Separation of presynaptic CaV2 and CaV1 channel function in synaptic vesicle exo- and endocytosis by the membrane anchored Ca2+ pump PMCA

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Differential functions of Cav2 and Cav1 channels in neurotransmitter release versus other Ca2+-dependent presynaptic processes can hardly be explained just by different coupling distances to SVs, since there are also situations where loose coupling is predominant (4, 10). Moreover, compared with CaV2.1 and CaV2.2 in vertebrates) remains spatially restricted to a few hundred nanometers due to the limited abundance and brief opening of the channels and the presence of endogenous CaV2.2 buffers (5, 6). It is thus conceivable that Ca2+ signals originating within presynaptic terminals but outside AZs are engaged to tune SV recycling and plastic changes according to changes in activity.

Apart from the need for fast activating and inactivating Cav2 channels for SV release, other types of VGCCs have been implicated in presynaptic plasticity. In GABAergic synapses, pharmacological blockade of Cav1 channels does not affect AP-induced SV release but converts posttetanic potentiation into synaptic depression (7). In hippocampal CA3 mossy fiber boutons (8–10) or in synapses of the lateral amygdala (11), Cav2.3 and Cav1.2 channels are required for presynaptic long-term plasticity but are unable to trigger SV release (9, 11).

Significance

Synaptic vesicle (SV) release from presynaptic terminals requires nanometer precise control of action potential (AP)-triggered calcium influx through voltage-gated calcium channels (VGCCs). SV recycling also depends on calcium signals, though in different spatiotemporal domains. Mechanisms for separate control of SV release and recycling by AP-triggered calcium influx remain elusive. Here, we demonstrate largely independent regulation of release and recycling by two different populations of VGCCs (CaV2, CaV1), identify CaV1 as one of potentially multiple calcium entry routes for endocytosis regulation, and show functional separation of simultaneous calcium signals in the nanometer space of a presynaptic terminal by the plasma membrane calcium ATPase (PMCA). The CaV2/CaV1/PMCA functional triad may provide conserved means for independent control of different vital presynaptic functions.

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**Neuronal network function critically depends on the tight control of synaptic vesicle (SV) release probability at chemical synapses over wide ranges of activity regimes. At the same time, synaptic gain remains adjustable to render network function flexible. To maintain synaptic function over time, SV recycling rates must be matched to vastly different activity patterns and synaptic gains. While SV release and recycling as well as their plasticity-related adjustments all include Ca2+-dependent steps, they operate in parallel but on different time scales. A tight spatial and temporal coordination of presynaptic Ca2+ signals and their effectors is thus needed for both the induction of changes in synaptic strength and the maintenance of robust synaptic function. However, the mechanisms that effectively separate Ca2+ signals in time and space (e.g., through different voltage-gated calcium channels [VGCCs]) to allocate these to different presynaptic functions are not well understood.

SV release probability depends on the sensitivity of the vesicular Ca2+ sensor and the positioning of VGCCs inside active zones (AZs) (1). Various mechanisms that can tune release probability by modulating their precise localization or kinetic properties have been uncovered (2–4). Irrespective of such modulation, efficient Ca2+-triggered SV release through presynaptic VGCCs (mainly
CaV2.1 and CaV2.2, CaV1 channels display higher conductances (12), suggesting that additional mechanisms are required to allocate CaV1-related Ca2+ signals to specific presynaptic functions while avoiding interference with SV release. SV recycling also includes regulation by presynaptic Ca2+ signals but operates mostly at different subsynaptic sites and at slower time scales than Ca2+-triggered SV release (13–15). We hypothesize that activity-dependent regulation of SV recycling employs CaV1-dependent Ca2+ entry and that active mechanisms exist to regulate the relative contributions of CaV2 and CaV1 channels to SV release versus recycling. We address these hypotheses at the Drosophila larval neuromuscular junction (NMI), an established model for glutamatergic synapse function (16–18).

Results

AP-Triggered Presynaptic Ca2+ Influx through Spatially Separated CaV2 and CaV1 Channels. AP-induced neurotransmitter release at the NMI depends on close proximity of CaV2 channels to readily releasable SVs in AZs established by interactions with the cytomatrix protein Bruchpilot (Brp) (17). While confirming the colocalization of Brp and the Drosophila CaV2 homolog, Dmca1A (cachephy), at presynaptic AZs (Fig. L4), we newly identified the localization of the CaV1 channel homolog, Dmca1D, in presynaptic axon terminals (Fig. 1B). Antibody specificity has previously been demonstrated by Western blotting and selective labeling of control neurons in CaV1 null mosaic mutants (19). Quantitative image analysis yielded a Pearson correlation coefficient (PCC) of 0.655 ± 0.028 SD (n = 8) for CaV2/Brp (SI Appendix, Fig. S1A and C). The Mander’s coefficients (M1, M2) indicated that by far most of the Brp label is positive for CaV2 (M2 = 0.842 ± 0.042 SD), whereas ~40% of the CaV2 label remains negative for Brp (M1 = 0.608 ± 0.066 SD, SI Appendix, Fig. S1D). By contrast, CaV1 localizes predominantly outside Brp-labeled AZs (Fig. 1B), although some CaV1 label also reaches into AZs (Fig. 1B, white arrows). Specifically, the PCC for Brp/CaV1 is 0.297 ± 0.027 SD (n = 10) and >70% of the CaV1 label remains Brp negative (M1 = 0.275 ± 0.032 SD), whereas ~60% of the Brp label displays co-occurrence of CaV1 (M2 = 0.611 ± 0.034, SI Appendix, Fig. S1B–D).

