Mobility of Calcium Channels in the Presynaptic Membrane

Highlights

- Calcium channels (VDCC) are mobile yet confined within the presynaptic membrane
- Intracellular Ca$^{2+}$ chelation by BAPTA or EGTA reduces VDCC mobility
- Increasing VDCC surface expression by $\alpha_2\delta$1 leaves synaptic VDCC density unaffected
- Simulations suggest mobility-dependent equalization of synaptic release probability

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In Brief

By single-molecule tracking, Schneider et al. demonstrate the dynamic organization of presynaptic calcium channel populations in hippocampal neurons. The data suggest that channel mobility promotes cooperativity of calcium influx and serves as a potential means to control vesicle release probability.
Mobility of Calcium Channels in the Presynaptic Membrane

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SUMMARY

Unravelling principles underlying neurotransmitter release are key to understand neural signaling. Here, we describe how surface mobility of voltage-dependent calcium channels (VDCCs) modulates release probabilities (Pr) of synaptic vesicles (SVs). Coupling distances of <10 to >100 nm have been reported for SVs and VDCCs in different synapses. Tracking individual VDCCs revealed that within hippocampal synapses, ~60% of VDCCs are mobile while confined to presynaptic membrane compartments. Intracellular Ca2+ chelation decreased VDCC mobility. Increasing VDCC surface populations by co-expression of the α2δ1 subunit did not alter channel mobility but led to enlarged active zones (AZs) rather than higher channel densities. VDCCs thus scale presynaptic scaffolds to maintain local mobility. We propose that dynamic coupling based on mobile VDCCs supports calcium domain cooperativity and tunes neurotransmitter release by equalizing Pr for docked SVs within AZs.

INTRODUCTION

The molecular architecture of active zones (AZs) ensures the precision and adjustability of synaptic vesicle (SV) release. This involves a tight coupling of presynaptic voltage-dependent calcium channels (VDCCs) and calcium sensor proteins on docked SVs. Experimentally, differential staining of intracellular calcium domains by chelators such as EGTA and BAPTA has been used to assess channel-sensor coupling and modeling has led to various testable hypotheses (Eggermann et al., 2012). Protein-protein interactions that directly or indirectly link VDCCs to SVs are described (Catterall, 1999; Davydova et al., 2014; Kaeser et al., 2011; Liu et al., 2011; Wong et al., 2014). However, the precise modalities of channel-sensor coupling, in particular its dynamic aspects, remain unclear. Tight coupling of 10–20 nm was uncovered for cortical GABAergic Schaffer collateral and glutamatergic cerebellar synapses (Bucurenciu et al., 2008; Schmidt et al., 2013). In this scenario, a single channel opening can trigger vesicular fusion and release probabilities (Pr) may be rather uniform as long as channels are evenly distributed within AZs and exceed the number of docked vesicles (Ermolyuk et al., 2013; Holderith et al., 2012; Scimemi and Diamond, 2012). Loose coupling of 100 nm along with higher VDCC numbers were reported for other types of synapses and probably contributes to presynaptic plasticity (Borst and Sakmann, 1996; Meinrenken et al., 2002; Vyleta and Jonas, 2014). Pr even varies considerably between synapses in single axons, i.e., for a given type of synapse. Such fluctuations can result from differences in the number of readily releasable SVs but also from synapse-specific rates of Ca2+ influx due to maturation- or plasticity-related differences in the subtype composition, activity state, local density, and positioning of Ca2+ channels (Holderith et al., 2012; Ermolyuk et al., 2012; Li et al., 2007; Reid et al., 1997).

Assuming a low to moderate density of VDCCs within AZs, we wondered whether VDCC mobility contributes to a dynamic mode of channel-sensor coupling. Using single particle tracking-photoactivated localization microscopy (sptPALM), we monitored mEOS2-tagged α1 calcium channel subunits at high spatio-temporal resolution in cultured hippocampal neurons. We found that a substantial fraction of CaV2.1 (P/Q)- and CaV2.2 (N)-type channels is mobile within presynaptic areas. We assessed various parameters for their impact on channel mobility and found that it is largely unaffected by α2δ1 subunit-mediated increase in synaptic VDCC abundance and by an acute depletion of the readily releasable pool (RRP) of SVs. In contrast, intracellular calcium chelation uncovered Ca2+-dependent regulation of channel mobility. To integrate these findings, we performed computational modeling, which suggests that the interplay between channel densities, mobility, and Ca2+ influx...
determines calcium domain cooperativity between adjacent channels and thus serves as a potential means to control $P_r$.

RESULTS

Expression of Tagged VDCCs Preserves Normal Synaptic Phenotypes

The surface dynamics of presynaptic VDCCs has been addressed by tracking the $\alpha_2\delta$ subunit (Mercer et al., 2011) or extracellularly tagged $\alpha_1$ subunits (Di Biase et al., 2011). These insightful studies were limited by promiscuous associations of $\alpha_2\delta$ subunits with $\alpha_1$ subunits (Hoppa et al., 2012), compromised channel function, or reduced accessibility for fluorophores under physiological conditions. To overcome these obstacles, we used sptPalm (Manley et al., 2008). We fused the photoconvertible fluorophore mEOS2 or GFP to the cytoplasmic N termini of Ca$_{\alpha_2\delta}$1 and Ca$_{\alpha_2\delta}$2 $\alpha_1$ subunits and validated channel function in HEK cells (Figures S1A–S1F). Next, we assessed synaptic targeting of tagged $\alpha_1$ subunits in hippocampal neurons by co-staining for Rab3 interacting molecule (RIM 1/2) and Bassoon, two prominent components of the cytomatrix at the AZ (CAZ) involved in presynaptic localization of VDCCs (Davydova et al., 2014; Kaeser et al., 2011). Tagged VDCCs co-localized with both RIM and Bassoon, similar to endogenous channels in non-transfected cells (Figures S1G–S1I).

Patch-clamp recordings from synaptically coupled hippocampal neurons in the presence of isofrom-specific channel blockers revealed that the relative contribution of Ca$_{\alpha_2\delta}$1 and Ca$_{\alpha_2\delta}$2 to transmission remained unaffected when tagged Ca$_{\alpha_2\delta}$1 was expressed, whereas expression of Ca$_{\alpha_2\delta}$2 resulted in Ca$_{\alpha_2\delta}$2-dominated synapses (Figure S1J). Using an antibody specific for rodent but not human Ca$_{\alpha_2\delta}$1 (Figures S2A and S2B), we found that endogenous Ca$_{\alpha_2\delta}$1 was completely replaced by Ca$_{\alpha_2\delta}$1::GFP at about 60% of synapses and partially replaced at remaining synapses (Figures S2C and S2D). Consistent with previous reports (Cao and Tsien, 2010; Hoppa et al., 2012), expression of Ca$_{\alpha_2\delta}$2::GFP virtually abolished the contribution of Ca$_{\alpha_2\delta}$1 to synaptic transmission (Figure S1J). Paired-pulse ratios (PPRs) from synaptically connected neurons remained unaffected by expression of either Ca$_{\alpha_2\delta}$1::GFP or Ca$_{\alpha_2\delta}$2::GFP (Figure S1K). Expression of tagged Ca$_{\alpha_2\delta}$1 thus allows monitoring of its presynaptic distribution under conditions close to the mature state of natural synapses whereas the predominance of Ca$_{\alpha_2\delta}$2 upon expression of tagged Ca$_{\alpha_2\delta}$2 mimics channel composition of synapses in young hippocampal neurons (Scholz and Miller, 1995).

Calcium Channels Display Confined Mobility within Presynaptic Membranes

To assess the mobility of individual channels, we applied sptPalm on cells expressing mEOS2-tagged $\alpha_1$ subunits. Experiments on fixed COS7 cells revealed a localization accuracy of 21.4 ± 4 nm for a 30 ms integration time per frame, and similar accuracy (27.7 ± 10 nm) was obtained for immobile molecules in living neurons (Figures S2E–S2G). Spontaneous uptake of fluorescently labeled antibodies against synaptotagmin-1 (Syt1) uncovered active synapses (see Supplemental Information).

