and improves temporal precision of transmission. The fact that the mutation-induced bidirectional manipulation of Ca2+-phospholipid binding to the Munc13-1 C2B domain leads to corresponding bidirectional changes in SVR rates demonstrates the existence of a Ca2+-phospholipid-sensing regulatory process in SV priming within intact...
Figure 8. Munc13-1-dependent SV priming affects the AP timing in MNTB principal neurons during recovery from synaptic depression and accelerates the rebound from synaptic depression at hippocampal Schaffer collateral-CA1 synapses

(A–F) Recordings in brainstem slices obtained in the absence of kyn.

(A and B) Cell-attached recordings of postsynaptic APs triggered in MNTB PNs in response to afferent fiber stimulation during a 100-Hz (25 stimuli) train (A1 and B1) and at various recovery intervals (A2 and B2) in a WT DN (A) and a muDN (B) synapse. All eight recovery intervals (0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 s) were tested in a single sweep to shorten the duration of the recording protocol. APs recorded during recovery from synaptic depression are shown superimposed after alignment to the stimulus onset. Traces shown in B2 are temporally slightly offset to align the negative peak of the AP recorded after 16 s recovery (dotted line) in the muDN with that of the corresponding WT DN synapse to facilitate latency fluctuation comparison.

(C) Similar experiment as in (A) and (B), but postsynaptic APs were recorded intracellularly under current-clamp conditions during the 100-Hz trains (left) and for various test intervals (superimposed, right).

(D) Latency fluctuations for APs recorded 0.25 s (left) and 16 s (right) after conditioning 100-Hz trains. 21 consecutive trials are shown superimposed.

(E and F) Summary data for latencies of intracellularly recorded postsynaptic APs (E) and fluctuations of AP timing (F) plotted versus recovery interval. Data from WT synapses of both KI lines were similar and therefore pooled. AP latencies are expressed relative to the timing of the AP peak for 16 s recovery interval (tAP16 s) (E). Changes in latency fluctuations are expressed as the ratio of the SD of tAP relative to the SD of tAP16 s (F).

(G and H) Recordings in P16–P21 hippocampal slices, obtained using 2 mM Ca2+ and 1 mM Mg2+ in the bath solution and in the absence of kyn.

(G) Recovery from STD induced by 10-Hz stimulus trains (20 APs) at hippocampal SC/C-CA1 synapses of a C2B WT KI (G1) and a C2B muKI (G2) mouse (left panels). Recovery was probed by recording a single eEPSCtest evoked at increasingly longer recovery intervals (0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 s), which are shown superimposed (right panels).

(H) Average time course of eEPSC amplitudes during conditioning 10-Hz stimulation and the subsequent recovery from STD, obtained from WT KI (H1) and muKI (H2) SC/C-CA1 synapses. At least three trials were averaged for each synapse tested. Smooth traces represent a model of synaptic plasticity fitted to the data. The model consisted of SV pool depletion and a stimulus-induced augmentation of the release probability, which slowly decayed in the absence of AP firing. Estimated SV pool recovery time constants \( \tau_r \) were 2.40 s (WT KI) and 1.76 s (muKI). Assuming that pool recovery has completed after 16 s, the relative pool occupancy at 2 s recovery was estimated as the ratio of \( R_{P2} / R_{P16} \) for a subset of WT KI and muKI synapses (H3).

Data depict mean ± SEM.
circuits and identifies Munc13-1 as its major target. We propose that Munc13-1 integrates Ca$^{2+}$ and PI$_P$ signaling to tune SVR speed according to the requirements imposed on the release machinery. We demonstrate that this regulatory process is relevant in calyx of Held and Schaffer collateral/commissural-CA1 synapses. Given that Munc13-1 is expressed in essentially all neurons of the brain (Augustin et al., 1999a), this regulatory principle is likely to operate in many brain circuits to control information processing.

**The Munc13 C$_{2B}$ domain**

Five aspartic acid residues of the Munc13 C$_{2B}$ domain coordinate two Ca$^{2+}$ ions (Shin et al., 2010). Binding assays with the isolated C$_{2B}$ domain indicate an apparent Ca$^{2+}$ EC$_{50}$ of 5.5 µM to promote phospholipid binding. Such high [Ca$^{2+}$], is likely only reached near open VGCCs (Eggermann et al., 2011; Neher and Sakaba, 2008) or during high-frequency AP firing (Korogod et al., 2005; Lin et al., 2017). Our findings, showing that Ca$^{2+}$-dependent phospholipid binding to the C$_{2B}$ domain promotes SVR, imply that the C$_{2B}$ domain of Munc13s “sees” high [Ca$^{2+}$], levels during AP firing.

The lipid binding properties of the Munc13-1 C$_{2B}$ are rather unique, with PI$P$ and PI$_P$ binding preferentially and equally well. This may allow the domain to respond to subtle temporal and spatial changes in phosphoinositide (PI) second messenger levels, which occur during ongoing presynaptic activity, e.g., via Ca$^{2+}$-dependent regulation of phospholipases or PI kinases/phosphatases or upon activation of cell-surface receptors (Brown and Sihra, 2008). These considerations, and the fact that Munc13-1 acts as an AZ organizer (Sakamoto et al., 2018), lead to the notion of a dynamic interplay between the lipid and protein composition of SV fusion sites that is regulated by synaptic activity.

While the RRP is completely eliminated upon Munc13 loss (Siksou et al., 2009; Varoqueaux et al., 2002), several mutations of Munc13-1 regulatory domains reduce RRP size. This was observed in KI neurons expressing a DAG-insensitive Munc13-1 (Rhee et al., 2002) and subsequently with C$_{2A}$ and C$_{2C}$ deletion mutant Munc13-1 variants. The latter finding led to the notion that Munc13-1 may not only regulate SNARE complexes but also bridge SV and AZ plasma membranes, involving the C$_{1}$-C$_{2}$B tandem (Liu et al., 2016; Quade et al., 2019). The present study did not yield evidence for RRP changes upon elimination or enhancement of C$_{2B}$-lipid interactions, indicating that the C$_{2B}$ mutations we introduced do not interfere with any membrane bridging function.

