Spatial distribution of primed vesicles in the calyx of Held is strongly skewed towards Ca$^{2+}$ channel clusters

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Abstract

Fast release of neurotransmitters from the presynapse is known to depend on the coupling between synaptic vesicles and presynaptic Ca\(^{2+}\) channels. While the average coupling distance has been estimated in a number of preparations, the degree of heterogeneity in vesicle positioning at the active zone and its role in synaptic transmission has not been addressed. We study this issue in the calyx of Held using a biophysical vesicle release model and a simple non-trivial vesicle distribution function which is characterised by the channel-vesicle coupling strength. We constrain this coupling parameter using experimentally determined release probabilities from literature and conclude that the distribution of primed vesicles at the calyx of Held active zone is strongly skewed, with most vesicles being within $\sim$100 nm of a Ca\(^{2+}\) channel cluster. In addition, we simulate other electrophysiological experiments to explore their dependence on the coupling parameter, including Ca\(^{2+}\) uncaging, paired-pulse action potential (AP)-stimulation and AP-stimulation in the presence of Ca\(^{2+}\) buffers.

6.1 Introduction

Ca\(^{2+}\)-evoked, phasic release of neurotransmitter from vesicles at mammalian CNS synapses is a precisely-timed process. There are two prerequisites for this tight regulation: (1) a brief and highly localised [Ca\(^{2+}\)] transient and (2) a molecular release machinery capable of rapidly transforming these concentration elevations into the fusion of a vesicle with the presynaptic plasma membrane [196]. Patch-clamp recordings at the calyx of Held, a giant glutamatergic synapse in the auditory system, have resulted in a very good understanding of the non-linear Ca\(^{2+}\)-dependence of phasic release, while EM studies have provided detailed information about the ultrastructural organisation of the presynapse [76, 207]. However, the precise localisation of Ca\(^{2+}\) channels within the active zone, and therefore the spatial coupling between channels and release-ready vesicles, remains subject of study [208, 209]. This coupling, or ‘positional priming’, is an intuitive and potent mechanism to control release probability, as the Ca\(^{2+}\) concentrations are higher at positions closer to the channel mouth, leading to increased action potential (AP)-evoked release [202].

The vesicular release probability ($p_{vr}$), a major determinant of short-term plasticity (STP), is known to vary from synapse to synapse and is modulated by synaptic activity [79]. This modulation could occur via the energy barrier for vesicle fusion and/or by intrinsic changes in the molecular composition of the release machinery (see previous chapters). Heterogeneity in $p_{vr}$ then originates from different distributions of energetic states of the release machinery. Alternatively, or additionally, $p_{vr}$ could be modulated by controlling the distance
between vesicles and Ca\textsuperscript{2+} channels. In this case, heterogeneity in \( p_{\nu r} \) originates from a distribution of vesicle positions [210]. Unfortunately, it is not feasible to experimentally determine the effect of the positioning of individual vesicles on \( p_{\nu r} \). A presynaptic correlate regulating this positional priming in the calyx of Held was found by Young and Neher, who concluded Synaptotagmin-2 (Syt-2) to be responsible for positioning vesicles with respect to Ca\textsuperscript{2+} channels [206]. Furthermore, a large presynaptic protein complex having as central components the proteins RIM, RIM-BP and Munc13 has been identified as being crucial in organising the coupling between Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} sensors [30, 77, 211]. The frog neuromuscular junction and the squid giant synapse were the first two synapses used for studying the average coupling distance between Ca\textsuperscript{2+} channel and vesicle [77, 212, 213]. Only recently, a modeling study addressed this issue in the mammalian CNS, by studying cortical synapses [214]. An earlier study in the calyx of Held already stressed the key differences between describing the coupling distance using a distribution of primed vesicles at the active zone on the one hand, and using a single average (coupling) distance from the Ca\textsuperscript{2+} source to characterise all primed vesicles on the other [215]. It does not seem likely that all vesicles are positioned at exactly the same distance to the Ca\textsuperscript{2+} source: either by having all vesicles placed in a ring around a Ca\textsuperscript{2+} channel cluster (CCC) — or vice versa, by having a ring of Ca\textsuperscript{2+} channels around each single vesicle [216, 217]. Hence, an alternative to the conventionally used measure of ‘mean coupling distance’ is required. Therefore, in this study we propose a non-trivial vesicle distribution with minimal complexity, which depends on the active zone area and a single parameter characterising the channel-vesicle coupling strength. We use this distribution in detailed simulations to theoretically study how heterogeneous vesicle positioning affects release evoked by single APs, Ca\textsuperscript{2+} uncaging and during paired-pulse stimulation. Furthermore, we propose methods to constrain the coupling parameter using experimental data and we provide an estimate of this parameter in the calyx of Held based on vesicular release probability data from literature.