Epifluorescence and sptPalm revealed that prominent clusters of Ca$_{\alpha_2\delta}$1::mEOS2 or Ca$_{\alpha_2\delta}$2::mEOS2 were mostly associated with Syt1-positive boutons, whereas extrasynaptic channel distribution appeared largely diffuse (Figures 1A and 1B). SptPalm also revealed that outside clusters most channels are mobile. Inside synaptic clusters both immobile and mobile molecules were found (Figures 1A and 1B). Plotting the mean square displacement (MSD) over time intervals clearly indicated that the majority of Ca$_{\alpha_2\delta}$1::mEOS2 and Ca$_{\alpha_2\delta}$2::mEOS2 molecules are freely mobile outside synapses and either mobile (yet spatially confined) or immobile within synapses (Figures 1C and 1D). Comparing diffusion coefficients from synaptic and extrasynaptic channel populations revealed clear differences (Figures 1F and 1G). Within synapses, 35%–45% of the channels are immobile (median diffusion coefficient < 0.002 μm$^2$/s), whereas elsewhere along axons only about 20% of channels are immobile. While all remaining channel molecules are mobile, they move faster outside synapses (Figures 1F and 1G). Notably, synaptic Ca$_{\alpha_2\delta}$1::mEOS2 and Ca$_{\alpha_2\delta}$2::mEOS2 displayed similar mobility (median diff.–coeff. Ca$_{\alpha_2\delta}$1-mEOS2 0.01 μm$^2$/s inter-quartile range [IQR] 0.005–0.03 μm$^2$/s, median diff.–coeff. Ca$_{\alpha_2\delta}$2-mEOS2 0.014 μm$^2$/s IQR 0.006–0.04 μm$^2$/s; Figures 1F, 1G, and 2C). The short lifetime of mEOS2 fluorescence precluded direct assessments of dwell times for synaptic channel populations. However, single molecules exchanging between synaptic and extrasynaptic membrane compartments could be observed (Figures 1A and 1B). To test whether the mobility profiles are characteristic for VDCCs or a general feature of presynaptic membrane proteins, we monitored tagged syntaxin-1A, a presynaptic t-SNARE. Syntaxin-1A::mEOS2 displayed high mobility and, in contrast to VDCCs, no confinement within synapses (Figures 1E and 1H). Consistent with previous findings (Ribaut et al., 2011), the synaptic exchange rate for syntaxin-1A molecules appeared high and a fraction of ~15% immobile syntaxin-1A::mEOS2 molecules could reflect temporal immobilization of syntaxin-1A in synapses. These experiments demonstrate that despite the need for tight channel-sensor coupling, a large population of channels (~60%) is dynamic but confined within presynaptic membranes. This observation was corroborated by calculating the radius of confinement as a measure of the mean surface area explored by channel molecules in the presynaptic compartment (Ca$_{\alpha_2\delta}$1::mEOS2, median $r_{\text{conf}}$ 107.2 nm, IQR 73.32/157.5 nm; Ca$_{\alpha_2\delta}$2::mEOS2, median $r_{\text{conf}}$ 133.7 nm, IQR 93.4/211.4 nm; Figure 2E). These measures clearly exceeded coupling distances deduced from electrophysiological data (Borst and Sakmann, 1996; Bucurenciu et al., 2008; Meirenken et al., 2002; Schmidt et al., 2013; Scimemi and Diamond, 2012; Vyleta and Jonas, 2014).

Channel Mobility Is Unaffected by Channel Numbers within the Presynaptic Membrane

We reasoned that as a determinant for SV release, the mobility of synaptic VDCCs might be influenced by local channel density, RRP size, Ca$^{2+}$ influx, and Ca$^{2+}$ buffering. To manipulate these parameters, we first co-expressed tagged Ca$_{\alpha_2\delta}$1 or Ca$_{\alpha_2\delta}$2 with the $\alpha_2\delta$1 subunit to increase presynaptic channel density and $P_r$ (Hoppa et al., 2012). Indeed, anti-GFP fluorescence intensities (Figures S3A–S3E) and the counting of synapse-confined
CaV2.2::mEOS2 - localisation
Syntaxin1A synap/g415c

CaV2.2::mEOS2 - trajectories

Expression of CaV2.1::GFP did not increase synapsin fluorescence rather pointed to a concomitant enlargement of the affected. Inspection of Bassoon- and RIM-specific immunofluorescence revealed that synaptic channel densities had remained largely unaltered. Modeling channel dynamics within a confined area predicts that an increase in channel density reduces channel mobility (see below; Figures S4A and S4C). However, co-expression of z2b1 did not alter channel mobility for CaV2.1::mEOS2 and only slightly decreased it for CaV2.2::mEOS2 (Figures 2C and 2D). The radius of confinement did not change for CaV2.1 and decreased only moderately for CaV2.2 (Figure 2E). This suggested that synaptic channel densities had remained largely unaffected. Inspection of Bassoon- and RIM-specific immunofluorescence rather pointed to a concomitant enlargement of the CAZ. Quantitative analysis showed that overexpression of z2b1 alone increased synaptic Bassoon and RIM levels (Figures S3F and S3G). Expression of CaV2.1::GFP did not increase synapse/synaptic mobile trajectories (%)

traces as a relative measure for channel numbers (Figures 2A and 2B) revealed that z2b1 co-expression led to a substantial increase in the number of synaptic VDCCs and to markedly increased synaptic activity (Figure S3G).

Modeling channel dynamics within a confined area predicts that an increase in channel density reduces channel mobility (see below; Figures S4A and S4C). However, co-expression of z2b1 did not alter channel mobility for CaV2.1::mEOS2 and only slightly decreased it for CaV2.2::mEOS2 (Figures 2C and 2D). The radius of confinement did not change for CaV2.1 and decreased only moderately for CaV2.2 (Figure 2E). This suggested that synaptic channel densities had remained largely unaffected. Inspection of Bassoon- and RIM-specific immunofluorescence rather pointed to a concomitant enlargement of the CAZ. Quantitative analysis showed that overexpression of z2b1 alone increased synaptic Bassoon and RIM levels (Figures S3F and S3G). Expression of CaV2.1::GFP did not increase synaptic Bassoon or RIM levels, whereas CaV2.2::GFP alone exerted a strong effect. Co-expression with z2b1 further increased synaptic Bassoon and RIM fluorescence for each of the tagged channel isoforms, reaching maximum levels when CaV2.2::GFP and z2b1 were co-expressed (Figures S3F and S3G). Elevated Bassoon or RIM immunofluorescence could reflect higher synaptic protein density or enlarged CAZs. We therefore used stimulated emission depletion (STED) microscopy to determine the spatial extension of Bassoon and RIM clusters by 2D Gauss fittings (Figures 3A and 3B). This revealed that Bassoon and RIM clusters indeed became enlarged due to expression of CaV2.1::GFP, CaV2.2::GFP, and/or z2b1 (Figures 3A–3C). We propose that the CAZ and its scaffold proteins define an area of confinement for synaptic calcium channels which scales with channel number in a manner that leaves synaptic channel density and mobility largely constant.

Synaptic VDCC Mobility Is Regulated by Free Intracellular Calcium

To assess effects of presynaptic Ca2+ on channel mobility, we used two different calcium chelators, BAPTA-AM and EGTA-AM. Altering cytosolic calcium buffer capacities by exogenously applied chelators strongly influences P, (Awatramani et al., 2005; Ernokyuk et al., 2013; Williams et al., 2012). We monitored changes in P, in neurons preloaded with BAPTA-AM or EGTA-AM by measuring mEPSCs. Both chelators strongly increased the interevent interval but did not alter mEPSC amplitudes.