Certain Munc13 C$_{2B}$ mutations change presynaptic [Ca$^{2+}$]$_i$ transients in cultured neurons, presumably via VGCC modulation (Calloway et al., 2015). We observed no defects in calyceal I$_{Ca(T)}$ of C$_{2BDN}$ and C$_{2BKW}$ mutants. Likewise, AP-evoked global [Ca$^{2+}$]$_i$ transients in dye-preloaded calyces were unaltered, indicating that the major spatial aspects of AZ organization and AP-evoked presynaptic Ca$^{2+}$ influx are intact in mut$_{DN}$ and mut$_{KW}$ synapses.

**Phospholipid-dependent regulation of SV priming and fusion**

Phospholipids are presumed to regulate Ca$^{2+}$-dependent vesicle fusion in neurons and neuroendocrine cells. Many presynaptic proteins have lipid-binding domains (Pinheiro et al., 2016), and PI$_P$ is a major component of the plasma membrane at release sites (Kabachinski et al., 2014; Milosevic et al., 2005; Walter et al., 2017). PI$_P$ likely clusters syntaxin (Honigmann et al., 2013; van den Bogaart et al., 2011) and other AZ components (de Jong et al., 2018), acts as a regulator of synaptotagmin-driven SV fusion (van den Bogaart et al., 2012), and is involved in presynaptic clathrin-mediated endocytosis (Jung and Hauske, 2007), also in the calyx of Held (Eguchi et al., 2012).

A major challenge in studying phospholipid-dependent regulation of synaptic protein function is that the dynamic lipid composition at AZs is unknown and difficult to manipulate experimentally. Most relevant data were obtained with in vitro analyses of neuroendocrine cells, where vesicle fusion occurs over the entire plasma membrane, and by pharmacologically manipulating phospholipid composition. The time course of such manipulations is slow, and they often lack specificity. Fast-acting, light-activated lipid compounds exist, but their activation is difficult to restrict to AZs (Frank et al., 2016; Walter et al., 2017). To circumvent these challenges, we chose a genetic approach, i.e., the mut$_{KW}$, initial synaptic strength, PPR, and the rate of eEPSC depression during the onset of high-frequency trains are unaltered, indicating that PPR is unchanged. However, for all but the three highest stimulus frequencies (50–200 Hz), we determined increased steady-state replenishment rate constants $\tilde{k}_R$. Elevated PI$_P$ affinity of the mutated Munc13-1 C$_{2BDN}$ domain thus augments SVR. During high-frequency stimulation, the elevated PI$_P$ affinity of the mutated C$_{2BKW}$ domain may become less important because elevated [Ca$^{2+}$]$, thus$ enhances phospholipid binding to the C$_{2B}$ domain also in WT synapses. Indeed, for high-frequency stimulation, enhancing AP-evoked presynaptic Ca$^{2+}$ influx pharmacologically only moderately speeds up eEPSC recovery, indicating that $\tilde{k}_R$, during 100- and 200-Hz conditioning is close to maximum even in WT synapses.

The C$_{2BDN}$ mutation perturbs Munc13-1 function by replacing two aspartic acid residues, abolishing Ca$^{2+}$$-CaM$ binding (Shin et al., 2010). In mut$_{KW}$ synapses, initial synaptic strength, PPR, and the rate of eEPSC depression during the onset of high-frequency trains are unaltered, indicating that PPR is unchanged. However, for all but the three highest stimulus frequencies (50–200 Hz), we determined increased steady-state replenishment rate constants $\tilde{k}_R$. Elevated PI$_P$ affinity of the mutated Munc13-1 C$_{2BKW}$ domain thus augments SVR. During high-frequency stimulation, the elevated PI$_P$ affinity of the mutated C$_{2BKW}$ domain may become less important because elevated [Ca$^{2+}$]$, thus$ enhances phospholipid binding to the C$_{2B}$ domain also in WT synapses. Indeed, for high-frequency stimulation, enhancing AP-evoked presynaptic Ca$^{2+}$ influx pharmacologically only moderately speeds up eEPSC recovery, indicating that $\tilde{k}_R$, during 100- and 200-Hz conditioning is close to maximum even in WT synapses.

The C$_{2BDN}$ mutation perturbs Munc13-1 function by replacing two aspartic acid residues, abolishing Ca$^{2+}$$-CaM$ binding (Shin et al., 2010). We observed higher initial synaptic strength in mut$_{DN}$ synapses as compared to WT. This is likely caused by higher $\tilde{k}_R$, as PPRs are lower in Munc13-1 C$_{2BDN}$ synapses, and synaptic depression during high-frequency trains proceeds faster while RRP is unaffected. An explanation for this unexpected finding is that the C$_{2BDN}$ variant shows significant membrane binding in the absence of Ca$^{2+}$ (Shin et al., 2010), which may lead to increased Munc13-1 C$_{2BDN}$ activity at resting [Ca$^{2+}$]$_i$.

**The interplay of regulatory domains of Munc13s**

Three Ca$^{2+}$- and second-messenger-sensitive regulatory domains allow Munc13s to respond to a wide range of [Ca$^{2+}$]$_i$ and lipid second messengers. Ca$^{2+}$-CaM binding to Munc13s occurs...
Multiple previous studies focused on Munc13 C2B function, and discrepancies regarding the notion of Munc13s as a regulatory domain of SVR. It is therefore likely that the regulatory domains of Munc13s operate in a successive, cooperative, and synergistic fashion rather than redundantly.