6.2 Results

6.2.1 Vesicle positioning and stochasticity in reactions determine EPSC amplitude triggered by deterministic [Ca\textsuperscript{2+}] transients

We set out to construct a biophysically accurate spatiotemporal model of the calyx of Held. Using anatomical and biophysical data, Meinrenken et al. [215] inferred in a detailed modeling study that, at each active zone (AZ) of the calyx of Held, release of most vesicles is governed by a single cluster of about 12
Ca\textsuperscript{2+} channels. The (radially symmetric) topology of our model is based on their findings, with a single Ca\textsuperscript{2+} point source in the centre of the AZ, at the base of the 0.8 × 0.8 × 0.4 µm reaction volume. Vesicles are positioned on a circular area, with radius \( r \) ranging between \( r_0 = 20 \text{ nm} \) (the estimated size of the SNARE complex [218]) and \( r_{\text{max}} = 400 \text{ nm} \) (fig. 6.1A).

Release was modeled using an adaptation to the vesicle release model for chromaffin cells from [128] to the calyx of Held (Walter et al., unpublished), since many properties of both model systems are conserved [63]. The total number of 70000 vesicles was based on a detailed ultrastructural study of the calyx of Held [207]. A detailed description of the model, which treats the vesicle states as stochastically evolving entities using the stochastic simulation algorithm (SSA) [219], is provided in the appendix.

Ca\textsuperscript{2+} diffusion and buffering during AP-stimulation were simulated in a realistic spatiotemporal fashion using the ‘calcium calculator’ tool (CalC [220]; see appendix). Depending on the distance towards a CCC, the \([\text{Ca}^{2+}]\) transient not only displays a difference in peak concentration (20 nm: \([\text{Ca}^{2+}]_{\text{max}} \approx 85 \mu\text{M}; 400 \text{ nm: } [\text{Ca}^{2+}]_{\text{max}} \approx 0.7 \mu\text{M} \), but also in the delay of arrival — with the time-to-peak \([\text{Ca}^{2+}]\) at a distance of 400 nm being about 220 \( \mu\text{s} \) later than at 20 nm (fig. 6.1B). A longer distance to a CCC thus affects the synaptic delay via a diffusion component. The vesicular release triggered by these \([\text{Ca}^{2+}]\) transients was convolved with a typical miniature excitatory post synaptic current (mEPSC) to obtain an EPSC [221, 222]. For simplicity, we first simulated a system in which the distance \( r_{\text{RRP}} \) to a CCC is exactly the same for all primed vesicles, so that each vesicle experiences the same \([\text{Ca}^{2+}]\) transient. EPSC amplitudes range from about 0.2 nA for vesicles positioned at a distance of 100 nm from a CCC, to about 15 nA at 20 nm (fig. 6.1C). For vesicles further away (≥100 nm), AP-induced release becomes increasingly more unreliable, with more and more stimuli resulting in EPSC failures.