Differential localization of CaV2 and CaV1 channels in and around AZs have also been described in hippocampal neurons (20), but the functional implications of this organization remain unknown. Both chronic and acute manipulation of CaV1 reveal a structure, arguing against developmental effects (24), but the channel identity and Ca2+ entry route for endocytosis regulation remain unknown. We find that CaV1 channels localize mainly around AZs (Fig. 1B), underlie slowly inactivating L-type current (19), and contribute significantly to AP-triggered presynaptic Ca2+ influx (Fig. 1 E–H). Therefore, CaV1 localization and properties are well suited for activity-dependent modulation of SV endocytosis. In line with this hypothesis, we find increased synaptic depression during sustained activity upon CaV1-kd, both in low (Fig. 2 A and B) and in high external Ca2+ (SI Appendix, Fig. S3 A and B). With CaV1-kd (Fig. 2B) or knockout (SI Appendix, Fig. S3 C–E), synaptic depression is increased already after 5 s, and, after 15 to 20 stimuli, a significantly reduced steady state PSC amplitude is reached (Fig. 2B and SI Appendix, Fig. S5E). This, however, could still reflect an involvement of CaV1 in multiple steps of the SV cycle. We therefore conducted additional experiments to prove our hypothesis that CaV1 functions in SV endocytosis regulation.

First, we tested whether endocytosis was required to see the effect of CaV1-kd on synaptic depression. Here, we repeated the experiments in Fig. 2A and B under acute blockade of endocytosis with dynasore (25). This led to increased synaptic depression during 1 Hz stimulation in control, whereas there was no additional effect on depression for CaV1-kd (Fig. 2C). Moreover, the difference observed between control and CaV1-kd after 5 s (Fig. 2A and B) is largely abolished with dynasore (Fig. 2C), and, for both CaV1-kd and control, PSC amplitudes decline to ~65% of their initial amplitude within 75 s of stimulation (Fig. 2C). Therefore, the effect of CaV1-kd on synaptic depression is highly dependent on ongoing SV endocytosis, thus indicating a role of CaV1 in SV endocytosis regulation. A slightly faster decline of PSC amplitudes for CaV1-kd in between ~10 and 30 s of stimulation (Fig. 2C, Inset) might point to an additional fast function of CaV1, such as SV recruitment to release sites or priming. However, compared with the difference in synaptic depression that requires endocytosis, this effect of CaV1-kd is small and was not further investigated.

Second, we tested whether CaV1-kd altered the size of the readily releasable pool (RRP) of SVs. We estimated RRP size in control and with motoneuronal CaV1-kd by cumulative PSC charge analysis during brief high-frequency stimulation (1 s, 60 Hz, Fig. 2 D–F) in 2 mM external Ca2+. Cumulative PSC charge was calculated by back-extrapolation of a linear fit from the last 15 stimuli of the cumulative PSC integrals to time point zero (Fig. 2E). RRP size was estimated by dividing cumulative PSC charge by the average mPSC charge (26). RRP sizes were not statistically different (P = 0.33, t test) between control (3,052 ± 281 SEM) and CaV1-kd (2,786 ± 360 SEM, Fig. 2F). Therefore, increased synaptic depression with CaV1-kd is unlikely a consequence of altered RRP size.