We previously examined functional effects of a point mutation (Munc13-1W464R, mutW464R) that renders Munc13-1 Ca2+-insensitive (Lipstein et al., 2013). MutW464R calyces show much slower SV pool recovery following low presynaptic depolarizations, but recovery of AP-evoked eEPSCs following STD is less perturbed. The latter finding led to the conclusion that additional, possibly more effective, molecular pathways for [Ca2+]i-dependent SVR regulation must exist (Lipstein et al., 2013).

The present study identifies Ca2+-dependent phospholipid binding to Munc13-1 as such a pathway. The loss of fast eEPSC recovery in mutDN synapses demonstrates that it plays a major role in Ca2+-dependent SVR. This mechanism likely operates in concert with Ca2+-CaM and DAG binding to adjust presynaptic function on demand, stressing the notion of Munc13s as a regulatory hub in SV priming.

The function of the Munc13 C2B domain—consensus and discrepancies

Multiple previous studies focused on Munc13 C2B function, establishing it as a Ca2+-phospholipid binding domain that controls Munc13 priming function and thus dynamically regulates transmitter release (Kabachinski et al., 2014; Michelassi et al., 2017; Shin et al., 2010). Beyond this, Munc13 C2B interactions with AZ scaffold proteins (Brockmann et al., 2020) and VGCCs (Calloway et al., 2015) were reported. Unfortunately, a direct comparison of previous studies with ours is complex as different organisms were studied.

Nevertheless, at least three general commonalities emerge. First, effective and specific blockade of Ca2+-phospholipid binding to Munc13 C2B reduces SV priming activity and attenuates synaptic transmission, albeit to different degrees and under different stimulation conditions (Kabachinski et al., 2014; Michelassi et al., 2017; Shin et al., 2010). Second, certain mutations in the Ca2+-phospholipid-binding pocket of Munc13 C2B can cause increased transmitter release, likely due to an increase in Ca2+-independent phospholipid binding (Kabachinski et al., 2014; Michelassi et al., 2017; Shin et al., 2010). Third, Ca2+-phospholipid-dependent regulation via the C2B domain can modulate the dynamics of synaptic transmission upon prolonged high-frequency stimulation (Shin et al., 2010; present study), with different consequences for short-term plasticity. The remaining differences in the context of these commonalities likely arise due to differences in the neuronal membrane lipid composition present in the various preparations and synapse types studied.

Equally important are the facets that distinguish the present findings from previously published ones. Most importantly, the conclusions drawn from our work versus that of Shin et al. (2010) differ fundamentally. Whereas Shin et al. (2010) did not report evidence for a regulation of SVR rate by Munc13 C2B, we provide clear evidence for such a regulation. Aspects of this discrepancy might be due to different experimental conditions.

Synaptic depression and recovery at the calyx of Held synapse

Several mechanisms, including pool depletion, negative feedback via presynaptic metabotropic receptors, and postsynaptic receptor desensitization, contribute to synaptic depression (Zucker and Regehr, 2002). Synaptic transmission at post-hearing onset calyx synapses shows only limited sensitivity to auto-inhibition via mGluRs (Renden et al., 2005) or AMPARs desensitization and saturation (Taschenberger et al., 2002). Because we recorded eEPSCs in the presence of 1 mM kyn, further attenuating possible postsynaptic effects, we assume pool depletion as the principal cause for synaptic depression in our experiments.

STD is determined by a balance of SV recruitment and consumption. Recovery from synaptic depression is slow (τ = 4 s) at calyx synapses when measured after stimuli that do not strongly increase global [Ca2+]i (Iwasaki and Takahashi, 2001; von Gersdorff et al., 1997). Considering Kc = 1 = 0.25 s−1 and a total number of SV docking sites (N) that approximately equals the size of the FRP (1,000–2,000 SVs), the SVR rate would be limited to 250–500 SV/s. For 200-Hz trains and q ~ 60 pA (Chang et al., 2015), this equates to an eEPSCs of only 75–150 pA (~8–17 pA for recordings in 1 mM kyn), which is much less than experimentally observed. In fact, we estimated a 16-times-higher kFRP of ~4 s−1 to predict the observed steady-state release rates during 200-Hz trains. Our kFRP estimate rests on the assumption that N is approximately equal to the FRP size, i.e., that the occupancy (ρocc) of docking sites in resting calyces is close to 100%. If ρocc were substantially lower (Malcagni et al., 2020), we would need to postulate a larger N and, consequently, lower kFRP.

Assuming a strongly upregulated kFRP during high-frequency conditioning, which then decays to its basal value after cessation of stimulation, we expect a biphasic eEPSC recovery time course. During low-frequency conditioning, kFRP increases much less, which explains the absence of a fast component of eEPSC recovery. However, the mere existence of a biphasic eEPSC recovery alone is insufficient proof for a Ca2+-dependence of SVR (Hallemand et al., 2010), and for some synapses, experimental interference with either [Ca2+]i or its sensor binding did not indicate a Ca2+-dependence of SVR (Miyano et al., 2019; Ritzau-Jost et al., 2018).

Nevertheless, the findings that, in calyx synapses, eEPSC recovery is accelerated upon augmenting presynaptic Ca2+ influx (Wang and Kaczmarek, 1998) and sensitive to...
manipulations that interfere with Ca²⁺ binding to CaM (Sakaba and Neher, 2001a), with Ca²⁺-CaM binding to Munc13-1 (Lipstein et al., 2013), or with Ca²⁺-phospholipid binding to Munc13-1 (present study) support the notion that the fast component of pool recovery is regulated by elevated [Ca²⁺].