We further studied how the stochastically evolving vesicle states affect the variability of EPSC amplitudes, by performing 10 simulations for each distance \( r_{\text{RRP}} \). For \( r_{\text{RRP}} \) smaller than about 50 nm, we found this effect to be negligible, while release at more distant sites became increasingly more variable (fig. 6.1D). This effect became more pronounced for vesicles further away from the CCC, when AP-evoked EPSC amplitudes are of the order of a single mEPSC. At 400 nm, all 10 stimuli led to EPSC failures (reducing the variability back to 0). We thus conclude that both positioning of the vesicles with respect to the Ca\textsuperscript{2+} source as well as the inherent stochasticity of the system affect the amplitude of AP-evoked EPSCs, although the latter is negligible for more tightly coupled vesicles.
Figure 6.1: The simulation geometry and stochasticity affect AP-evoked EPSCs. (A) Cartoon of the simulated system. \([\text{Ca}^{2+}]\) transients originate from the \(\text{Ca}^{2+}\) channel cluster in the centre of the active zone, which is modeled as a point source. The distance \(r\) between vesicles and \(\text{Ca}^{2+}\) channel cluster is governed by a coupling constant, a minimal distance \(r_0\) and the radius of the active zone \(r_{\text{max}}\). (B) AP-evoked \([\text{Ca}^{2+}]\) transients at various distances from a CCC, for an inward \(\text{Ca}^{2+}\) current of magnitude \(I_{\text{Ca}} = 1.34\) pA. The peak amplitude decays from 85 \(\mu\)M at 20 nm to 0.7 \(\mu\)M at 400 nm. (C) Effect of the \([\text{Ca}^{2+}]\) transients from figure B on release, when all RRP-vesicles are located at the same distance \(r_{\text{RRP}}\) from a CCC. NRP-vesicles are situated at \(r_{\text{NRP}} = 200\) nm in all simulations. (D) The stochastic behaviour of the system affects EPSC amplitude, as can be seen from the normalised mean ± SEM EPSC amplitude of 10 simulations at each \(r_{\text{RRP}}\)-value. The effect plays a negligible role for vesicles positioned closer to the \(\text{Ca}^{2+}\) source (≤50 nm) but becomes increasingly more pronounced for vesicles further away (≥100 nm), as illustrated by the EPSCs in the inset — up until the point at which the vesicles are so far away (~400 nm) that all 10 stimuli lead to EPSC failures, thus decreasing the variability to 0.
6.2.2 AP-evoked release depends on positional heterogeneity whereas Ca\textsuperscript{2+} uncaging-evoked release does not

Next, we wanted to determine the effect of heterogeneity in the position of releasable vesicles with respect to a Ca\textsuperscript{2+} source during AP-evoked release. As shown in the appendix, we assume the cumulative density function (cdf), which gives the chance of finding a vesicle at distance \( r \) from the cluster, to depend on (1) the area of the circular AZ as well as on (2) a term describing the channel-vesicle coupling, resulting in the following probability density function (pdf) \( f(r) \):

\[
f(r) = \frac{(r - r_0) \cdot \lambda e^{-\lambda(r-r_0)}}{1 - e^{\lambda(r_{\text{max}}-r_0)} \cdot (r_{\text{max}} - r_0 + \frac{1}{\lambda})}
\] (6.1)

which is defined for \( r_0 \leq r \leq r_{\text{max}} \). The maximum value of this function can be seen to occur at

\[
r_{\text{peak}} = r_0 + \frac{1}{\lambda}
\] (6.2)

This relation provides an intuitive interpretation of the parameter \( \lambda \): it can be seen as a coupling strength between a vesicle and a CCC, which controls the location of \( r_{\text{peak}} \) (the mode of the pdf) and hence the average position of all primed vesicles (i.e. the ‘mean coupling distance’) \( r_{\text{mean}} \). In the following simulations, the size of the system will be as defined in figure 6.1A, with \( r_0 = 20 \) nm and \( r_{\text{max}} = 400 \) nm. Figure 6.2A shows the shape of the pdf for three different values of the coupling strength parameter \( \lambda \). For a strong coupling constant, the function is skewed towards the CCC, while a weak coupling constant results in a less skewed distribution. In the absence of coupling (\( \lambda \to 0 \) nm\textsuperscript{-1}), the distribution can be shown to reduce to one purely governed by the area of circular AZ (see appendix). Furthermore, the average vesicle position \( r_{\text{mean}} \) approaches \( r_0 \) for

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**Figure 6.2 (facing page):** Changes in the vesicle distribution at the active zone affect action potential-evoked release. (A) The probability density function plotted for three different values of the coupling constant \( \lambda \). (B) Kinetics of average EPSCs (\( n = 10 \)) resulting from distributions with different couplings are not strongly affected. The coupling values \( \lambda = 1/20, 1/50, 1/100 \) and \( 1/200 \) nm\textsuperscript{-1} correspond, in respective order, to a mean vesicle position \( r_{\text{mean}} = 60, 118, 182 \) and \( 229 \) nm. (C-E) The effect of strong \( (\lambda = 1/20 \) nm\textsuperscript{-1}; C1-E1) or weak \( (\lambda = 1/200 \) nm\textsuperscript{-1}; C2-E2) coupling constant on action potential-evoked release. (C) Typical distribution of the locations of all primed vesicles at the active zones right before the arrival of an action potential (AP). Histogram bin width is 1 nm. (D) EPSCs evoked by an AP from the vesicle distributions shown in C. (E) Distribution of the vesicles remaining (Post-AP) and released (Difference) from the distribution shown in C, 3 ms after application of the AP.
strong coupling, while weak coupling causes $r_{\text{mean}}$ to diverge in an exponential fashion towards $\sim 290$ nm in the ‘uncoupled’ situation (see appendix; suppl. fig. 6.1A-B).