Figure 1. Synaptic and Extrasynaptic Mobility of VDCCs and Syntaxin1A
(A and B) CaV2.1::mEOS2 and CaV2.2::mEOS2 (green) enrich at hippocampal synapses revealed by uptake of anti-Syt1 (magenta). Second row displays localization of channel molecules within a focal plane. Third row shows trajectories of channel molecules along axonal segments with selected regions enlarged below. Few channels escaping synaptic areas indicate exchange between compartments (arrows). Scale bars: 3 µm, 0.5 µm for zoomed examples. (C–E) Plot of mean square displacement (MSD) over time intervals for axonal, synaptic mobile, and immobile CaV2.1::mEOS2 (C; n = 32/105/60 for axonal/synaptic mobile/synaptic immobile trajectories), CaV2.2::mEOS2 (D; 425/420/227), and Syntaxin1A::mEOS2 (E; n = 538/130 for axonal/synaptic mobile trajectories). (F–H) Normalized distribution of diffusion coefficients for CaV2.1::mEOS2 (F; n = 612/3281 for axonal/synaptic trajectories), CaV2.2::mEOS2 (G; 5046/22681), and Syntaxin1A (H; 5849/30139). Dotted lines mark threshold for immobile fractions (D < 0.002 µm²/s). Data in (C)–(H) are from 2–5 neuronal cultures 14–21 DIV.
At the same time, both chelators decreased the mobility of CaV2.1::mEOS2 and CaV2.2::mEOS2 and increased the fraction of immobile channels. EGTA-AM primarily affected CaV2.2::mEOS2 mobility, whereas BAPTA-AM affected both channel isoforms, increasing the rate of immobile synaptic channels to 55%–60% (Figures 2C and 2D). The radius of confinement dropped below 100 nm (CaV2.1::mEOS2, median rconf 82.1 nm, IQR 57.7–121.8 nm; CaV2.2::mEOS2, median rconf 92.5 nm, IQR 62.4–130.8 nm; Figure 2E). In contrast, the mobility and radius of confinement of syntaxin-1A increased, whereas its immobile fraction remained unaltered (Figures 2C–2E).

Considering the negative effect of Ca2+ chelation on synaptic channel mobility, we wondered whether increased Ca2+ influx would act oppositely. KCl-triggered depolarization increased mEPSC frequency during the first minutes (Figures S4E–S4H) but left overall synaptic mobility of CaV2.1 unaltered and modestly decreased CaV2.2 mobility (Figures 2C and 2D). This implies that a boost of fusion events leading to the depletion of the RRP has little effect on the mobility of VDCCs. We thus conclude that acute global changes in [Ca2+]i within the physiological range do not significantly affect the dynamics of VDCCs. In contrast, altered Ca2+ buffering capacity reduces spontaneous activity, impacts on the shape of the Ca2+ domain upon channel opening, and may also change the dynamics and relative positioning of synaptic molecules such as VDCCs and syntaxin-1A, either by Ca2+ buffering or reduced activity and hence lower membrane turnover.

Modeling the Contribution of VDCC Mobility to Synaptic Transmission

Our observations suggest that the local stochastic mobility of VDCCs is tightly controlled within the synapse and might contribute to activity-related Ca2+ fluctuations. To illustrate this hypothesis, we used Monte Carlo simulations to model the Ca2+ signal while considering the stochastic motion of VDCCs and setting the open probability for the channel population to 0.5 within synapses. Vesicular release depends on local changes of [Ca2+]i within less than 100 nm around SVs (Eggermann et al., 2012) and at a depth of 5–10 nm. We thus wondered whether the observed stability of local channel density relates to [Ca2+]i fluctuations. Comparing [Ca2+]i fluctuations of small, intermediate, or large channel populations within the same area of

Figure 2. Calcium Channel Surface Expression and Mobility under Varying Conditions

(A and B) Effect of α2δ1 expression on CaV::mEOS2 surface expression, exemplified for synaptic CaV2.1 in (A) and quantified for both channel subtypes in (B). Scale bar: 1 µm. (B) Synaptic CaV2::mEOS2 trajectories > 8 points in 2 cultures, CaV2.1::mEOS2 n = 97 synapses, +α2δ1, n = 77; CaV2.2::mEOS2 n = 51, +α2δ1, n = 50). (C) Diffusion coefficient of mobile fractions (D > 0.002 µm²/s) of CaV2.1::mEOS2, CaV2.2::mEOS2, and Syntaxin1A::mEOS2 under conditions as indicated (CaV2.1::mEOS2: nconf = 1,794/ nKCl = 1,280/ nα2δ1 = 7,681/ nEGTA = 5,275/ nBAPTA = 2,487; CaV2.2::mEOS2: nconf = 1,734/ nKCl = 2,518/ nα2δ1 = 11,087/ nEGTA = 8,870/ nBAPTA = 2,487; Syntaxin1A::mEOS2: nconf = 21,289/ nBAPTA = 26,661, data from 2–5 cultures, probed by Kruskal-Wallis test followed by Dunn’s test). (D) Immobile fractions of CaV2.1::mEOS2, CaV2.2::mEOS2, and Syntaxin1A::mEOS2 under various conditions. (E) Radius of confinement of mobile channels and syntaxin1A within synapses given as medians and interquartile range. Data from 2–5 cultures, probed by one-way ANOVA test followed by Dunn’s test. (CaV2.1::mEOS2: n = 153 synapses, +α2δ1 n = 278, +EGTA-AM n = 293, +BAPTA-AM n = 97; CaV2.2::mEOS2: n = 512, +α2δ1 n = 247, +EGTA-AM n = 96, +BAPTA-AM n = 40; syntaxin1A: n = 105).
confined by a reduction in channel mobility unaccompanied large differences in the coefficient of variation (CV) for local presynaptic Ca\textsuperscript{2+} transients (Figure 4A). The amplitude of total calcium increases with channel numbers. The CV, however, peaked for intermediate channel densities where channel mobility is close to the one determined experimentally (Figure 4B). Increasing channel density induces a decrease in channel mobility due to molecular crowding effects (Figure S4C). The model implies that channel density and channel dynamics are adjusted close to maximal CV of local Ca\textsuperscript{2+} transients within the presynaptic compartment. This correlation holds true for different membrane surfaces without changes in channel density (Figure 4B). Estimating synaptic channel density by taking the median size of Bassoon clusters as average surface area of AZs and measured numbers of trajectories as a measure for channel numbers yielded \( \sim 175 \) channels per \( \mu m^2 \). Considering that this estimation is biased by the blinking of mEOS2 (Figure S2E) and persistent endogenous VDCCs (Figures S2C and S2D), channel densities for the maximal CV in our model get close to those determined experimentally (Figure 4B, inset). It is conceivable that channel density is optimized to ensure local cooperativity between intracellular calcium domains from adjacent channels, as suggested by others (Matveev et al., 2011; Schneggenburger et al., 2012).

In order to convert channel density and mobility into more physiological parameters, we calculated the P\textsubscript{r} of readily releasable vesicles (RRVs; for parameters see Experimental Procedures). P\textsubscript{r} depended on channel density and reached maximum in a surface of 0.09 \( \mu m^2 \) with one RRV and channel densities between 220 and 300 channels per \( \mu m^2 \) (Figures 4C and 4D). Allowing repositioning of channels over time resulted in a graded increase in P\textsubscript{r}, which started at much lower channel densities. Channel mobility is particularly important for small synapses, where channel numbers are low and diffusion of channels induces high variability of P\textsubscript{r} indicated by large standard deviations. Such variability of P\textsubscript{r} has been reported for hippocampal synapses even along one axon (Ernolyuk et al., 2012). Immunolizing channels in a random distribution caused a steep change in P\textsubscript{r} (Figure 4D). Reducing channel mobility similar as measured for BAPTA-AM-loaded cells induced a similar shift of P\textsubscript{r} as for statically distributed channels. Increasing RRVs (5–10) shifted the P\textsubscript{r} curve toward lower channel densities but kept variability of P\textsubscript{r} for particular channel densities (Figure S4D). This modeling approach supports our hypothesis that a dynamic arrangement within the presynaptic membrane optimizes the density of VDCCs required for a given P\textsubscript{r}. It indicates that mobile VDCCs equalize P\textsubscript{r} for each individual synaptic vesicle independent from the positioning of the vesicle itself, which may modulate short-term plasticity.