For 200-Hz stimulation of WT synapses, we estimated a $R_0$ of ~4 pools/s at steady state. Assuming a linear relationship between global [Ca²⁺] and SVR rate constant according to $R = k_{basal} + \alpha \times [Ca^{2+}]$, with $k_{basal} = 0.1 \text{ s}^{-1}$ and a slope factor $\alpha$ of ~1 pool/($\mu$M $\times$ s) (Hosoi et al., 2007), this corresponds to [Ca²⁺] of ~3.9 $\mu$M. Such supra-micromolar global [Ca²⁺] values of ~1.4 $\mu$M and in the range of 3–10 $\mu$M were previously reported for voltage-clamped WT calyces stimulated with 200-Hz AP waveform trains (Lin et al., 2017) and for dye-preloaded WT calyces during prolonged 100-Hz firing (Korogod et al., 2005), respectively.

Munc13-1-controlled SV recruitment and information processing in single synapses and synaptic circuits

Our analyses of postsynaptic AP latency and jitter show that altered SVR can affect information transfer at calyx synapses. Consistent with the “failsafe” operation of calyx synapses (Guinan and Li, 1990; Lorteije et al., 2009), we never observed spike failures during or following AP trains. In comparison to WT, mutDN synapses show longer AP latencies and reduced temporal precision of transmission following conditioning trains. In contrast, muKvw synapses show improved temporal precision and shorter AP latencies. This demonstrates that the SVR speed determines postsynaptic AP timing after bouts of presynaptic activity. This may have only subtle functional consequences at the calyx of Held, particularly with faster SVR at physiological temperature (Kushmerick et al., 2006). However, profound effects on the reliability of postsynaptic firing are likely at the temperature (Kushmerick et al., 2006). However, profound effects on the reliability of postsynaptic firing are likely at the

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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- Mouse Maintenance

METHOD DETAILS

- Western Blot Analyses
- Co-Immunoprecipitation Experiments
- Immunostaining
- Slice Preparation
- Electrophysiology
- Ca²⁺ Imaging in Nearly Unperturbed Terminals

QUANTIFICATION AND STATISTICAL ANALYSIS

- eEPSC Trains and Paired-Pulse Ratios (PPR)
- Readily-Relieasable SV Pool and Release Probability
- Steady-State Replenishment Rate Constant ($R_0$)
- STP at Hippocampal SC/C-CA1 Synapses

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.neuron.2021.09.054.

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AUTHOR CONTRIBUTIONS

N.B. initialized the project. N.L. generated and validated Munc13-1 C₂B KIs. N.L., S.C., and H.T. designed and performed postsynaptic recordings in brainstem slices. K.-H.L. designed and performed presynaptic recordings and Ca²⁺ imaging in brainstem slices, with assistance of H.T. F.J.L.-M. designed and performed recordings in hippocampal slices and Munc13-1 immunolabeling analyses of brainstem slices, with assistance of H.T. N.L., S.C., K.-H.L., F.J.L.-M., and H.T. analyzed and interpreted data. H.T. supplied software routines and performed simulations. N.B. and E.N. provided conceptual input and advice. N.L., H.T., and N.B. wrote the manuscript. All authors provided text edits.

DECLARATION OF INTERESTS

The authors declare no competing interests. N.B. is a member of the Neuron advisory board.

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SUPPORTING CITATIONS

The following references appear in the supplemental information: Chanda and Xu-Friedman (2010); Foster et al. (2002); Neher and Sakaba (2001); Wong et al. (2003).

REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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**Chemicals, peptides, and recombinant proteins**

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**Experimental models: Cell lines**

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(Continued on next page)
### RESOURCE AVAILABILITY

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nils Brose (Brose@em.mpg.de).

**Materials availability**

Mouse lines will be shared upon request within the limits of the respective material transfer agreements.

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Mouse Generation
The Munc13-1 knock-in (KI) mice were generated by homologous recombination in 129/ola embryonic stem cells (Hooper et al., 1987). A targeting vector containing UNC13A exons 13-26 with an insertion of an FRT-Puromycin-FRT cassette before exon 18 (Figure 1A) was cloned. To create the C2BDN line, three nucleotide exchanges were included, two leading to the exchange of aspartic acid residues in position 705 and 711 of Munc13-1 to asparagine (GAC to AAC), and one that eliminates a nearby BamHI site (GGA to GGC) but preserves the glycine residue (pTKPuroFRT-UNC13A Exons 13-26 Exon 18/C2BDN; Figures 1A and 1D). To create the C2BKW line, the targeting vector included two nucleotide exchanges, one leading to the replacement of a lysine residues in position 706 of Munc13-1 by a tryptophan (AAG to TGG), and one to eliminate a nearby BamHI site (GGA to GGC), preserving the glycine residue (pTKPuroFRT-UNC13A Exons 13-26 Exon 18/C2BKW; Figures 1A and 1D). The resulting vectors were used to electroporate stem cells, and the correct genomic integration of the cassette was identified by the acquisition of puromycin resistance (Thomas and Capecchi, 1987), and by long-range PCR amplification and DNA sequencing. Positive clones were amplified, injected into blastocysts, and the resulting mice were screened for germline transmission via long-range PCR amplification. To eliminate the puromycin resistance cassette, mice were crossed with Gt(Rosa)26So(Pmr1)Dym mice (Farley et al., 2000). Offspring were analyzed using genotyping PCR (Sense prime: 5’-GGGTAGCTGCAGGATTTATTGTAT-3’, Antisense prime: 5’-TGTGCCAGGTATTTATGATC-3’), and sequencing (Figures 1B–1D), and animals in which a successful cre recombination had occurred were selected for further breeding with C57BL/6N mice for three generations. Mice were then cross-bred to produce homozygous and wt littermates for experiments.