To test a potential role of CaV1 in SV endocytosis more directly, we employed synaptopHluorin imaging to measure SV
left, overlay of 20 consecutive PSP traces, average in gray) indicate no differences. (K–M) Responses to single and paired stimuli. Evoked PSCs recorded in voltage clamp mode from M6/7 (K) in controls (left, black trace) and with motoneuronal Cav1-kd (L). Paired pulse facilitation (K, control, black trace, Cav1 RNAi, blue trace) is significantly increased upon Cav1-kd (M). (N) In temperature-sensitive Cav2 mutants (ca2+), evoked EPSCs require not Ca2v (i) Bath application of tyramine has no obvious effect on evoked PSCs. (J) EPSPs do not require Ca1, control and Ca1-RNAi. (K) Evoked EPSCs require not Ca1, control and Ca1-RNAi. (L) SP ampl. PP ratio. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. (O) Evoked EPSCs require Ca2, control and Ca2. (P) SP ampl. PP ratio. (Q) PP ratio. In mosaic Cav2 null mutants generated with the FLP-stop method, PSC amplitude is reduced by >90% at Cav2 knock out synapses (red traces) but normal at control NMJs (black traces). (R) Quantification of single pulse (SP) amplitudes and paired pulse ratios (Q) in Cav2 null versus control synapses. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. (S) Bath application of tyramine has no obvious effect on evoked PSCs. (J) EPSPs do not require Ca1, control and Ca1-RNAi. (K) Evoked EPSCs require not Ca1, control and Ca1-RNAi. (L) SP ampl. PP ratio. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant. (O) Evoked EPSCs require Ca2, control and Ca2. (P) SP ampl. PP ratio. (Q) PP ratio. In mosaic Cav2 null mutants generated with the FLP-stop method, PSC amplitude is reduced by >90% at Cav2 knock out synapses (red traces) but normal at control NMJs (black traces). (R) Quantification of single pulse (SP) amplitudes and paired pulse ratios (Q) in Cav2 null versus control synapses. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
Fig. 2. Activity dependent Ca\textsuperscript{2+} influx through presynaptic Ca\textsubscript{V1} augments SV recycling. (A) Representative recordings of PSCs from muscle M6/7 during 1 min of motoneuron stimulation at 1 Hz in control (black trace) and following Ca\textsubscript{V1} RNAi in motoneurons (blue trace) indicate increased synaptic depression with Ca\textsubscript{V1} RNAi. (B) For quantification, PSC amplitude was normalized to the first PSC and averaged over 6 control (black circles) and 6 motoneuronal Ca\textsubscript{V1}-kd animals (blue triangles). Lines indicate single exponential fits, and gray shaded areas represent the SEM. Steady-state synaptic depression is reached after ∼25 stimuli and increased by 45% upon Ca\textsubscript{V1}-kd. (C) Similar experiment as in B but with endocytosis blocked by dynasore (80 μM). Normalized PSC amplitudes during 1 Hz motoneuron stimulation for 2 min in control (black trace) and following Ca\textsubscript{V1} RNAi decline to identical values of steady-state synaptic depression. The time course of PSC amplitude decline is faster within Ca\textsubscript{V1}-kd (see inset C). (D) Representation EPSC trains in response to a stimulus train of 1 s duration at 60 Hz (2 mM [Ca\textsuperscript{2+}]). Cumulative EPSC charge was calculated for control (black line) and Ca\textsubscript{V1}-kd by back-extrapolation of a linear fit (dotted lines) to the last 15 stimuli of the cumulative EPSC integrals to time point zero. (F) RRP size was estimated by dividing cumulative EPSC charge by the average mEPSC charge for control (black circles) and Ca\textsubscript{V1}-kd (blue squares). Mean values are indicated by horizontal bars and SD by error bar. (G) Representative false color-coded snapshots of synaptopHluorin imaging from boutons on M6/7 before, during, and at different time intervals after RRP depletion with a 1 s train of 60 Hz (2 mM [Ca\textsuperscript{2+}]). Fluorescence decline is fitted with single exponentials (lines). Time constant of decay is significantly increased by Ca\textsubscript{V1}-kd from 4.4 to 7.8 s. (H) Quantification reveals significantly reduced dye load with Ca\textsubscript{V1}-kd (blue circles) compared with control (black circles). ΔF/F at increasing time intervals shows significantly reduced SV recycling with Ca\textsubscript{V1}-kd (blue) compared with the control (black). Between the 1,475 and 17,775 ms intervals, fluorescence decline is fitted with single exponentials (lines). Time constant of decay is significantly increased by Ca\textsubscript{V1}-kd from 4.4 to 7.8 s. (I) Representative PSCs during RRP depletion induced by 60 Hz stimulation train for 1 s, followed by single evoked responses at 11 different time points after the train (at 25, 75, 175, 475, 1,475, 4,500, 7,700, 10,275, 12,275, 15,275, and 17,775 ms). Train and first 5 PSCs after RRP depletion (∼25) are shown representative images of FM1-43 dye taken up into recycled SVs in axon terminals on M6/7 after stimulation with high K\textsuperscript{+} (20 mM) for 3 min followed by 3 min wash in Ca\textsuperscript{2+} free saline in a control (Left) and with Ca\textsubscript{V1}-kd (Right). Restimulation for 5 min in high K\textsuperscript{+} causes nearly complete unloading of labeled SVs (Lower panels) in control (Left) and with Ca\textsubscript{V1}-kd (Right). (N) Quantification reveals significantly reduced dye load with Ca\textsubscript{V1}-kd (blue circles) compared with the control (black circles). By contrast, activity-induced SV release (unload) is not significantly affected. **P < 0.01; ns, not significant.
aciddification after RRP depletion with high-frequency stimulation (1 s train at 60 Hz; Fig. 2 G and H). In both control and Cav1-kd, SV release during the stimulus train caused synaptic pHluorin fluorescence increases (Fig. 2 G, Left), followed by fluorescence decreases due to SV recycling and acidification, which was measured at 8 different time points (between 175 and 17,775 ms after stimulation with increasing time intervals between measurements). With Cav1-kd, the time course of SV acidification was significantly slower (τ = 7.8 ± 1.1 s, n = 6) compared with the control (τ = 4.4 ± 1.1 s, n = 7; P < 0.01, t test). A significant difference with Cav1-kd was observed already ~5 s after the stimulation train, thus matching the timing of the effect of Cav1-kd on synaptic depression during low-frequency stimulation (Fig. 2 A and B). These data lend further support to a role of Cav1 in augmenting SV endocytosis.

To compare the time courses of SV acidification and PSC amplitude recovery after presynaptic RRP depletion, we measured PSC amplitude at the same 8 time points after stimulation trains of 1 s duration and 60 Hz frequency (Fig. 2 I and J). The stimulation train caused synaptic depression by ~80% in control and Cav1-kd condition (Fig. 2K). Following RRP depletion, PSC amplitude recovery follows a fast and a substantially slower time course (26). The fast recovery time constant of ~50 ms was not significantly affected by Cav1-kd (Fig. 2J). By contrast, the slow recovery time constant was significantly increased by Cav1-kd (36.6 s) compared with the control (14.5 s). Finally, a role of Cav1 in SV endocytosis is also supported by imaging the uptake and release of the styryl dye FM1-43. Following bath application of high K+ (20 mM) for 3 min, FM1-43 uptake into recycled SVs was significantly reduced by Cav1-kd (Fig. 2 L–N). By contrast, unloading of labeled SVs by reactivating synaptic transmission in high K+ was not affected (Fig. 2 L–N).