In order to sample the positions of all primed vesicles from the above pdf, we make use of the acceptance-rejection sampling method (see appendix) [223]. This enables us to study the effect of coupling strength on AP-evoked release through a set of randomly selected vesicle positions. Interestingly, while the distance to a CCC (and thus the coupling strength) determines the amount of release (as shown in fig. 6.1C for a fixed distance $r_{\text{RRP}}$), the release kinetics appear to be relatively unaffected by positional heterogeneity. Figure 6.2B shows AP-evoked EPSCs normalised to their peak current for 4 different values of the coupling parameter, averaged over 10 simulations each. While the decay is invariant, the time-to-peak is slightly longer and the peak is a little broader in the less tightly coupled situation. Presumably, this is partly due to the $[\text{Ca}^{2+}]$ transients taking more time to reach vesicles further away (as in fig. 6.1B). Also — since the (loosely coupled) vesicles further away result in EPSC amplitudes of the order of a single mEPSC (as in fig. 6.1D) — the failure or delayed release of a single vesicle (caused by stochastic fluctuations) will profoundly affect the EPSC shape, resulting in a delayed and/or broadened average EPSC.

Subsequently, we compared the responses evoked by single APs and $\text{Ca}^{2+}$ uncaging, for populations of strongly and weakly coupled vesicles. For strong coupling, we used $\lambda = 1/20 \text{ nm}^{-1}$, while for weak coupling, we took $\lambda = 1/200 \text{ nm}^{-1}$ (comparable to [224]). A typical example of the vesicle distribution for both cases before AP-stimulation is shown in figure 6.2C1-2 (compare with the red and black continuous distributions in fig. 6.2A). Due to larger $[\text{Ca}^{2+}]$ transients upon arrival of an AP at the presynaptic terminal, the vesicles closest to the $\text{Ca}^{2+}$ channel fuse most efficiently (as shown before in fig. 6.1C). Therefore, the AP-evoked EPSC is much larger for the tightly coupled distribution of vesicles than for the loosely coupled one (compare fig. 6.2D1-2). The distribution of the primed vesicles that remained 3 ms after the arrival of the stimulus showed that indeed most of the vesicles responsible for the (fastest phase of the) EPSC were those closest to the $\text{Ca}^{2+}$ source (within about 80 nm of a CCC; fig. 6.2E1-2). A comparable reduction in AP-induced EPSCs (fig. 6.2D1-2) accompanied

**Figure 6.3 (facing page):** Changes in the vesicle distribution at the active zone do not affect $\text{Ca}^{2+}$ flash-induced release. (A-C) The effect of strong ($\lambda = 1/20 \text{ nm}^{-1}$; A1-C1) or weak ($\lambda = 1/200 \text{ nm}^{-1}$; A2-C2) coupling constant on $\text{Ca}^{2+}$ flash-evoked release. (A) Typical distribution of the locations of all primed vesicles at the active zones right before the arrival of the $\text{Ca}^{2+}$ flash. Histogram bin width is 1 nm. (B) EPSCs evoked by a $\text{Ca}^{2+}$ flash from the vesicle distributions shown in A. (C) Distribution of the vesicles remaining (Post-Flash) and released (Difference) from the distribution shown in A, 10 ms after application of the $\text{Ca}^{2+}$ flash.
by an increased time-to-peak (fig. 6.2B) has been observed experimentally in a study on RIM1/2-deficient calyces, in which the mean coupling distance was concluded to be affected [211].