Mouse Maintenance
Mutant mouse generation and animal experiments were approved by the responsible authorities of the local government (Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; permit 33.19-42502-04-15/1817). Animals were maintained in groups in accordance with European Union Directive 63/2010/EU and ETS. Animal health was controlled daily by caretakers and by a veterinarian, and a quarterly health monitoring was done according to FELASA recommendations with either NMRI sentinel mice or animals from the colony (serological analyses; microbiological, parasitological, and pathological examinations). Abnormal findings were not made during the period of the study. Mice were kept in individually ventilated cages, under specific pathogen-free conditions, 21 ± 1°C, 55% relative humidity, 12 h/12 h light/dark cycle). Food and tap water, as well as bedding and nesting material, were provided ad libitum, and cages were changed once a week. P14–17 littermate homozygous Munc13-1 KI mice and wt controls of either sex were analyzed. Littermates were used to minimize effects of genetic background, although only minor differences were observed between wt lines (Table S1). Mice were routinely genotyped by PCR (Figures 1B–1D). We did not observe large deviations from the expected Mendelian ratios among offspring, although for C2BKW mice a slightly lower than expected fraction of homozygous KI mice was obtained, possibly indicating a slightly increased perinatal lethality. C57BL/6N mice were used for the experiments shown in Figures 6D, S3B, S3C, and S7.

METHOD DETAILS

Western Blot Analyses
P2 crude synaptosomal fractions were obtained from cortical brain tissue of P16 KI mice and wt littermates as previously described (Lipstein et al., 2013). Between 2-20 μg protein were separated on 4%-12% gradient Bis-Tris polyacrylamide gels (Invitrogen) and blotted onto nitrocellulose membranes. The protein load per lane was quantified by a reversible membrane staining protocol (MemCode; Pierce) and quantified using ImageJ. The following antibodies were subsequently used to identify the indicated proteins: rabbit polyclonal (rp) anti-Munc13-1 (40), anti-ubMunc13-2, and anti-bMunc13-2 (Varoqueaux et al., 2005), rp-anti-Doc2 (SySy; 174 103), rp-anti-CAPS-1 (SySy 262 013), rp-anti-Munc18-1 (SySy; 116 002), mouse monoclonal (mm)-anti Synapsin 1A/B (SySy 110 011), mm-anti-Synaptobrevin 2 (SySy 104 211), mm-anti-Synaptotagmin (SySy 105 001), rp anti-Rim (lab antibody; Q703), mm anti- SNAP25 (SySy; 111 011). After incubation with corresponding secondary antibodies ( Peroxidase-AffiniPure Goat Anti-Mouse IgG or peroxidase-AffiniPure Goat Anti-Rabbit IgG antibody), the western blot signal was detected with an INTAS imager (INTAS Science Imaging), quantified using ImageJ, and normalized to the protein load. Between 3-4 independent analyses were preformed per condition. Quantification is presented as mean ± SEM.

Co-Immunoprecipitation Experiments
Co-immunoprecipitation was performed according to Lipstein et al. (2013). Briefly, crude synaptosomal fractions obtained from cerebral corti of adult (8–11 weeks) wt and KI mice were solubilized and ultracentrifuged using a Beckmann L-70 at 100,000 g to remove
insoluble material. A sample (‘input’) was collected, and the remaining fraction was incubated with a rp-anti-Munc13-1 (40). Sepha-
rose-Protein G beads (Invitrogen) were added to capture the antibody and associated proteins, washed to remove background, and
eluted using denaturing Laemmli buffer (‘IP’ samples). The samples were loaded on 4–12% gradient Bis-Tris polyacrylamide gels and
blotted according to a modified version of the Sigma protocol (product C7055) with mm-anti-Calmodulin (Upstate Biotech-
nology). Munc13-1 was blotted using the mm-anti Munc13-1 (SH5; Betz et al., 1998).

Immunostaining
Immunostaining experiments were performed on P15 coronal brainstem sections using primary antibodies against Munc13-1,
Bassoon, and MAP-2. Brains were rapidly frozen in isopentane (−35 °C). Sixteen μm-thick coronal cryosections of the MNTB region
were cut and mounted on Superfrost slides, air-dried for 15 min, and immersion-fixed in ice-cold 4% paraformaldehyde solution (4% PFA in 0.1 M PB, pH 7.4) for 5 min at room temperature (RT). To ensure similar fixation and labeling conditions between genotypes,
sections of mutant and corresponding wt mice were mounted together. Sections were incubated for 90 min at RT in blocking solution
(0.1 M PB, 5% normal goat serum, 0.1% cold water fish skin gelatine, 0.5% Triton X-100, pH 7.4) before being treated overnight at 4 °C
with the primary antibodies rp-anti-Munc13-1 Ab (SySy 126 103, 1:400), mm-anti-Bassoon (Enzo Life Sciences SAP7F407 1:400), and
chicken polyclonal anti-MAP2 (Novus NB300-213, 1:600), diluted in incubation buffer (0.1 M PB, 3% normal goat serum, 0.1% cold
water fish skin gelatine, 0.3% Triton X-100, pH 7.4). After washing in PB, sections were incubated for 2 h at RT in the dark with the
fluorescent secondary antibodies Alexa 488-coupled goat anti-rabbit, Alexa-555-coupled goat anti-mouse, and Alexa-633-coupled
goat anti-chicken (Invitrogen, 1:1000) diluted in incubation buffer. Covells were slips with Aqua-PolyMount (Polysciences).
Confocal laser scanning micrographs of presynaptic compartments surrounding MNTB PNs were acquired with a Leica TCS SP5
I confocal microscope. An HCX PL APO lambda blue 63 x water immersion objective (NA = 1.2) and a pinhole setting of 0.38 AU
were used to obtain single-plane micrographs (512 x 512; x-y pixel spacing = 48.1 nm) in sequential scanning mode. Laser power
and gain were adjusted to ensure that signals were in the linear range of detection. Confocal images were subjected to deconvolution
using two ImageJ (National Institutes of Health; Bethesda, MD) plugins: point spread functions were generated using -Diffraction PSF
3D plugin, and deconvolution was performed using DeconvolutionLab plugin (Biomedical Imaging Group, EPFL; Lausanne,
Switzerland). Individual Munc13-1 immunoreactive puncta were detected using a thresholding algorithm (ImageJ). For all Munc13-1-positive puncta within ~1 μm wide regions of interest (ROIs) drawn around the perimeter of MNTB PNs and approximately
delineating the presynaptic compartments, the center of mass was determined and those coordinates were exported for further pro-
cessing. For each ROI, the average density of Munc13-1-positive puncta per μm perimeter was obtained and the mean nearest-
neighbor distance was calculated as a parameter characterizing the distribution of Munc13-1-positive puncta within a given ROI.