**DISCUSSION**

Using sptPALM we could show that about 60% of presynaptic Ca\textsuperscript{2+} channels in hippocampal neurons are mobile yet confined within AZs. While we cannot rule out that the immobile fraction represents VDCCs tethered to SVs, this interpretation can hardly be reconciled with our finding that channel mobility was unaffected by KCl-induced changes in AZ membrane topography. In fact, loose channel-sensor coupling prevails at hippocampal synapses that harbor similar numbers of Ca\textsubscript{2,1} and Ca\textsubscript{2,2} channels (Ernolyuk et al., 2013; Vyleta and Jonas, 2014). Consistently, we found that the mobility is very similar for both channel types and does not mirror their differential assignment to nano- or microscale coupling at calyx of Held synapses (Fedchynsh and Wang, 2005).

Limited by image acquisition frequency and duration, we cannot discriminate whether immobile and mobile channels represent separate subpopulations or temporal fluctuations of individual channels as predicted by our model. Immobilization within the AZ could reflect short-term nanoscale coupling with SVs or tight interactions with CAZ proteins. Various interactions between presynaptic VDCCs and scaffold proteins are known but some are mutually exclusive or involve discrete channel variants only (Davydova et al., 2014; Han et al., 2011; Kaeser et al., 2011; Liu et al., 2011; Maximov et al., 1999). The availability of scaffold proteins may be limited due to their engagement in
competing interactions. Otherwise identical channels may therefore be embedded differently within a given AZ. Consistent with previous observations (Cao and Tsien, 2010), we found a prominent Ca V2.1 splice variant, which lacks established RIM/Mint1- and RBP-binding motifs (Chaudhuri et al., 2004; Davydova et al., 2014) to compete with endogenous channels for synaptic localization. For the mobile fractions of Ca V2.1 and Ca V2.2 channels, we calculated \( r_{\text{conf}} \) above 100 nm. Presuming a stretched conformation, the cytoplasmic tails of VDCCs (Wong et al., 2014) can explore a substantial area of the AZ while being linked to the CAZ. We used a modeling approach to assess the impact of this degree of freedom on \( P_r \). At low channel densities, \( P_r \) increase with channel mobility. Same time channel density is predicted to reciprocally affect mobility. Thus, to ensure a certain \( P_r \), the number of synaptic VDCCs may be kept low if their mobility is high enough. Our data suggest that the channel density is

Figure 4. Modeling of Calcium Fluctuations as Function of Channel Number and Mobility
(A) Impact of channel density on intracellular calcium fluctuations at 5 and 10 nm distances from the membrane. Channel dynamics is color coded for three ranges of diffusion coefficient (gray, \( D > 0.01 \, \text{µm}^2/\text{s} \); pink, \( D = 0.001–0.01 \, \text{µm}^2/\text{s} \); red, \( D < 0.001 \, \text{µm}^2/\text{s} \)). [Ca D] is color coded as indicated in the scale bar from 0 to 200 µM. (B) The CV for [Ca D] fluctuations plotted against channel numbers within depicted areas of 300 × 300 nm (blue), 400 × 400 nm (red), 500 × 500 nm (green). A median diffusion coefficient of 0.02 µm²/s was assumed and channel open probability set to 0.5; for other parameters, see Supplemental Information. Inset shows channel density at maximal calcium variability versus AZ size. (C) Within a 300 × 300 nm area, one vesicle (black spot) was randomly placed 5 nm above the membrane. Channel distribution was monitored for static and mobile channel populations at time points as indicated and triggered for activation (see Supplemental Information for parameters). A colored vesicle indicates successful release due to random rearrangement of channels. (D) \( P_r \) plotted against channel density for populations of mobile channels (blue), immobile channels (black), or reduced mobility in presence of BAPTA-AM (red). Note that the sudden jump of the \( P_r \) is caused by the parameter of one RRV (see also Supplemental Information for parameters). Arrow indicates the density corresponding to the example distributions in (C). Points represent mean \( P_r \) ± SD of 100 iterations for given densities.
optimized to the size of the AZ, keeping the majority of channels mobile. Accordingly, increased channel expression did not result in higher synaptic channel density. In addition, the immobilizing effect of BAPTA suggests that next to changing intracellular Ca\(^{2+}\) transients, channel positioning contributes to the \(P_r\) reducing effect of BAPTA. Following this principle, non-uniform channel density across an AZ is counterbalanced by local mobility changes, resulting in an AZ-wide homogeneous contribution of VDCCs to \(P_r\). Clustering of synaptic VDCCs within AZs has been detected by immuno-EM (Holderith et al., 2012; Indriati et al., 2013) in different synapses and was consistent with calculating realistic \(P_r\) (Ermolyuk et al., 2013). In contrast, immunogold labeling for Rim1/2 revealed a more uniform distribution for these VDCC-binding CAZ proteins (Holderith et al., 2012). Snapshots from mobile channel simulations occasionally appear cluster-like but reflect states of random distribution (Figure S4B). Our model suggests that the fractions of mobile and immobile channels are transient within the AZ, collectively represented as fractions of immobile and mobile channels within the experiments.

An obvious question is whether channel mobility is a fixed parameter for a given type of synapse or is subject to plasticity-related modulations. Our Ca\(^{2+}\) chelation experiments suggest the latter, demonstrating that reduced baseline levels of free [Ca\(^{2+}\)], lead to a significant decrease in channel mobility. Synaptotagmin-1A behaved oppositely arguing against a generic effect. Baseline [Ca\(^{2+}\)] can be regulated via signaling through G protein-coupled receptors, altered Ca\(^{2+}\) buffering, or extrusion. VDCCs themselves can add to baseline [Ca\(^{2+}\)], in response to elevated resting potentials, thereby considerably increasing \(P_r\) (Awatramani et al., 2005). This raises the interesting possibility of a feedforward mechanism by which an increased rate of spontaneous VDCC opening within AZs translates into enhanced channel mobility and thus, according to our results, enhanced \(P_r\). In view of the reported size of an intracellular calcium domain caused by single channel opening, which is tuned by endogenous calcium binding proteins (Tadross et al., 2013), the observed dynamics seem to be even more relevant. The dynamics of stochastically opening Ca\(^{2+}\) channels also offers a mechanism for the substantial contribution of high voltage-gated calcium channels to spontaneous transmitter release (Ermolyuk et al., 2013; Williams et al., 2012), in particular when considering the rather stable positioning of docked and primed vesicles in the presynaptic membrane (Lee et al., 2012; Lemke and Klingauf, 2005). To what extent channel densities vary between terminals remains to be explored, but in light of our study high channel densities could account for the nanodomain coupling observed in several synapses (Bucurenciu et al., 2008; Schmidt et al., 2013).

This study uncovers the existence and potential relevance of ongoing channel fluctuations within AZs. Regulatory modes that truly alter local synaptic channel densities rather than mean channel numbers remain to be identified. Our attempt to increase local synaptic channel density by co-expressing tagged \(\alpha1\) subunits with \(\alpha2\)/\(\alpha3\) led to increased synaptic VDCC numbers accompanied by enlargement of the CAZ. This observation is in accordance with the linear scaling of various presynaptic proteins with AZ size (Holderith et al., 2012; but see Hoppa et al., 2012). As an important new facet, we propose that VDCCs, beyond their documented permissive role in presynaptic assembly at neuromuscular junctions (Chen et al., 2011), can play an instructive role in scaling AZs at hippocampal synapses. In addition, the variation of the distance between channel and sensor within very short timescales may introduce variability to short-term plasticity. Hence, differential effects of BAPTA could be interpreted as different modes of a dynamic interplay between calcium channel and calcium sensor (Wyleta and Jonas, 2014). The proposed equilibration of individual \(P_r\) might contribute to short-term plasticity changes, tuned by the dynamics of VDCC in the presynaptic compartment. Apart from synapses, mobility of VDCCs may play a role in modulation of the intrinsic excitability of neurons by adjusting the interplay between calcium channels and calcium-activated potassium channels.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

The experiments were conducted in primary hippocampal rat neurons prepared from embryonic Wistar rats (embryonic day 18) as described (Davydova et al., 2014). The detailed procedure and methods of transfection are described in detail in Supplemental Experimental Procedures.