While the positioning of a vesicle with respect to a Ca\textsuperscript{2+} channel is clearly of importance during AP-induced release, it is expected to play no role for the release in response to Ca\textsuperscript{2+} uncaging. This stimulation method is thought to lead to a spatially homogenous elevation of the intracellular Ca\textsuperscript{2+} concentration [59] and was therefore used to probe the Ca\textsuperscript{2+}-dependency of synaptic vesicle release [76, 132]. As this implies that all releasable vesicles ‘see’ the same Ca\textsuperscript{2+} concentration, we tested whether this expected homogeneity was reproduced by our spatiotemporal release model. To this end, we compared the effect of a Ca\textsuperscript{2+} flash — modeled as an instantaneous and homogeneous step in Ca\textsuperscript{2+} concentration — to 10 µM on a strongly and weakly coupled vesicle population (fig. 6.3A1-2). As opposed to AP-induced release, one would expect no difference in evoked release between the two populations. Indeed, the resulting EPSCs were extremely similar (with small differences presumably due to the stochasticity of the system), with a comparable amplitude and the same time-to-peak (fig. 6.3B1-2). Comparison of both vesicle populations before and 10 ms after stimulation confirms that vesicles at all locations were equally likely to fuse, both for strong and weak coupling (fig. 6.3C1-2). Our model is thus in line with the idea that Ca\textsuperscript{2+} uncaging-evoked release is independent of the channel-vesicle coupling strength.

### 6.2.3 Paired-pulse protocol in systems with strong and weak channel-vesicle coupling

We proceeded to study the effect of different channel-vesicle coupling strengths on responses to a paired-pulse stimulation protocol. Like many other preparations, the calyx of Held is known to display facilitation under conditions of low initial release probability (caused by e.g. a decrease in the extracellular Ca\textsuperscript{2+} concentration), while under physiological conditions depression dominates [81, 210, 225, 226]. Since our simulations of AP-evoked [Ca\textsuperscript{2+}] transients were based on electrophysiological measurements of the calyx under physiological conditions (see appendix), we expected the simulations to show paired-pulse depression. Even though weakly coupled vesicles in some simulations at a 10–50 ms inter-stimulus interval (ISI) displayed a paired-pulse ratio (PPR) larger than 1 (i.e. facilitation), on average their PPR was less than 1, so that the overall tendency was depression (fig. 6.4). The apparent slight increase in average PPR at 50 ms ISI for weak coupling is due to 2 (out of 10) simulations having a PPR of about 1.4, which greatly uplift the average PPR of the remaining 8 simulations (0.76). Strongly coupled vesicles showed more depression than did weakly
coupled ones for all ISIs. As the amount of paired-pulse depression depends on the value of the coupling constant, the experimentally observed magnitude and time constant of depression could be of use in constraining this parameter.

![Graph showing the paired-pulse ratio (PPR) depending on the coupling strength parameter.](image)

**Figure 6.4:** The paired-pulse ratio (PPR) depends on the coupling strength parameter. Pairs of AP-evoked stimuli were simulated with various inter-stimulus intervals (ISIs). The data points are the mean ± SEM of 10 simulations each, for strong ($\lambda = 1/20 \text{ nm}^{-1}$) and weak ($\lambda = 1/200 \text{ nm}^{-1}$) coupling values.

Since the first AP stimulus readily depletes a large fraction of the RRP in the strongly coupled case (fig. 6.2E1), too little vesicles remain to result in facilitated release. On the other hand, very few vesicles are released initially in the weakly coupled situation, leaving the RRP virtually unchanged (fig. 6.2E2). The slight reduction in RRP might be offset temporarily (for short ISIs) by an increased residual Ca$^{2+}$ concentration, which is generally thought to be responsible for facilitation [210] — however, here we find an overall tendency towards depression (fig. 6.4). We thus conclude that a more weakly coupled system (with vesicles being on average further away) by itself cannot produce paired-pulse facilitation. Sources of facilitation which we did not study, such as the binding of residual calcium to a secondary sensor, buffer saturation, or Ca$^{2+}$ current facilitation, nevertheless could combine with distance-dependent effects to produce facilitation [47].

### 6.2.4 Concentration-dependent effects of BAPTA on AP-evoked release can be used to constrain the channel-vesicle coupling parameter

Next, we simulated the effects of the fast Ca$^{2+}$ buffer BAPTA on AP-evoked release, to show that our model makes a set of clear predictions that can be
tested experimentally, and that a combined experimental and computational approach can be used to determine the value of the coupling parameter. To experimentally study the average distance between \( \text{Ca}^{2+} \) channels and the \( \text{Ca}^{2+} \) sensor responsible for vesicle release, \( \text{Ca}^{2+} \) chelators with different \( \text{Ca}^{2+} \) binding kinetics but comparable affinities are used [77]. In particular, the fast \( \text{Ca}^{2+} \) buffer BAPTA and the slow buffer EGTA are used in the calyx of Held [215].

Depending on the coupling strength, buffer concentration and type of buffer, an AP-evoked EPSC can be inhibited to varying degree. For strong channel-vesicle coupling, only sufficiently high concentrations of BAPTA will be able to inhibit release, whereas in the case of weaker coupling, the slower EGTA will also affect the response.