Together, these data indicate that activity-dependent Ca2+ influx through Cav1 facilitates SV recycling. However, Cav1-kd does not eliminate PSC amplitude or synaptophysin signal recovery after RRP depletion but slows the recovery time constant. Therefore, Ca2+ entry through Cav1 is not mandatory for SV endocytosis, but it has an augmenting effect. Moreover, the effects of Cav1-kd on synaptic depression (Fig. 2 A and B), synaptophysin signal (Fig. 2 G and H), and PSC amplitude recovery (Fig. 2 I and J) manifest within 5 s. Full membrane recycling, new SV formation, and vesicle filling is difficult to reconcile with this speed, unless ultrafast recycling and very fast SV reformation take place. It has been demonstrated, however, that endocytic proteins can also function in release site clearance (27). Therefore, reduced endocytosis in Cav1-kd could potentially increase synaptic depression or slow recovery from RRP depletion by reducing the speed of release site clearance. Either

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**Fig. 3.** PMCA separates presynaptic Ca1 and Ca2 channel function. (A–C) Representative confocal Z-projection of terminal motor axons stained for neuronal surface by a-HRP (blue), A2B with b-Hrp (magenta), and Venus-tagged plasma membrane calcium ATPase PMCA (green). Dotted white box demarks area enlarged and shown as single optical sections in (A, i–C, i). (D–F) Evoked PSCs in 0.5 mM external Ca2+ as multiple sweeps (black traces) and average (red) in a representative control (D), following PMCA-kd in motoneurons (E), and with PMCA, Cav1 double knockdown (F). (G) Overlay of averages shows increased amplitude in PMCA-kd (red) versus control (black) and restoration by concomitant Cav1,1-kd (green). PSC amplitude (H), area (I), and time constant of decay (J) are significantly increased with presynaptic PMCA-kd (red circles) compared with the control (black triangles). Effects of PMCA-kd on evoked synaptic transmission are rescued by concomitant Cav1,1-kd (green squares). (K) PSC rise time is not affected. (L–N) Imaging with GCaMP6s coupled to Brp reveals local AP-induced Ca2+ signals at presynaptic A2B before, during, and after stimulation (10 APs at 100 Hz) for control (L, Upper images) and with PMCA-kd (L, Lower images). (M and N) Quantification reveals no differences in evoked A2 Ca2+ signal amplitudes upon Cav1,1-kd (blue) but a nearly twofold, significant increase with PMCA-kd (red) compared with the control (black). (O) PMCA-kd in motoneurons increases muscle tone thus shortening body length (P) and is rescued by Cav1,1-kd. (Q) Compared with the control (black triangles), crawling speed is significantly decreased by PMCA-kd (red circles) but not affected by Cav1,1-kd (blue triangles). Cav1,1-kd in PMCA-kd (green squares) partially rescues reduced locomotion speed. (R and S) Mild paired pulse facilitation as observed in controls (black) is converted to mild paired pulse depression by PMCA-kd (red). Concomitant Cav1,1-kd yields partial rescue (green). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

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way, Ca\textsuperscript{2+} influx through Ca\textsubscript{1} and Ca\textsubscript{2} channels independently control two fundamental aspects of synapse function; Ca\textsubscript{2} is essential for AP-triggered SV exocytosis due to the central position within the AZ, whereas Ca\textsubscript{1} augments SV recycling and modulates the dynamic coding properties of the synapse from its more peripheral location around AZs. However, we cannot rule out the possibility that Ca\textsuperscript{2+} influx through Ca\textsubscript{2} also contributes to SV endocytosis regulation.

Functional Separation of Ca\textsubscript{2} and Ca\textsubscript{1} Channel at the Presynapse.

Synaptic coding reliability likely requires some independence of evoked release from Ca\textsuperscript{2+} signals regulating endocytosis. But how do Ca\textsuperscript{2+} signals evoked by the same AP(s) yet mediated through Ca\textsubscript{1} and Ca\textsubscript{2} in adjacent subregions of the presynaptic membrane remain functionally separated? It has been demonstrated that the local Ca\textsuperscript{2+} signals through Ca\textsubscript{2} that trigger release at AZs are confined in their spatiotemporal extent by endogenous buffers (1).