**Plasmids**

Expression constructs for tagged VDCC \(\alpha1\), \(\alpha2\)/\(\alpha3\), and \(\beta3\) subunits were generated by standard cloning strategies or kindly provided by various colleagues (see Supplemental Experimental Procedures).

**Life Imaging**

sptPALM experiments were conducted as described (Manley et al., 2008) using mEOS2-tagged constructs. Acquisition and analysis are described in detail in Supplemental Experimental Procedures.

**Statistics**

Statistical values are given as median with interquartile range (IQR) or mean ± SEM unless stated otherwise (see Supplemental Experimental Procedures for details).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.03.050.

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Mobility of Calcium Channels in the Presynaptic Membrane

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Supplementary information

supplementary Figures 1-4
Supplementary Figure 1:
S1 related to Figure 1, 2 and 3

Functional characterisation and synaptic expression of N-terminal tagged CaV2.1 and CaV2.2 channels

(A) Barium currents elicited by a depolarisation step from -90 mV to 0 mV for 150 ms in HEK-cells transfected with CaV2.1 + β3 + α2δ1 (black) or CaV2.1::GFP + β3 + α2δ1 (red). (B) IV relationship for CaV2.1 + β3 + α2δ1 (grey) and CaV2.1::EOS + β3 + α2δ1 (orange) displayed as current density at different membrane potentials, values are not corrected for liquid junction potential (n = 13 for CaV2.1 + β3 + α2δ1 and n = 16 for CaV2.1::mEOS2 + β3 + α2δ1). (C) Calculated peak voltage and half maximal activation for CaV2.1 (grey, n = 20) CaV2.1::GFP (green, n = 13) and CaV2.1::EOS (orange, n = 9) do not differ significantly (one-way ANOVA). (D) Barium currents elicited by a transient depolarisation step from -90 mV to 0 mV for 150 ms in HEK-cells transfected with of CaV2.2 + β3 + α2δ1 (black) and CaV2.2::GFP + β3 + α2δ1 (red). (E) IV relationship for CaV2.2 + β3 + α2δ1 (grey) and CaV2.2::mEOS2 + β3 + α2δ1 (orange) displayed as current density at different membrane potentials, values are not corrected for liquid junction potential (n = 22 for CaV2.2 + β3 + α2δ1 and n = 5 for CaV2.2::mEOS2 + β3 + α2δ1). (F) Calculated peak voltage and half maximal activation for CaV2.2 (grey, n = 17), CaV2.2::GFP (green, n = 10) and CaV2.2::mEOS2 (orange, n = 9) do not differ significantly (one-way ANOVA). (G) Overlay of immunofluorescence images for CaV2.1::GFP (green)/bassoon (red)/RIM (blue). Boxed areas are enlarged to illustrate the co-localisation of CaV2.1::GFP with the two presynaptic scaffold proteins, RIM and Bassoon. Arrows indicate localised clusters for all three proteins. (H) Same experiment as in (G), but for CaV2.2::GFP. Dimension of the scale bars are 10 µm for the overlay pictures and 2 µm for the enlarged boxed areas for both (H) and (G). (I) Relative co-localisation for endogenous and overexpressed CaV2.1 or CaV2.2 with Bassoon and RIM, differences are not significant (Bassoon: nCaV2.1endo = 2929 synapses, nCaV2.1::GFP = 1516 synapses, nCaV2.2endo = 2783 synapses, nCaV2.2::GFP = 510 synapses; RIM: nCaV2.1::GFP = 1157 synapses, nCaV2.2::GFP = 719 synapses). Scale bars in (G) and (H) represent 10 µM for the overlay and 2 µM for the zoomed area. (J) Postsynaptic currents recorded from coupled pairs of cultured hippocampal neurons, expressing CaV2.1::GFP or CaV2.2::GFP were compared to the wild type situation. First, contribution of expressed channel isoforms was measured by cumulative block of CaV2.1 and CaV2.2 (n = 3) or single block of CaV2.2 with 1 µM α-Conotoxin GIVA (n = 10) and CaV2.1 with 0.5 µM α-Agatoxin IVA (n = 11). Expression of CaV2.1::GFP did not alter the synaptic fraction of CaV2.1 within the transfected synapse (n = 5), whereas expression of CaV2.2::GFP (n = 4) led to a complete dominance of CaV2.2 channels within the synapse as revealed by enhanced sensitivity to α-Conotoxin. (K) Paired-pulse ratio upon stimulations with an interval of 50 ms were not significantly different between non-transfected (n = 16) and transfected neurons (CaV2.1::GFP, n = 4; CaV2.2::GFP, n = 10). All quantified data are given as mean ± SEM.
Supplementary Figure 2

A. Diagrams of human- and mouse- CaV2.1 channels.

B. Immunofluorescence images of mouse and human CaV2.1 channels.

C. Images showing localization of human and rat CaV2.1-GFP proteins.

D. Bar graph comparing total and partial replacement of synapses.

E. Graph showing frequency of localization accuracy over time.

F. Histogram showing frequency distribution of localization accuracy.

G. Histogram showing frequency distribution of localization accuracy.
**Supplementary Figure 2:**

S2 related to Figure 3 and experimental procedure (S2E, F; sptPALM)

Expression level of tagged calcium channels in presynaptic boutons and characterisation of fluorescence signals from mEOS2 tagged calcium channels

(A) Schematic representation of binding domains for anti GFP-antibody and the rodent-specific antibody against the II-III intracellular loop in Ca\textsubscript{v}2.1. (B) Images of HEK-cells expressing mouse Ca\textsubscript{v}2.1::GFP + β3 + α2δ1::HA or the human isoform of Ca\textsubscript{v}2.1::GFP + β3 + α2δ1::HA show clear anti-GFP labelling for both, whereas anti-Ca\textsubscript{v}2.1 immunoreactivity is restricted to the mouse isoform. Scale bar represents 10 µm. (C) Co-labelling of endogenous Ca\textsubscript{v}2.1 channel population and expressed human Ca\textsubscript{v}2.1::GFP together with Bassoon uncover two populations of synapses, one expressing only human Ca\textsubscript{v}2.1::GFP (open arrows) and a second population expressing human Ca\textsubscript{v}2.1::GFP and the endogenous rat Ca\textsubscript{v}2.1::GFP. Scale bar 10 µm. (D) Percentages of synapses expressing only human Ca\textsubscript{v}2.1::GFP (total replacement) versus synapses with both endogeneous Ca\textsubscript{v}2.1 and human Ca\textsubscript{v}2.1::GFP, 10 DIV after transfection (2 cultures, 739 synapses). (E) Examples of fluorescence events of single mEOS2-tagged Ca\textsubscript{v}2.1-channels expressed in COS7-cells. (F) Distribution of localisation accuracy calculated for Ca\textsubscript{v}2.1::mEOS2 channels in fixed cells (n = 119), mean accuracy 21.4 ± 4 nm. (G) Distribution of localization accuracy of Ca\textsubscript{v}2.1::mEOS2 channels expressed in hippocampal neurons 14 DIV, mean accuracy 27.7 ± 10 nm (n = 100). All quantified data are given as mean ± SEM.
Supplementary Figure 3:

S3 related to Figure 3

Changes in expression level of calcium channels, Bassoon and RIM induced by expression of tagged calcium channels with and without the α2δ1-subunit.