As noted before, an AP-evoked EPSC in our model is mostly made up of vesicles that were within an 80 nm radius of a CCC (fig. 6.2E1-2). Therefore, BAPTA will be fast enough to buffer \( \text{Ca}^{2+} \) ions before they reach the \( \text{Ca}^{2+} \) sensor of some of these vesicles, but EGTA will not. Hence, we will only consider BAPTA-induced inhibition of AP-evoked release. Application of BAPTA will reduce the radius within which vesicles are likely to be released upon AP-stimulation, and thus the average EPSC amplitude, in a concentration-dependent manner. This concentration-dependent effect can be seen in figure 6.5A1-2 for strong and weak coupling, in the presence of 0, 100 and 500 \( \mu \text{M} \) BAPTA. Comparison of the histograms in figure 6.5B1-2 with those in figure 6.2E1-2 shows that the amount of vesicles released is clearly reduced, resulting in smaller EPSC amplitudes. The vesicles that are released are typically positioned closer — within a radius of about 50 nm — to a CCC.

We used our model to simulate AP-evoked EPSCs in the presence of various

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**Figure 6.5 (facing page):** Concentration-dependent effects of the fast \( \text{Ca}^{2+} \) buffer BAPTA on AP-induced release can be used to experimentally determine the coupling parameter. (A-B) The concentration-dependent effect of BAPTA on strongly (\( \lambda = 1/20 \text{ nm}^{-1} \); A1-B1) and weakly (\( \lambda = 1/200 \text{ nm}^{-1} \); A2-B2) coupled vesicles during AP-induced release. (A) EPSCs evoked by an AP in the presence of 0, 100 and 500 \( \mu \text{M} \) BAPTA. (B) Typical distribution of the locations of all primed vesicles at the active zones right before application of the AP (Pre-AP), as well as the distributions of those remaining 3 ms after the stimulus (Post-AP) and of those that were released (Difference), in the presence of 500 \( \mu \text{M} \) BAPTA. Histogram bin width is 1 nm. (C) AP-evoked EPSCs at 0, 10, 100, 250 and 500 \( \mu \text{M} \) BAPTA were normalised to the average amplitude at 0 \( \mu \text{M} \) BAPTA. The data points are the mean ± SEM of 10 simulations each. After fitting these data points one can interpolate the IC\(_{50}\) (334 \( \mu \text{M} \) for strong coupling and 198 \( \mu \text{M} \) for weak coupling), which clearly depends on the value of the coupling parameter. Hence, an experimentally obtained IC\(_{50}\) can be used to constrain the coupling parameter. The fits were performed using a double-exponential function. Inset: zoom of the leftmost part of the graph.
Heterogeneity in vesicle positioning

Chapter 6

A1

1nA
1ms

no BAPTA

100µM BAPTA

500µM BAPTA

A2

1nA
1ms

no BAPTA

100µM BAPTA

500µM BAPTA

B1

Number of vesicles

r (nm)

0 100 200 300 400
10
20
30

0 10 20 30

B2

Number of vesicles

r (nm)

0 100 200 300 400
10
20
30

C1

EPSC amplitude (norm.)

BAPTA conc. (mM)

0 0.1 0.2 0.3 0.4 0.5

0 0.2 0.4 0.6 0.8 1.0

0 0.02 0.04 0.06 0.08 0.1

0 0.02 0.04 0.06 0.08 0.1

C2

EPSC amplitude (norm.)

BAPTA conc. (mM)

0 0.1 0.2 0.3 0.4 0.5

0 0.2 0.4 0.6 0.8 1.0

0 0.02 0.04 0.06 0.08 0.1

0 0.02 0.04 0.06 0.08 0.1

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concentrations BAPTA (0–500 μM), for strong and weak coupling. The amount of inhibition of the average EPSC was plotted against the BAPTA concentration to find a model-predicted IC$_{50}$ (the concentration at which the response is inhibited by 50%; fig. 6.5C1-2). The weakly coupled system already shows a significant reduction in response of 16% at very low BAPTA concentrations (10 μM), whereas the reduction for strong coupling is more gradual. The value of IC$_{50}$ is seen to increase for stronger channel-vesicle coupling (334 μM for strong coupling versus 198 μM for weak coupling, based on double-exponential fits to the data). When additional simulations for a range of other coupling values are generated these can be compared against experimentally obtained values to constrain the coupling parameter [77].