Slice Preparation
Acute brainstem slices of postnatal (P14–P17) mice of either sex were prepared as previously described (Chang et al., 2015). After
decapitation, brains were immersed in ice-cold low-Ca2+ artificial CSF (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 0.1 CaCl2, 3
MgCl2, 25 glucose, 25 NaHCO3, 1.25 Na2HPO4, 0.4 ascorbic acid, 3 myoinositol, and 2 Na-pyruvate (pH 7.4, bubbled with 95% O2,
5% CO2). The brainstem was glued onto the stage of a VT1000S vibratome (Leica), and 200 μm-thick coronal slices containing the
medial nucleus of the trapezoid body (MNTB) were cut. Slices were incubated for 40 min at 35 °C in a chamber containing normal
aCSF (identical to low-Ca2+ aCSF, except that 3 mM MgCl2 and 0.1 mM CaCl2 were replaced with 1.3 mM MgCl2 and 1.7 mM CaCl2).
Slices were kept at RT (21–24 °C) for up to 5 h after recovery.
Acute parasagittal hippocampal slices (300 μm thick) were prepared from P16–P21 mice of either sex using a VT1000S vibratome and
ice-cold low-Ca2+/high-Mg2+ sucrose-based cutting solution containing (in mM): 120 sucrose, 64 NaCl, 25 NaHCO3, 2.5 KCl, 1.25
Na2HPO4, 10 glucose, 0.5 CaCl2 and 7 MgCl2 (pH 7.4, bubbled with 95% O2, 5% CO2). Slices were incubated for 40 min at 35 °C in a chamber filled with normal aCSF containing 1 mM MgCl2 and 2 mM CaCl2. Slices were kept at RT for up to 5 h after recovery.

Electrophysiology
Whole-cell patch-clamp recordings were made from calyx of Held terminals and principal neurons (PNs) of the MNTB at RT using an
EPC-10 amplifier controlled by Pulse or PatchMaster software (HEKA Elektronik). Patch-pipettes (Science Products) were coated
with dental wax in order to minimize fast capacitive transients during voltage-clamp experiments and to reduce stray capacitance.

For postsynaptic recordings, pipettes with an open-tip resistance of 2.5–4 MΩ were filled with a solution containing (in mM): 100
K-glutamate, 60 KCl, 5 Na2-phosphocreatine, 10 HEPES, 5 EGTA, 0.3 Na2-GTP, and 4 ATP-Mg, pH 7.3, with KOH. During experi-
ments, slices were continuously perfused with normal aCSF solution containing 1.3 mM MgCl2 and 1.7 mM CaCl2 and supple-
mented with 5 μM strychnine, to block glycinergic inputs. Cells were visualized by infrared-differential interference contrast microscopy
through a 40X water-immersion objective using an upright BX51WI microscope (Olympus). All experiments were performed at RT.
A bipolar stimulation electrode was used to evoke presynaptic APs (stimulus intensity ≤ 20 V, 100 μs duration). Series resistance (Rs)
was < 8 MΩ and compensated ≥ 82%. Holding potential (Vh) and leak current were ~70 mV and ≤ 200 pA, respectively. Sampling
interval and low-pass filter settings were 20 μs and 5.0 kHz, respectively, eEPSC peaks measured in P14-17 mouse calyx syn-
apses frequently exceed amplitudes of 10 nA (Chang et al., 2015; Joshi and Wang, 2002). Assuming a maximum Rm = 8 MΩ that is
80% compensated by the amplifier Rr-compensation circuitry, such peak amplitudes cause a transient voltage escape that corre-
sponds to a > 20% drop in driving force at Vm = ~70 mV for a synaptic conductance having a reversal potential of ~0 mV. To reduce
eEPSC amplitudes for improved voltage-clamp and in order to attenuate postsynaptic AMPAR saturation and AMPAR desensitization, all experiments were performed in the continuous presence of 1 mM of the low-affinity GluR antagonist kynurenic acid (kyn), unless explicitly stated otherwise. The blocking ratio eEPC \text{Kyn}/eEPC \text{Ctrl} is shown for a subset of the experiments in Figure S1. Furthermore, voltage-clamp errors caused by remaining uncompensated Rs were corrected offline by a software routine (see offline analysis).

Presynaptic voltage-clamp recordings were performed using patch pipettes with an open-tip resistance of 3–4.5 MΩ. Series resistance was ≤ 15 MΩ and Rs was compensated 60%–65%. For measuring ICa(V) and membrane capacitance (ΔCm), pipettes were filled with a Cs-glucuronate based solution containing (in mM): 100 Cs-glucuronate, 30 TEA-Cl, 30 CsCl, 10 HEPES, 0.05 EGTA, 5 Na2-phosphocreatine, 4 ATP-Mg, 0.3 GTP, pH 7.3 with CsOH. The bath solution was supplemented with 1 μM TTX, 1 mM 4-AP, and 40 mM TEA-Cl to suppress voltage-gated sodium and potassium currents. Calyx terminals were visualized by oblique illumination (Dodt gradient contrast) through a 60 × water-immersion objective using an upright BX51WI microscope (Olympus). All experiments were performed at RT. The size of the readily-releasable pool of SVs was estimated by monitoring ΔCm using the sine + DC technique (Lindau and Neher, 1988) using the software lock-in amplifier implemented in PatchMaster (HEKA Elektronik) by adding a 1 kHz sine-wave voltage command (peak-to-peak amplitude ± 35 mV) to Vh = −80 mV. Presynaptic recordings with a leak current > 200 pA were excluded from the analysis. In eEPSC train recordings and during recordings of eEPSC recovery from depression, ≥ 3 repetitions per protocol were recorded for each cell included in the final analysis.

Whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons were made at RT with patch-pipettes (open-tip resistance 2.5–3.5 MΩ) filled with a solution containing (in mM): 130 K-glucuronate, 10 KCl, 2 MgCl2, 2 Na2ATP, 10 HEPES, 10 EGTA, pH 7.3 with KOH. During experiments, slices were continuously perfused with normal ACSF solution containing 1 mM MgCl2 and 2 mM CaCl2, supplemented with 25 μM bicuculline methiodide (HelloBio) to block GABAAR-mediated IPSCs. Schaffer collateral/commissural fibers were stimulated with a glass electrode filled with aCSF and placed in stratum radiatum ≥ 80 μm away from the cell body. Transmitter release was evoked by applying brief electrical pulses (100 μs, 10-50 V). Stimulus intensity was adjusted to obtain peak EPSC amplitudes in the range of 80-300 pA (mean values 161 ± 18 pA, n = 13, and 172 ± 23 pA, n = 13, for wt KW and mut KW synapses, respectively) when stimulated with single APs. Assuming a mean unitary EPSC amplitude of 10-20 pA, this corresponds to the recruitment of 4-30 Schaffer collateral/commissural fibers.

**Ca2+ Imaging in Nearly Unperturbed Terminals**

For measuring AP-evoked Ca2+ transients in nearly unperturbed calyces, terminals were preloaded with the low affinity Ca2+ dye Cal520-FF (Kd = 9.8 μM). Presynaptic patch pipettes were filled with a K-glucuronate based solution containing (in mM): 100 K-glucuronate, 60 KCl, 10 HEPES, 5 EGTA, 5 Na2-phosphocreatine, 4 ATP-Mg, 0.3 GTP, pH 7.3 with KOH, to which 400 μM Cal520-FF were added immediately before arrival of the i+1 th AP can be calculated recursively according to

\[ i_{t+1} = \frac{i_t - \Delta C_m}{h} \]

\[ h = \frac{E_{rev} - V_{h}}{g_{Ca}} \]

\[ g_{Ca} = \frac{a_{Ca} \cdot [Ca^{2+}]_0}{K_d + [Ca^{2+}]_0} \]

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Time series image stacks were analyzed offline using ImageJ (Schneider et al., 2012) and Igor Pro (Wavemetrics). Offline analysis of electrophysiological data was performed using Igor Pro, R (R Project for Statistical Computing) or Microsoft Excel. Voltage-clamp errors caused by remaining uncompensated Rs were fully compensated by applying a software correction procedure similar to that described in Traynelis (1998) and courtesy provided by E. Neher. Statistical analysis was conducted using Igor Pro, R and Microsoft Excel. Data are expressed as mean ± standard error of the mean (SEM). Error bars in all graphs indicate SEM. SEM and 95% confidence intervals for FRP that described in Traynelis (1998) and courtesy provided by E. Neher.

If not stated otherwise, a two-tailed Welch–Satterthwaite t test was used to test for statistical significance of differences between sample means (see p values in Table S1). In Table S1, the number of animals used in each experiment is indicated by a capital N, whereas the number of cells tested is indicated by a lowercase n.

**eEPSC Trains and Paired-Pulse Ratios (PPR)**

For a train of presynaptic APs, the prediction of a simple depletion model featuring a constant SV recruitment rate constant \( k_s = \frac{1}{N_o} \) for the number of occupied sites \( N_o \) immediately before arrival of the \( i+1 \)th AP can be calculated recursively according to

\[ i_{t+1} = \frac{i_t - \Delta C_m}{h} \]

\[ h = \frac{E_{rev} - V_{h}}{g_{Ca}} \]

\[ g_{Ca} = \frac{a_{Ca} \cdot [Ca^{2+}]_0}{K_d + [Ca^{2+}]_0} \]
where $N_{\text{total}}$ is the sum of all docking sites, $p_i$ is the release probability at AP arrival, $\Delta t$ is the inter-stimulus interval, and $\tau_i$ is the time constant of recovery.

For simplicity, we assume here $N_1 = N_{\text{total}}$.

Assuming that $p$ increases immediately following an AP from its current value by a constant fraction $a$ toward a maximum value of 1, we can express $p_i$ recursively as

$$p_{i+1} = (p_i + a \times (1 - p_i) - p_0) \times e^{-\Delta t/\tau_i} + p_0; \text{ for } i \geq 1$$

where $p_0$ is the release probability at rest, $\tau_i$ is the time constant of synaptic facilitation, and $\Delta t$ is the inter-stimulus interval.

For the paired-pulse ratio (PPR) of the first two consecutive eEPSCs we can therefore write

$$PPR = \frac{EPSC_2 - N_2 \times p_2}{EPSC_1 - N_1 \times p_1} = \left(1 - p_0 \times e^{-\Delta t/\tau_0}\right) \times \left(1 + b \times e^{\Delta t/\tau_0}\right); b = a \times \left(\frac{1}{p_0} - 1\right)$$