(A-D) Fluorescence detection of tagged Ca\textsubscript{V}2::GFP channels and the presynaptic scaffold protein Bassoon in cultured hippocampal neurons with (B, D) or without (A, C) co-expression of HA-α2δ1. Insets display synaptic clusters of Ca\textsubscript{V}2.1::GFP or Ca\textsubscript{V}2.2::GFP with Bassoon next to synapses from non-transfected cells with Bassoon clusters only. Scale bars represent 10 µm for overviews and 2 µm for zoomed regions. (E) Quantification of fluorescence intensity for N-terminal tagged calcium channels with and without α2δ1-subunit (Ca\textsubscript{V}2.1::GFP: n = 1661 synapses, + α2δ1: n = 1854 synapses; Ca\textsubscript{V}2.2::GFP: n = 634 synapses, + α2δ1: n = 1555 synapses, from 3-4 independent cultures 14-21 DIV). (F) Fluorescence intensities for Bassoon in the presence of different combinations of channel subunits, normalized to intensities in non-transfected cells; statistical analysis by one-way ANOVA test followed by a Dunnett-test, data derived from 2-3 independent cultures 14-21 DIV (Ca\textsubscript{V}2.1::GFP/Bassoon n = 787 synapses, Ca\textsubscript{V}2.2::GFP/Bassoon n = 367 synapses, α2δ1::HA/Bassoon n = 239 synapses, Ca\textsubscript{V}2.1::GFP + α2δ1::HA/Bassoon n = 137 synapses, Ca\textsubscript{V}2.2::GFP + α2δ1::HA/Bassoon n = 1043 synapses). (G) Fluorescence intensities for RIM in the presence of different combinations of channel subunits, normalized to intensities in non-transfected cells; statistical analysis by one-way ANOVA test followed by a Dunnett-test, data derived from 2-3 independent cultures 14-21 DIV (Ca\textsubscript{V}2.1::GFP/RIM n = 1137 synapses, Ca\textsubscript{V}2.2::GFP/RIM n = 600 synapses, α2δ1::HA/RIM n = 303 synapses, Ca\textsubscript{V}2.1::GFP + α2δ1::HA/RIM n = 936 synapses, Ca\textsubscript{V}2.2::GFP + α2δ1::HA/RIM n = 1436 synapses). (H) Activity dependent uptake of fluorescently labelled anti-synaptotagmin1 antibody incubated for 20 min at 37 °C. Anti-synaptotagmin1 fluorescence intensity is normalized to average uptake of non-transfected synapses within the same preparation, we compared for α2δ1::HA (5162 synapses); Cav2.2::GFP (2328 synapses); Cav2.2::GFP + α2δ1::HA (2328 synapses); Cav2.1::GFP (3263 synapses) and Cav2.1::GFP + α2δ1::HA (6663 synapses) from 2-3 independent cultures 14-21 DIV. All quantifications are given as mean ± SEM.
**Supplementary Figure 4:**

S4 related to Figure 4 and experimental procedures (S4E-G)

Illustrated model assumptions for assessing the impact of spatial confinement on the mobility of VDCCs/changes in synaptic activity under KCl depolarisation or intracellular calcium buffering

**(A)** Snapshot of the distribution of 30 channels within a 300 x 300 nm area starting with an initial diffusion coefficient of 0.02 \( \mu \text{m}^2/\text{s} \). Note the segregation in very low/immobile channels (labelled dark pink, diff.-coeff. < 0.001 \( \mu \text{m}^2/\text{s} \)), mobile (labelled pink, diff.-coeff. = 0.001-0.01 \( \mu \text{m}^2/\text{s} \)) and highly mobile (labelled red, diff.-coeff. > 0.01 \( \mu \text{m}^2/\text{s} \)) channels, which is only caused by the spatial confinement over time. Indicated by the quadrant borders marked with dashed lines channels appear occasional clustered. **(B)** Three snapshots at different time-points of simulation are given to illustrate the variability in channel arrangements. Appearing tendencies of clusterisation of molecules are not different from random distribution as indicated in the cumulative distributions beside the examples. **(C)** Frequency distributions of diff.-coeff. within a defined membrane compartment (300x300 nm) for three different channel populations: 10 (red), 30 (blue) and 50 (green) channels. The increase in the immobile fraction as well as the left shift of the distribution indicates the effect of molecular crowding. The dashed line indicates the border for mobile and immobile fractions as used for the experimental data. Medians of the diffusion coefficients are indicated by arrows above the distributions. **(D)** Plotted are release probabilities (\( P_r \)) against channel density for different numbers of docked vesicles, as indicated one ready releasable vesicle (RRV, blue), five RRV (green) and ten RRV (red). Each curve represents the mean \( P_r \pm \text{SD} \) calculated from 100 iterations for each density and vesicle number. **(E)** Example traces for mEPSCs under control conditions, after incubation with BAPTA-AM (20 \( \mu \text{M} \) for 20 min). **(F)** Cumulative distribution of inter event interval for 40 mM KCl (red), control (black) EGTA-AM (green) and BAPTA-AM (blue). **(G)** Mean \( \pm \text{SEM} \) of interevent interval for the different conditions. **(H)** Mean \( \pm \text{SEM} \) for mEPSC amplitude for the conditions as indicated. Data are from 2 independent cultures 14-16 DIV (control: 15 cells, KCl: 8 cells, EGTA-AM: 6 cells, BAPTA-AM: 7 cells). Differences between data were tested by one-way ANOVA followed by Bonferroni post-test, \( p < 0.0005 \) ***.
Supplementary experimental procedures

Cell culture/transfection

Dissociated primary rat hippocampal cultures were prepared from embryonic Wistar rats day E18 as described previously (Frischknecht et al., 2008). Briefly, cell suspensions obtained after dissociation with trypsin were plated onto poly-L-lysine (Sigma)-coated 18 mm glass coverslips (Menzel-Glaeser, Braunschweig, Germany) at a density of 30,000 cells per coverslip. After 1-2 h in Dulbecco's Modified Eagle Medium (DMEM) plus fetal bovine serum at 37 °C, five coverslips were transferred into a 35 mm dish containing a 70 - 80% confluent monolayer of astrocytes in neurobasal medium supplemented with B27 and 5 mM glutamine. Cultures were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO₂. At 3 days in vitro (DIV) AraC was added to the cells to a final concentration of 1.4 μM.

Neurons were transfected at 4-5 DIV using the calcium phosphate method. Prior to transfection, cells were placed in a 12 well dish with 1 ml 37°C Optimem media (Life Technologies). To prepare the precipitate, 150 μl of transfection buffer in mM: 274 NaCl, 10 KCl, 1.4 Na₂HPO₄, 15 Glucose, 42 HEPES, pH 7.04-7.1 was added drop wise to a solution containing 5 μg of DNA and 250 mM CaCl₂, under gentle stirring. The resulting mix was placed for 20 min at RT; 60 μl of the mix was added per well, and neurons were placed in the incubator for 30 to 60 min. Medium was exchanged for 2 ml 37°C pre-warmed Neurobasal medium, followed by two times exchanging 1.5 ml. Finally, after this procedure cells were placed back in the stored dishes in conditioned culture media. Experiments were performed between 14 and 21 DIV.

Transient expression of tagged VDCCs in COS-7 cells or human embryonic kidney cells (HEK-293) was achieved by co-transfection of constructs for tagged α1-subunits together with β3- and α2δ1-encoding constructs at a 1:1:1 ratio using the FuGENE® X-tremeGENE 9 DNA transfection reagent (Roche) according to manufacturer’s protocol. For comparison with non-tagged subunits we either transfected non-tagged subunit combinations in case of CaV2.1 or used a stable cell line expressing CaV2.2 (kindly provided by the lab of D. Lipcombe; (Lin et al., 2004)). Transiently transfected cells were measured 24-48 h after transfection. Current amplitudes >1 nA were considered to result from successful co-transfection of all three subunits (α1, β3 and α2δ1) as further confirmed by simultaneous detection of the GFP-tag fused to β3- and the extracellular HA epitope in α2δ1::HA (data not shown).