In conditions that cause a severe but still nonlethal knockdown of PMCA (PMCA-kd) in motoneurons (SI Appendix, Fig. S4 D–E), no overt malformation of NMJs is observed. Quantitative analyses nonetheless reveal decreased AZ number and density in parallel with moderately increased postsynaptic glutamate receptor (GluR) levels (SI Appendix, Fig. S5). If these structural changes manifested physiologically, decreases in SV release due to lower AZ density would co-occur with increases in postsynaptic responses due to increased GluR levels and, thus at the most, cause mild changes in synaptic transmission. Presynaptic PMCA-kd, however, considerably increases the amplitude and duration of evoked PSCs (Fig. 3 D, E and H–K). These effects are unlikely caused by a redistribution of Ca\textsubscript{1} toward AZs upon PMCA-kd, because the localization of Ca\textsubscript{1} relative to Bp is similar with presynaptic PMCA-kd (PCC, 0.329 ± 0.027; M1 = 0.335 ± 0.195; M2 = 0.593 ± 0.134, n = 5) compared with the control (PCC, 0.297 ± 0.027; M1 = 0.275 ± 0.032; M2 = 0.611 ± 0.034; n = 10, SI Appendix, Fig. S1 C and D). Both effects are dependent on Ca\textsuperscript{2+} influx through Ca\textsubscript{1} channels, as they are reverted when PMCA and Ca\textsubscript{1} are knocked down concomitantly (Fig. 3 D–G). To rule out that this is due to reduced strength of GAL4-driven UAS-PMCA-kd in the presence of a second UAS-transgene, we coexpressed the PMCA-kd together with UAS-GFP-RNAi. This does not reduce PSC amplitude (SI Appendix, Fig. S6 A and B). As an additional control, we also expressed Ca\textsubscript{1}-kd in a hypomorphic basigin (bsg\textsubscript{SH1217}) mutant background. Fly basigin is the single ortholog of the vertebrate Ig domain proteins basigin and neurolastin, which have been identified as binding partners essential for PMCA stability and function (29, 32, 33). Moreover, in Dro\textit{sophila}, bsg is required presynaptically to inhibit asynchronous evoked SV release (34). Our recordings not only confirm these data but also show that Ca\textsubscript{1}-kd partially rescues PSC amplitudes and fully rescues PSC area in bsg\textsubscript{SH1217} mutants (SI Appendix, Fig. S6 C–E). Loosely coupled calcium channels have previously been considered a potential cause for increased PSC areas in bsg\textsubscript{SH1217} mutants (34). In fact, both PMCA-kd and reduced PMCA function in bsg mutants can be rescued by Ca\textsubscript{1}-kd. Together, these data suggest that the low capacity, high affinity Ca\textsuperscript{2+} extrusion pump PMCA operates as a membrane-bound buffer that is strategically localized within the presynaptic membrane to prevent spill-over into AZs of Ca\textsuperscript{2+} entering through Ca\textsubscript{1} channels.

As a second control, we estimated the resting Ca\textsuperscript{2+} levels in presynaptic boutons with bicistronic expression of tdTomato and GCaMP5G for ratiometric cytosolic [Ca\textsuperscript{2+}] measurement under UAS control (36). No significant differences were observed in tdTomato, GCaMP5G, or normalized GCaMP fluorescence intensities between control and PMCA-kd (SI Appendix, Fig. S6 H). Therefore, the significant increases of PSC amplitude and area in PMCA-kd are unlikely a result of differences in resting calcium. Moreover, increased synaptic transmission as caused by PMCA-kd can be acutely rescued by bath applying the membrane permeable, slow Ca\textsuperscript{2+} buffer EGTA-AM (SI Appendix, Fig. S6 F and G), further ruling out effects of structural changes (SI Appendix, Fig. S5) and corroborating the impact of activity-dependent, peripheral Ca\textsuperscript{2+} signals onto SV release when PMCA abundance is reduced. This constitutive function of PMCA is behaviorally relevant. PMCA-kd in motoneurons increases muscle tone such that body length of L3 larvae is reduced by ~25% (Fig. 3 O and P), and their locomotion is significantly slower (Fig. 3 Q). Morphological and behavioral effects of PMCA-kd can be fully (body tone, Fig. 3 P) or partially rescued (locomotion, Fig. 3 Q) by concurrent Ca\textsubscript{1}-kd. Ca\textsubscript{1}-kd alone has no effect (Fig. 3 Q). Therefore, postural and locomotor defects as observed with PMCA-kd are caused by increased synaptic transmission amplitudes through functional coupling of Ca\textsubscript{1} channels to SV exocytosis.
events but lower probabilities of two- or threefold amplitudes (Fig. 4 J, black bars). Increases in spontaneous release upon PMCA-kd and rescue by concomitant Ca$_1$-kd is also confirmed in higher external Ca$^{2+}$ (2 mM, SI Appendix, Fig. S7). Integer multiples of unitary amplitudes likely reflect release from adjacent AZs, eliciting a fused Ca$^{2+}$ signal by the sensor, which localizes to the SSR around GluR fields. Colabeling of presynaptic AZs supports this interpretation (SI Appendix, Fig. S8). With presynaptic PMCA-kd (Movie S4), the frequency distribution is shifted toward higher integer multiples (Fig. 4K). This supports the interpretation that PMCA keeps SV release probability in check. The effect of PMCA-kd is rescued by concomitant knockdown of Ca$_1$ (Fig. 4 L and Movie S5), but Ca$_1$-kd alone has no significant effect as compared to control (Fig. 4 J).

The functional coupling of multiple adjacent AZs upon presynaptic PMCA-kd significantly increases mean quanta content during evoked release. In controls, a presynaptic AP on average elicits calcium transients on two to three distinct postsynaptic sites underneath each presynaptic type 1b bouton (Fig. 4 M, closed circles; Movie S6). With 10 to 20 AZs per bouton (37), this results in an estimated release probability of 0.2, meaning that on average about 150 of the roughly 780 AZs release a single SV upon each AP. This matches the mean quanta content of 150 as estimated also by whole muscle fiber electrophysiology, thus indicating that postsynaptic Ca$^{2+}$ imaging is a reasonable measure of release probability at single synaptic sites. Single-site release probability was clearly increased upon presynaptic PMCA-kd (Fig. 4 N, closed circles; Movie S7). In particular, we observed coactivation of many neighboring sites during any given stimulus, indicating that the isolation of presynaptic AZs from Ca$^{2+}$ spillover from their neighbors is lost. This proximity effect complicated accurate counts of active sites per stimulus. Therefore, per AP we normalized total postsynaptic fluorescence for each bouton to bouton size. PMCA-kd causes a more than twofold increase of evoked responses imaged at the level of single boutons, and this effect is rescued by concomitant knockdown of presynaptic Ca$_1$ channels (Fig. 4 O and Movie S7). This underscores our interpretation that PMCA protects AZs from AP-triggered Ca$^{2+}$ influx though Ca$_1$, while allowing for Ca$_1$-mediated augmentation of SV endocytosis outside AZs.