Readily-Releasable SV Pool and Release Probability

The whole entity of SVs that can be released within about 50 ms in response to strong and prolonged presynaptic [Ca\textsuperscript{2+}] elevations elicited by presynaptic Ca\textsuperscript{2+} uncaging or direct presynaptic depolarizations can be subdivided into two main SV subpools – ‘fast releasing’ (FRP) and ‘slowly releasing’ (SRP) SVs. The SRP only marginally contributes to AP-evoke release (Sakaba, 2006) such that pool estimates derived from AP-evoked eEPSCs primarily represent the FRP. There is good experimental evidence showing that the FRP itself is functionally inhomogeneous (Lee et al., 2013; Taschenberger et al., 2016). For simplicity, we neglect such functional heterogeneity of the FRP here and report average p values ($\overline{p}$) instead. Assuming, for example, that the FRP of calyx terminals is composed of two population of SVs having different $p$s, we can write

$$EPSC = q \times N \times \overline{p} = q \times \left(N_{\text{high}} \times p_{\text{high}} + N_{\text{low}} \times p_{\text{low}}\right) = q \times N \times \left(\frac{N_{\text{high}}}{N} \times p_{\text{high}} + \frac{N_{\text{low}}}{N} \times p_{\text{low}}\right)$$

where $N$ is the sum of occupied sites $N_{\text{high}} + N_{\text{low}}$, $q$ is the quantal size and the subscripts identify high- and low-$p$ release sites. In this case, $\overline{p}$ is simply a weighted average

$$\overline{p} = \frac{N_{\text{high}} \times p_{\text{high}} + N_{\text{low}} \times p_{\text{low}}}{N}.$$ 

During train stimulation, $N_{\text{high}}$ decreases more rapidly than $N_{\text{low}}$ because $p_{\text{high}} > p_{\text{low}}$. This causes $\overline{p}$ to decrease. This decrease is partially compensated by synaptic facilitation. For simplicity, we assume here that $\overline{p}$ remains approximately similar throughout the trains.

We obtained an estimate for the FRP from cumulative eEPSCs measured in response to high-frequency trains (50, 100 and 200 Hz). The cumulative eEPSC amplitude was corrected for SV recruitment by fitting a line to the final 4 eEPSCs representing the steady state (Figures 4 B and S5C).

Steady-State Replenishment Rate Constant ($\overline{R}$)

At steady state, the average rate of release is balanced by the average rate of replenishment $r_\overline{R} = r_\overline{R}_s$, with $r_\overline{R}_s = N_{ss} \times \overline{p} \times f_{\text{stim}}$ and $R_\overline{R} = (N_{\text{total}} - N_{ss}) \times \overline{p}$, where $N_{\text{total}}$ is the total number of sites (occupied or empty), $N_{ss}$ is the number of occupied sites at steady state immediately before AP arrival, and $N_{ss}$ and $\overline{R}$ are the average number of occupied sites and the average replenishment rate constant.
during the inter-stimulus interval at steady state, respectively. For high-frequency stimulation that leads to strong SV pool depletion, a majority of docking sites is empty such that the difference between $N_{ss}$ and $\bar{N}_{ss}$ is negligible in comparisons to the number of empty sites $\bar{N}_{total} - \bar{N}_{ss}$ and we can write $\bar{K}_e = \frac{N_{ss}}{(N_{total} - N_{ss})} \times \bar{p}_{ss} \times f_{stim}$.

With $EPSC_1 = N_{total} \times p_1 \times q$ and $EPSC_{ss} = N_{ss} \times p_{ss} \times q$, and if we further assume that $p_{ss} \approx p_1$, then we obtain $\bar{K}_e = EPSC_{ss} \times f_{stim} \times \frac{EPSC_{ss}}{EPSC_1}$. $\bar{K}_e$ is accurate only within the limits of the simplifications described above but nevertheless serves as a useful parameter for comparing the functional differences among C2BDN, C2BKW and WR mutant mice.

**STP at Hippocampal SC/C-CA1 Synapses**

Synaptic responses ($R$) at SC/C-CA1 synapses were modeled as being proportional to the product of a depletable resource ($N$) and a probability by which this resource is consumed by an AP ($p$):

$$R_i \sim N_i \times p_i$$

Postsynaptic sensitivity to glutamate ($q$) was assumed to be invariant. All eEPSC train amplitudes were normalized to the respective peak amplitude of the first eEPSC during the conditioning 10 Hz stimulus trains. Changes in synaptic strength during conditioning trains and during subsequent recovery from depression were simulated using a simple model considering depletion of $N$ and a slowly decaying augmentation of $p$. A contribution of fast decaying paired-pulse facilitation was small under our experimental conditions and therefore neglected. The magnitude of $N$ immediately before arrival of the $i+1^{th}$ AP was calculated according to:

$$N_{i+1} = (1 - p_i) \times N_i + \left(1 - p_i \times (N_{total} - (1 - p_i) \times N_i)\right) \times \left(1 - e^{\frac{-\Delta t}{\tau_A}}\right)$$

$$p_{i+1} = (p_i + a \times (1 - p_i) - p_0) \times e^{\frac{-\Delta t}{\tau_A}} + p_0; \text{ for } i \geq 1$$

where $N_0$ designates the resting value of $N$ which for the analysis shown in Figure 8H equates to $1/p_1$ because of the normalization of all $R_i$ relative to $R_1$. $p_i$ is the release probability at arrival of the $i^{th}$ AP, $\Delta t$ is the inter-stimulus interval, and $\tau_A$ is the time constant of $N$. Assuming that $p$ increases immediately following an AP from its current value by a constant fraction $a$ toward a maximum value of 1, we can obtain $p$ recursively as:

$$p_{i+1} = (p_i + a \times (1 - p_i) - p_0) \times e^{\frac{-\Delta t}{\tau_A}} + p_0; \text{ for } i \geq 1$$

$$p_1 = p_0$$

where $p_0$ is the resting value of $p$, $\tau_A$ is the decay time constant of augmentation, and $\Delta t$ is the inter-stimulus interval. Fit results for $p_0$ and $a$ were 0.06 and 0.002 in wtKW and 0.08 and 0.003 in mutKW synapses, respectively. The time constant of augmentation $\tau_A$ amounted to $\sim 9$ s for both wtKW and mutKW synapses.