Plasmids/Molecular biology

Calcium channel α1-subunits used were mouse CaV2.1 (Addgene Plasmid 26578), human CaV2.1 (GenBank FJ040507), rat CaV2.2 (UniProtKB Q02294) and rabbit CaV2.2 (GenBank D14157). The N-terminal GFP labeled CaV2 constructs were described elsewhere (Szabo et al., 2006). Other calcium channel subunit cDNAs as β3 and α2δ1 were bought from Addgene (β3, Addgene 26574; α2δ1, Addgene 26575). For sptPALM experiments the N-terminal GFP was replaced by mEos2 using AvarI and RsrII restriction sites. The mEos2 cDNA was from Addgene (Plasmid 36236, generated by the lab of Robinson University of Delaware). AvarI and Rsfl restriction sites were added by PCR. The extracellularly Human influenza hemagglutinin based tag (HA-tag) -labeled mouse α2δ1 construct
was a gift from the lab of Gerald Obermair. Shortly after the signal peptide a double HA-tag was inserted between Phe27 and Pro28. All generated constructs were verified by sequencing. mEOS2::syntaxin1A was a generous gift from Dr. F. Meunier (Queensland Brain Institute, Australia).

**Electrophysiological recordings**

Whole-cell patch-clamp recordings on HEK cells were performed 24-48h after transfection at room temperature (RT). Patch pipettes were pulled from borosilicate glass with a pipette resistance between 2 and 4 MΩ. The pipette solution contained in mM: 130 CsCl, 3 MgCl₂, 0.66 CaCl₂, 11.7 EGTA, 10 HEPES (pH 7.4, 310 mOsm). Prior to experiments, 1 mM ATP and 0.1 mM GTP were added and pH was readjusted to 7.2 to 7.3 with CsOH. The extracellular solution contained in mM: 140 NaCl, 10 BaCl₂, 1 MgCl₂, 10 HEPES, 10 D-(+)-Glucose, pH 7.4 adjusted by adding NaOH, the osmolarity was between 300-310 mOsm. Barium currents were recorded by the use of an EPC10 patch-clamp amplifier (HEKA, Germany) and registered by the use of patch-master software 2.7 (HEKA, Germany). Leak currents have been subtracted online using a p/4 protocol running before the voltage protocol to trigger channel activation. Data analysis was performed using FitMaster 2.69 (HEKA), IGOR Pro 5.0 (WaveMetrics, USA) and Prism 5.0 (GraphPad, USA) software.

Paired whole cell recordings in cultured primary hippocampal neurons were performed after 14-21 DIV and at least 7 days after transfection. Primary hippocampal cultures were constantly perfused with extracellular solution containing in mM: 145 NaCl, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 10 Hepes, and 10 D-glucose (pH 7.4 adjusted with NaOH). Patch pipettes from borosilicate glass had a pipette resistance of 3-5 MΩ when filled with the intracellular solution of the following composition: 140 K-gluconate, 1 MgCl₂, 2 CaCl₂, 4 NaATP, 10 EGTA, 10 HEPES, and 0.4 GTP (pH 7.25 adjusted with KOH). Only patches with a series resistances <15 MΩ were analyzed. The membrane potential was clamped to -60 mV for the postsynaptic cell. In the presynaptic (transfected cell) action potentials were evoked by current pulses of 500 pA amplitude, 1 ms duration and 50 ms interpulse interval. Only pairs with latency between action potential and peak of EPSC shorter than 4 ms were considered as directly coupled. To test the contribution of Caᵥ2.2 and Caᵥ2.1 channels to evoked postsynaptic currents (EPSCs) the specific calcium channel blockers ω-Conotoxin GVIA (1µM) and ω-Agatoxin IVA (400 nM), both from Alomone Labs, Jerusalem, Israel, were added to the bath solution. Perfusion was stopped before toxin application. Relative changes of EPSC amplitude were recorded 8 minutes after the application of the first and 8 minutes after the application of the second toxin. The order of toxin application was altered between experiments. Residual calcium currents were blocked by application of 100 µM CdCl₂ at the end of the experiment (data not shown).

In order to access Short-term synaptic plasticity paired pulse ratio (PPR) was calculated as ratio between the second EPSC and the first EPSC from at least 10 responses. For recordings cells were kept at 30-32 °C.

mEPSCs were measured with similar solutions and temperature in the presence of 1 µM TTX, 10 µM APV and 10µM Picrotoxin to isolate AMPAR mediated synaptic currents. Patches with a serious resistance < 10 MΩ were analyzed using synaptosoft minianalysis (USA).

**Immunohistochemistry**
Primary antibodies were either applied to live cells before fixation to access the surface population of proteins or to fixed and permeabilized cells to visualize the total population of proteins. Live cell labelling was performed within cultured medium at 37°C and cells were fixed thereafter. As fixative we used 4% paraformaldehyde (PFA) in phosphate buffered salt solution (PBS) for 5 min starting at 37°C and permeabilized for 2 min with 0.3% TritonX in PBS. Afterwards unspecific immune reactivity was blocked by washing cells three times 10 min with a washing buffer containing 10% fetal calf serum (FCS), 25 mM Glycin and 2% bovine serum albumin (BSA) in PBS. Primary and secondary antibodies were applied consecutively for 1 h at RT. After additional washing steps cells were mounted on glass slides with Mowiol. The following primary antibodies were used: rabbit anti-GFP 1:1000 (Molecular Probes, Cat.-Nr. A6455); mouse monoclonal anti-GFP 1:1000 (Roche, Cat.-Nr. 11814460001, clone 7.1 and 13.1); rat anti-HA 1:1000 (Roche, Cat.-Nr. 11867423001, clone 3F10); mouse anti-HA 1:1000 (Covance, Cat.-Nr. MMS-101P, clone 16B12); guinea pig anti-Bassoon 1:1000 (synaptic system, Cat.-Nr. 141004 polyclonal); rabbit anti-RIM1/2 1:2000 (synaptic system, Cat.-Nr. 140203 polyclonal); fluorescently labelled anti-Synaptotagmin live 1:200 (synaptic system, Cat.Nr. 105103C3 polyclonal); fluorescently labelled mouse anti-Synaptotagmin live 1:200 (synaptic system, Cat.-Nr. 105311CpH monoclonal); rabbit anti-CaV2.2 1:1000 (Alomone Labs, Cat.-Nr. ACC-002); rabbit anti CaV2.1 1:1000 (synaptic system, Cat.-Nr. 152103); and secondary fluorescently labelled antibodies Alexa 488-, Cy3-, Cy5-antibodies from Jackson ImmunoResearch Laboratories.

Immunofluorescence images were either acquired on an upright microscope (Zeiss-Axio Imager A2, Germany) equipped with a PlanApochromat 63x/1.4 Oil objective (Zeiss, Germany), a Compact Light Source LQ-HXP 120 with a Mercury short arc reflector lamp (OSRAM HXP-R120W/45C Visitron Systems GmbH, Germany) and a CCD camera (CoolSNAP EZ, Photometrics, USA) under the control of VisiView software (Visitron Systems GmbH, Germany) or a confocal imaging setup based on a spinning disc mounted to a upright microscope (Olympus BX51WI). Here fluorescence signals were detected by an EM-CCD camera (Andor iXon 897D) controlled by IQ2-acquisition software (Andor, Ireland).

Acquired images were processed using MetaMorph software (Universal Imaging, USA). Synaptic puncta were defined as square regions of interest (ROI) with dimensions of about 0.9 x 0.9 μm around local intensity maxima using OpenView software (a gift from N.E. Ziv (Tsuriel et al., 2006)). Mean fluorescence intensities of transfected cells were normalized to the mean fluorescence intensities of non-transfected cells in the same region. Colocalisation of fluorescent spots was defined by matching ROI with the labelling for presynaptic calcium channels with scaffold proteins as Bassoon and RIM as quantified in Fig. S1I. The matched area was set to overlap at least 70% to be considered as colocalised.