**Discussion**

Our data show strict functional separation of AP-triggered neurotransmitter release by Ca$_2$ and activity-dependent modulation of SV recycling and short-term plasticity by Ca$_1$ VGCCs. Although task sharing and partial redundancy among Ca$_2$ isoforms is known for mammalian synapses (9, 38), and the dynamic regulation of their relative abundance within AZs can add to synaptic plasticity (39), insight into mechanisms that allow for the separate regulation of different aspects of presynaptic function by Ca$_2$ and Ca$_1$ channels is sparse.

**Division of Labor among Presynaptic Ca$_1$ and Ca$_2$ Channels, Peculiarity or General Principle?** Ultrastructural support for the coexistence of Ca$_2$ and Ca$_1$ channels has been obtained in rat hippocampal neurons, where Ca$_2$ localizes to AZs and Ca$_1$ outside AZs (20),

![Fig. 4.](image_url) PMCA confines spontaneous and evoked SV release. (A–C) Spontaneous PSCs (sPSCs) at the NMJ in TTX (10$^{-6}$) and 0.5 mM Ca$^{2+}$ in control (A), with motoneuronal PMCA-kd (B) and with PMCA, Ca$_1$ double-kd (C). Lower traces are at enlarged time scale. Asterisks mark smallest amplitude sPSCs, which are similar among genotypes (control, 0.6 ± 0.08 nA; PMCA-kd, 0.68 ± 0.16 nA, PMCA, Ca$_1$-kd, 0.64 ± 0.15 nA). (B) sPSC frequency is significantly increased by PMCA-kd (red) as compared to control (black) and reduced but not rescued by concomitant Ca$_1$-kd (green). (C) Mean sPSC amplitude is increased by PMCA-kd (red) and partially rescued by concomitant Ca$_1$-kd (green). (D) Similarly, cumulative sPSC amplitudes of controls (black) are significantly increased by PMCA-kd (red), which is partially rescued by concomitant Ca$_1$-kd (green), thus confirming the analysis of mean amplitudes (E) on the distribution level. (G–I) Imaging of responses to spontaneous SV release in TTX (10$^{-6}$ M) by postsynaptic GCaMP6m expression in control (G), with presynaptic PMCA-kd (H) and with presynaptic PMCA-ka, Ca$_1$-kd (I). Two left columns exemplify events at single time points, and the right column is a maximum projection over 0.1 s. (J) Frequency distribution of fluorescence intensities, analyzed separately for each postsynaptic site and binned to multiple integers of smallest amplitude unitary events, is dominated by unitary events in controls (black bars) and not significantly affected by Ca$_1$-kd in motoneurons (blue). (K) Presynaptic PMCA-kd shifts the distribution to the right. (L) This shift is rescued by concomitant Ca$_1$-kd. (M–O) Postsynaptic responses to AP-triggered SV release in control (M) and with presynaptic PMCA-kd (N). (M) In response to each AP (exemplified for 3 APs, stimuli 1 through 3), a different subset of postsynaptic sites (closed white circles) is activated while other sites remain silent (dotted white circles). The number of activated sites per bouton is increased by presynaptic PMCA-kd, with coactivation of neighbors. False color-coded images on the right show cumulative intensity distributions for 7 APs in control (M) and with PMCA-kd (N). (O) Quantification of total fluorescence normalized to bouton size reveals significantly increased responses in PMCA-kd (red circles) compared with the control (black triangles), and this is rescued by concomitant Ca$_1$-kd. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
largely as we find for Drosophila. Moreover, pharmacological data in mammals indicate that Cav1 and Ca2+ VGCCs separately control SV release and synaptic plasticity (40). In synapses of the amygdala, Cav1 is not required for SV release but for presynaptic forms of LTP (11); in GABAergic basket cells, Cav1 is not required for evoked release but for posttetanic potentiation (7); and at mouse neuromuscular synapses, anatomical (41) and physiological data (42) indicate the presence of both presynaptic Cav1 and Cav2 channels, but again with little contribution of Cav1 to evoked SV release (42). Therefore, studies of different synapse types in various species support the idea that multiple fundamental aspects of presynaptic function are executed in parallel on the basis of spatially separated VGCCs with different kinetics and conductances. This study provides a mechanism for functional separation in the small space of the axon terminal (see Figs. 3 and 4, and Discussion, last section).

The fast activation and inactivation kinetics of Cav2 channels in the AZ seem well suited for tight excitation-release coupling, and Cav2 activation mediates release mostly in an all or none fashion, though dynamic modulation of channel-SV coupling to adjust release probability is reported (9, 39, 43). By contrast, Cav1 channels typically have larger single-channel conductances and slower inactivation kinetics, suggesting that they are well suited to cope with the need for relatively high Ca2+ and the slow time course of endocytic vesicle retrieval.