6.2.5 Constraints on the channel-vesicle coupling strength parameter via the vesicular release probability

To study the relation between channel-vesicle coupling strength and release probability $p_{vr}$, we performed 10 simulations for a range of coupling strength values between $1/400 \leq \lambda \leq 1$ nm$^{-1}$. The release probability in the calyx of Held is typically found to be between 0.10 and 0.25 [132, 204], so we used this window to determine the corresponding interval of $\lambda$-values and thus constrain the typical distribution of vesicles at the active zone. This approach can be applied in conjunction with the method of using concentration-dependent effects BAPTA, to further constrain the coupling parameter.

In our simulations, we define release probability as the fraction of those vesicles that were initially present in the RRP and are released by a single action potential within 3 ms (comparable to [132]). Even though the $p_{vr}$ is variable, due to the stochastic nature of our simulations, a general relation between $p_{vr}$ and coupling strength can be observed by averaging over the 10 simulations per coupling value (fig. 6.6A). Our simulations match the experimentally found range in $p_{vr}$ for coupling parameters between (approximately) 0.02 ≤ $\lambda$ ≤ 0.04 nm$^{-1}$, as indicated by the dashed lines in figure 6.6A. If we express the mean vesicle location in terms of a mean coupling distance $r_{mean}$ (with the relation between $\lambda$ and $r_{mean}$ as shown in suppl. fig. 6.1A), these values correspond to an average position of primed vesicles between 69 nm and 115 nm from a Ca$^{2+}$ channel cluster (fig. 6.6B), which is comparable to the previously reported estimate of ∼100 nm (range from 30 to 300 nm) at the calyx of Held [215]. The amplitude of an AP-evoked EPSC clearly depends on the coupling parameter in a similar fashion to $p_{vr}$ (fig. 6.6C1). However, there appears to be no clear relationship between the variance in EPSC amplitudes and $\lambda$ (fig. 6.6C2), except for a positive correlation ($r = 0.61$): the variance increases for stronger couplings and (hence) larger EPSC amplitudes.
Figure 6.6: Constraints on the channel-vesicle coupling strength parameter. The data points in (A-C) are the mean ± SEM of the same 10 simulations, at coupling constants $\lambda$ between 1/400 and 1 nm$^{-1}$. (A) Relation between release probability and coupling constant. The grey line is a shape-preserving interpolant. Experimental observations of $p_{vr}$ being 0.10–0.25 constrain the value of $\lambda$ to the interval $0.02 \leq \lambda \leq 0.04$ nm$^{-1}$ indicated by blue and red dashed arrows. Inset: zoom of the leftmost part of the graph. (B) $p_{vr}$ constrains $r_{\text{mean}}$ between 69 nm and 115 nm. $r_{\text{mean}}$-values were obtained by averaging the positions of all primed vesicles over 10 simulations. (C1) EPSC amplitude versus coupling constant. (C2) The variance in EPSC amplitude versus coupling constant. Both quantities are positively correlated ($r = 0.61$, $p = 0.0196$). (D) The pdf plotted for the upper (red) and lower (blue) bound of the coupling parameter, as constrained by the experimental $p_{vr}$-values. Inset: typical examples of AP-evoked EPSCs resulting from both values of $\lambda$. 

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6.3 Discussion

In this study, we used a modeling approach to investigate the heterogeneity in spatial coupling between Ca\textsuperscript{2+} channel clusters and primed vesicles at the calyx of Held active zone. Although the importance of this positional heterogeneity has been noted before [210, 215], it has never been quantified in a continuous manner. Previous attempts to model its effects on vesicle release (in a number of preparations) were restricted to a limited number of (2–4) vesicle positions only (see e.g. [119,125–127]). In order to more faithfully model positional heterogeneity, we therefore randomly distributed the primed vesicles across the active zone according to a continuous probability density function. For this purpose we used a simple yet non-trivial pdf, characterised by a single channel-vesicle coupling strength parameter \( \lambda \) which describes the degree of positional heterogeneity. We investigated the effects of the resulting Ca\textsuperscript{2+}-dependent heterogeneity in release probability, in simulations of typical stimuli used in electrophysiological experiments. Using a range of typical release probability values from calyx of Held-literature, we constrained \( \lambda \). We suggested and simulated a number of different electrophysiological experiments which can be combined to increase the reliability of this estimate.