Quantification of the active zone size was performed by analyzing images acquired with a STED microscope (Leica TCS SP5). We imaged presynaptic Bassoon labeled with guinea pig anti-Bassoon antibody (synaptic system, Cat.Nr. 141004, polyclonal) and fluorescently labelled by anti-guinea pig ATTO 647N-conjugated secondary antibodies. Maximum projections of image stack where used to
determine the x-y dimension of the clusters by Gaussian fitting of the fluorescence signal. The fitting procedure is part of the MetaMorph based plugin PalmTracer also used for sptPALM analysis.

**sptPALM**

Cells were mounted in a Ludin chamber and imaged at 37°C using a TIRF microscope (IX81 microscope within an Xcellence system, Olympus, Germany). Laser lines were calibrated and adjusted individually by the use of the TIRF-multicolor condenser (Olympus, Germany). The microscope was equipped with a TIRF objective (100x 1.49 NA Olympus, Germany). Images were acquired using an Image EM C9100-13 digital monochrome back-thinned EM-CCD camera (Hamamatsu Photonics Germany). For photo-activation localization microscopy (PALM), cells expressing mEOS2-tagged constructs were photo-activated using a 405 nm laser and the resulting photo converted single-molecule fluorescence signal was excited with a 561 nm laser. Both lasers illuminated the sample simultaneously. In order to isolate the mEOS2 signal from auto fluorescence and background signals we used a triple beam splitter (zt UV-405/488/561, Chroma Technology Corp, USA) and Emission Bandpass filter (BP 605/40, Chroma Technology Corp, USA). The power was adjusted to keep the number of the stochastically activated molecules constant and well separated during the acquisition (405 laser used 3-5% of initial laser power (100mW), 561 nm laser was used 30-40% initial laser power (100mW)). Images were acquired by image streaming for up to 4000 frames at frame rate of 30 frames/s. Active synapses were identified by incubating hippocampal neurons 20 min before imaging with a fluorescently coupled antibody against the luminal domain of Synaptotagmin1 (Synaptic systems Cat.-Nr. 105311CpH for 20min in culture media diluted 1:200 within cultured media), which did allow the identification of active synapses (Kraszewski et al., 1995). In some experiments cells were pre-incubated with BAPTA- or EGTA-AM (20 µM) for 30 minutes.

Localization and trajectory reconnection of mEOS2 signals was performed by the use of a wavelet based algorithm (Izeddin et al., 2012). Single mEOS2 molecule fluorescence could be identified by occurrence of fluorescence in the red channel and the defined minimum duration of fluorescence. Trajectories of mEOS2-tagged molecules were reconstructed by a simulated annealing algorithm (Racine et al., 2006), taking into account molecule localization and total intensity. It has been described that mEOS2 molecules can show blinking-like behavior ([Annibale et al., 2011], Fig. S3). To avoid false reconnections between trajectories, all sub-trajectories of mEOS2 were analyzed as individual trajectories. The dynamic behavior of single molecules was computed from the MSD curves for all trajectories of at least 8 frames. Diffusion coefficients were calculated by linear fit of the first four points of the MSD plots. MSD plots of immobilized molecules (on fixed samples) revealed that under our imaging conditions only $D \geq 0.002 \mu m^2/s$ can be considered to be mobile. The calculation of the explored area for synaptic located molecules was performed on trajectories longer than 20 time points in order to fit the MSD according to the procedure described by (Kusumi et al., 1993).

**Monte-Carlo simulation**

A discrete space-time simulation based on random walk theory was developed using MatLab (Release 2013b – 8.2.0.701). The channel dynamics were modelled on a two-dimensional lattice.
basic lattice unit size was set to 10 nm corresponding to typical geometry values given in (Nadkarni et al., 2010; Ribault et al., 2011; Shahrezaei and Delaney, 2004). Ca^{2+} channels were modelled by a central unit of 10 x 10 nm, i.e. one lattice unit, surrounded by 8 boundary units. The dynamical movement of Ca^{2+} channels was simulated as a random walk in 2D, i.e. random moves into four possible directions with equal probability. This movement was limited due to two reasons. First, channels could not extend the movement beyond a given membrane boundary. The 2D membrane size was selected according to the microscopic observations and given values from the literature between 300 x 300 nm and 500 x 500 nm. Second, the channel movement was limited to free membrane spaces, i.e. channels could not overlap with each other. The time step of the simulation was chosen so that the median diffusion coefficient of 0.02 µm^{2}/s was observed for a freely moving channel. The initial channel positions were randomly selected under the condition that no channel overlap was observed. The channel positions were then tracked over a step period of about 50-100 simulation steps. The mean square deviation of every channel and, based on this, the diffusion coefficient for every channel was calculated from this data. The simulation procedure was repeated 100 times with a new set of random numbers each time to get a statistical average of the diffusion coefficient distribution. Different random number generators of MatLab (Mersenne, Twister, Congruential) were applied and no significant influence on the results could be observed.

The obtained channel positions after the movement served as the initial configuration for the second part of our Monte-Carlo simulation, the random walk simulation of Ca^{2+} ions above the membrane. Here every channel location served as the initial inserting location of a Ca^{2+} ion. The Ca^{2+} ion could then move as a random walker in 3D during a simulation time step, i.e. into six different directions. The step time was selected to match the Ca^{2+} diffusion coefficient of about 50 µm^{2}/s given by (Nadkarni et al., 2010). This movement was only limited by the membrane surface itself, i.e. the Ca^{2+} ions could not move below the membrane surface. We investigated the Ca^{2+} ion concentration in a vicinity of 10 nm above the membrane and used a lattice spacing of 1 nm for the unit size in z-direction. Ca^{2+} ions were released from every channel position according to a 50% probability and moved afterwards according to a random walker. The simulation was carried out until around 300-500 Ca^{2+} ions were released by each channel corresponding to the values given in (Shahrezaei and Delaney, 2004; Stanley, 1993). The random positions of all Ca^{2+} ions were recorded and used to calculate the ion concentration above the membrane. An averaging procedure has been used where all ions in a xyz-box of 10 x 10 x 5nm were summed up and transformed to a molar ion concentration. The levels reached compare well to the numbers of around 100-250 µM (Ribault et al., 2011). Based on this Ca^{2+} ion concentration a coefficient of variation CV_{A} was estimated. For this parameter, the average ion concentration value at the height of 5 nm above the membrane was compared to a local value of a coarse-grained lattice where 3 x 3 lattice units, i.e. 30 x 30nm, were taken together for the estimation of the discrete Ca^{2+} ion number in each such box. If the ion numbers are equal to the average everywhere above the membrane, the coefficient of variation is small and close to zero. If there are strong differences between the average value and the local values this parameter is large and closes to one. In order to calculate a corresponding vesicle release probability P, we randomly placed a number of vesicles from 1-10 at a distance of 5 nm above the membrane and estimated the number of Ca^{2+} ion in the local vicinity at a radius of about 20 nm around each vesicle. If the number of Ca^{2+} ions in the vicinity of the vesicle exceeds a threshold value of eight calcium ions, corresponding to a molar concentration of 250 µM, the release of the vesicle is triggered. The
Probability values are estimated based on 100 repeated simulations, the statistical value is given as mean value and standard deviation. Each simulation used a different set of random numbers and the random number generators provided by MatLab (Mersenne, Twister, Congruential) were used interchangeably in order to ensure independence of the applied pseudo-random number distributions.

**Statistic**

The statistical significance of the results was probed by various tests given in the figure legend and main text. We used GraphPad Prism 5.0 software to compare data. Data are represented either as means ± SEM, mean ± SD or medians with interquartile range (IQR) stated in the figure legends, depending on the data distribution. Significance level is marked as follow: p < 0.05 *, p < 0.005 **, p < 0.0005 ***.

**References:**