Presynaptic Cav1 Channels and Endocytosis Regulation. Endocytosis regulation by activity-dependent Ca2+ influx is discussed for mammalian and invertebrate synapses (14). At the Drosophila NMJ, separate Ca2+-entry routes for differential exo- and endocytosis regulation have been postulated (23), and the SV-associated calcium channel Flower has been suggested to contribute to this function (44). We identify Cav1 channels within the periphery of AZs as a distinct entry route for Ca2+-dependent augmentation of SV endocytosis. Although the precise underlying mechanisms remain to be investigated, an attractive hypothesis is that Cav1 may serve as an activity-dependent switch to direct recycling into different SV pools. In basket cells, Cav1-mediated Ca2+ influx has been speculated to mobilize vesicles into the releasable pool to maintain synaptic transmission during high-frequency bursting (7). Similarly, at the mouse NMJ, pharmacological blockade of L-type Cav1 channels decreases FM2-10 loading and quantal release upon high-frequency stimulation (45). This is in line with our findings of increased synaptic depression, reduced SV reacidification, decreased FM1-43 uptake, and reduced PSC recovery after RRP depletion upon reduction of presynaptic Cav1 function. However, the effects of Cav1-kd manifest within few seconds. Unless recycling and SV reformation are ultrafast, this seems too fast for SV reuse. In cultured hippocampal neurons, for example, SVs are not reused during the first 200 APs, irrespective of stimulation frequency between 5 and 40 Hz (27). However, given that endocytic proteins can also function in release site clearance (27), reduced endocytosis in Cav1-kd may increase synaptic depression and decrease recovery from RRP depletion indirectly as a result of reduced release site clearance. We can also not exclude additional effects of Cav1 channels on other steps in the SV cycle, such as SV priming.

For the mouse NMJ, it has been inferred that Cav1 activity directs recycled SVs into a high-probability release pool (45). Ultrastructural analysis of Drosophila synapses has also revealed two different recycling modes, one that depends on external Ca2+ and directs recycled SVs to AZs and another one that does not depend on external Ca2+ and replenishes other SV pools (46). Taken together, peri-AZ localization of presynaptic Cav1 channels as found in hippocampus (20) and at the Drosophila NMJ (this study) may provide a common control mechanism to direct SV recycling to different pools in an activity-dependent manner. Protection of AZs by the peri-AZ PMCA provides a mechanism to maintain mean quantal content, and thus coding reliability, in the face of Ca2+-mediated endocytosis regulation.

As in many mammalian neurons (47), in Drosophila motoneurons, Cav1 channels localize also to dendrites to boost excitatory synaptic input (19). Therefore, cooperative functions of Cav1 channels in different subneuronal compartments coordinate firing and SV recycling rates. Moreover, as in spinal motoneurons (47), Drosophila Cav1 channel function is modulated by biogenic amines (21), thus providing means for integrative regulation of motoneuron excitability and SV recycling rates in the context of internal state and behavioral demands.

PMCA Controls Release Probability by AZ Protection from Cav1 and is Adjustable. Here, we show that 1) axon terminal Cav1 segments into the peri-AZ compartment to augment SV endocytosis, and 2) PMCA, rather than directly acting on Ca2+ entering through Cav2, actively controls Cav1-dependent Ca2+ changes, thereby enabling side-by-side Ca2+ domains with profiles that meet the different requirements for SV release and recycling. This is consistent with reports on spatially restricted expression and/or regulation of PMCA in small T lymphocytes as a means to steer Ca2+-dependent processes specifically within cellular microdomains (32, 48). In consequence, we propose to expand the concept of controlling release probability by presynaptic Ca2+ buffering systems after nanodomain collapse, which has been scrutinized in many studies (1, 5), with the idea of nanodomain protection from presynaptic Ca2+ signals originating outside the AZ.

PMCA has high Ca2+ affinity (49) and can accelerate Ca2+ clearance on millisecond timescales (29, 31). While isolating AZs from Ca2+ influx through Cav1, PMCA otherwise does not affect the spatiotemporal properties of AZ Ca2+ nanodomains, because transmission amplitudes are not altered by PMCA-kd in the absence of Cav1 channels (Fig. 3 D–J). Instead, it ensures stable release probability in the face of presynaptic Ca2+ signals that augment SV recycling, shape APs (50), and control synaptic plasticity. In contrast to soluble Ca2+ buffers and fixed ones in the AZ (1), the membrane-bound peri-AZ PMCA can be regulated on short time scales (e.g., by downstream effectors of Ca2+ and phospholipids) (49, 51). In addition, release from auto-inhibition by binding of Ca2+/calmodulin, which is conserved across phyla (52), provides a molecular memory due to the slow time course of calmodulin release, allowing PMCA to persist in a pre-activated state and to respond instantaneously to the next Ca2+ signal (53). Therefore, PMCA-mediated control of SV release probability is likely adjusted by the local activity at the synaptic terminal. Our data show that changes in PMCA-dependent AZ protection largely impact SV release probability by allowing or preventing functional coupling of Cav1 channels with readily releasable SVs. We propose that the distinct localization of Cav1 channels and PMCA in between AZs enables effective and versatile regulation of synaptic strength on a short time scale. In fact, theoretical considerations (54) and recent studies on Ca2.1 dynamic coupling in hippocampal synapses (3, 10) and on differential spacing of Cav2 channels in cerebellar synapses (4) suggest that modulation of SV release probability favors loose coupling of VGCCs to SV. Thus, regulation of presynaptic PMCA activity emerges as an effective means to dynamically regulate plasticity and SV recycling rates downstream of Cav1.

Materials and Methods
Full methods and all genotypes used are available in SI Appendix.

Animals. Drosophila melanogaster were reared at 25 °C on a 12-h light-dark cycle on standard cornmeal diet (19). Third instar larvae of both sexes were used for experiments (see SI Appendix, Table S1 for complete list of all genotypes used).
Electrophysiology. Single-electrode current clamp recordings and two electrode voltage clamp recordings from larval muscle fibers were conducted at 25 °C (if not noted otherwise) with thin-walled borosilicate glass microelectrodes (Sutter, BF100-50-10) filled with 3 M potassium chloride and acquired with an Axoclamp 2A amplifier, a Digidata 1320, and PCclamp software (all Molecular Devices). All recordings were performed in HL3.1 saline containing (in mM) NaCl 70, KCl 2.5, MgCl2 2, CaCl2 0.5 or 2, NaHCO3 10, trehalose 5, sucrose 115 or 109 (when CaCl2 was 2), and Hepes 5 (pH adjusted to 7.24 to 7.25 with 1 N NaOH, mOsm: 310).

Live imaging. All presynaptic calcium signals at the NMJ were acquired in the presence of 7 mM extracellular glutamate to minimize movement. AP-triggered presynaptic Ca2+ signals in animals expressing UAS-GaMP6f under the control of vGlutOK371-GAL4 in motoneurons were imaged with an Orca Flash 4.0LT Camera Model C11440-42U CMOS camera (Hamamatsu) mounted on a fixed stage upright Zeiss Axio Examiner A1 epifluorescence microscope and controlled with HL5 software as previously described (21). For imaging of Ca2+ signals in presynaptic AZs, the UAS-bact-s-Mcherry-GaMP6f transgene (GaMP6f and mCherry fused to the AZ protein Brp) was expressed under the control of vGlutOK371-GAL4 to localize AZs in the membrane channel and GaMP6f fluorescent changes in AZs. The UAS-Nspy-bph transgene [Synaptophysin HuoJin, (55)] was expressed in motoneurons under the control of vGlutOK371-GAL4 to assess endocytosis by imaging ratiometric calcium at different time intervals after tetanic stimulation. Background was removed later by high-frequency motoneuron stimulation (60 Hz, 1 s). SV release and recycling were visualized by unloading and loading of the styryl dye Fluo FM1-43 for 3 min. Immediately after washing for 3 min in calcium- and flavin-43-free normal HL3.1 saline, the second image was taken to determine fluorescence intensity before FM1-43 loading into recycled SVs (Fload). Preparations were stimulated by perfusion with 0.5 mM calcium and 20 mM potassium saline and washed for an additional 3 min in calcium-free HL3.1 saline to determine fluorescence intensity in unloaded boutons after FM1-43 unloading (Funload). Background was subtracted from all images. ΔF/F was calculated as [F(load)−F(rest)]/[F(rest)] for unloaded boutons. For GaLA-GaMP6f-based transgene expression in motoneurons and simultaneous imaging of Ca2+ influx through postsynaptic glutamate receptors, we generated a constitutively muscle-expressed version of a previously described UAS-myrgCaMP5 reporter (56). Images were acquired with a CSU-X1 spinning disk (Yokogawa) on an upright Olympus BXS15 WM microscope and an EMCCD camera (Onyx+B97, Andor Technology). Recordings were obtained in normal HL3.1 saline in 0.5 and 2 mM Ca2+. Spontaneous events with and without 1 mM TTX were recorded at 20 Hz acquisition rate for a duration of 250 s (5,000 frames). Evoked responses were recorded without TTX and induced by motor nerve stimulation with a suction electrode. Presynaptic resting calcium levels were measured by expressing the P2A peptide to coexpress the red fluorescent protein tdTomato and the genetically encoded calcium indicator GaMP6f in motoneurons (36) in controls and in combination with PMCA-kd. Ratiometric imaging under a silicone immersion objective lense (100×, NA 1.35) was used to compensate for alteration of fluorescent intensities at different optical planes and expression levels. Image stacks were acquired with a Digital CMOS camera (ORCA-Flash4.0, Hamamatsu) and analyzed using custom-written routines in Fiji (NIH, https://imagej.net/software/fiji).

Immunocytochemistry. Triple immunostaining was conducted with rabbit α-GFP (1:400) for tagged Ca2+ channels, α-Ca1 (goat anti-Ca1.1, 1:200), and the AZ marker bruchpilot (mouse α-brp NCB2, 1:200). For quantification of synaptic structure in different genotypes motoneuron axon terminal bouton structure was visualized with α-HRP (horse radish peroxidase, 1:500), presynaptic AZs were labeled with α-NC82 (Brp), and glutamate receptor IC (GLURIC) was labeled by immunocytochemistry. PMCAVentus signal was enhanced with an Atto488 conjugated FluogTag4 anti-GFP nanobody (NanoTag Biotechnologies) incubated at 1:300 for 2 h at room temperature. Histology and CLSM imaging were conducted as previously described (19).

Statistical Analysis. Significance of normally distributed datasets was examined using two-sided unpaired or paired Student’s t tests or two-way ANOVA. For data not normally distributed, Mann-Whitney U test or Kruskal–Wallis ANOVA with post hoc groupwise comparison were used. Data are presented as mean ± SD or ± SEM.

Data Availability. All study data are included in the article and/or supporting information.

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