6.3.1 Coupling strength parameter captures both mean coupling distance and positional heterogeneity

We found that the vesicle distribution is strongly skewed towards CCCs, with the coupling strength parameter being in the interval \( 0.02 \leq \lambda \leq 0.04 \text{ nm}^{-1} \) (fig. 6.6D). For sake of comparison to the conventionally used measure of ‘mean coupling distance’ [77,214,216,217,224,227,228], we transformed the coupling parameter boundaries into an interval of \( r_{\text{mean}} \)-values (fig. 6.6B). It should be noted that this measure is crucially different from the coupling strength parameter \( \lambda \) in the way that a classical ‘mean coupling distance’ contains no spatial information about the heterogeneity. On the other hand, our distribution accounts both for the vesicle positions and the heterogeneity of vesicle placement with only a single parameter \( \lambda \).
The interval $p_{vr} = [0.10, 0.25]$ used in this study pertains to the young-to-slightly-matured calyx of Held [77,132,204]. In previous studies, the mean coupling distance was found to decrease during subsequent developmental stages from a ‘microdomain’ (>100 nm) to a ‘nanodomain’ (≤100 nm) coupling distance of ∼20 nm in mature calyceal synapses [77,216,227,228]. In agreement with these observations, we indeed find a mean coupling distance around the boundary of these regimes (69–115 nm; fig. 6.6B). Our pdf has $r_{mean} \to r_0 = 20$ nm as an asymptote for $\lambda \to \infty$ (suppl. fig. 6.1A): this implies that (nearly) all vesicles will be positioned at the same ∼20 nm distance from a CCC in mature calyces. The divergence towards infinity of $\lambda$ here depends on the choice of value of the minimal distance $r_0$, which we set equal to the size of a SNARE complex (20 nm) [218]: smaller $r_0$ would render $\lambda$ finite as $r_{mean} \to 20$ nm. Regardless of the exact value of $r_0$, our model predicts that, as the positional distribution of primed vesicles at the calyx of Held becomes increasingly more strongly-coupled in developing synapses, a larger fraction of the RRP is positioned at a similar distance from a CCC (close to $r_{mean}$). This renders the primed vesicle population increasingly more homogeneous in terms of coupling distance-dependent release probability.

6.3.2 Heterogeneity in $p_{vr}$ in recent calyx of Held models

Two recent studies incorporated a minimal distance between Ca$^{2+}$ source and vesicle similar to our parameter $r_0$, dubbed ‘exclusion zone’ in [208], and ‘perimeter coupling distance’ in [209]. The concept of an exclusion zone was shown in [208] to reduce the heterogeneity in $p_{vr}$ for release controlled by randomly distributed Ca$^{2+}$ channels or by sub-clusters of Ca$^{2+}$ channels. This finding was used to argue against a single CCC controlling release; such a setup would give rise to a large variation of $p_{vr}$-values across all primed vesicles. However, here we show that this heterogeneity can be controlled via the coupling parameter: as noted above, stronger coupling in our model results in a vesicle population more homogenous in terms of $p_{vr}$.

In [209], advanced EM techniques were used to estimate the number of Ca$^{2+}$ channels per CCC. The topographical arrangement of vesicles and CCC deemed most fitting to the experimental and modeling results found in that study corresponds to our setup: a cluster of Ca$^{2+}$ channels, with the vesicle(s) having the highest release probability being just outside this cluster. Where we used the distance between Ca$^{2+}$ source and vesicle to explain the heterogeneity in $p_{vr}$, [209] proposes a variable number of Ca$^{2+}$ channels per CCC and a fixed distance, amounting to the same result. Future research should investigate in which proportions these mechanisms contribute to the heterogeneity in $p_{vr}$.
6.3.3 Role of positional heterogeneity in short-term plasticity protocols

In this study, we simulate short electrophysiological stimuli that are predominantly governed by synchronous vesicle release from the RRP — importantly, kinetics of refill from upstream vesicle states or alternative Ca$^{2+}$ sensors play a negligible role. The main aspects governing the presented simulations of single AP-induced release are (1) ultrastructural properties of the calyx of Held; (2) characteristics of the Ca$^{2+}$ sensor for synchronous vesicle release; (3) Ca$^{2+}$- and Ca$^{2+}$ buffer-dynamics; and (4) heterogeneity in positioning of primed vesicles with respect to the Ca$^{2+}$ source. The first three have been subject of previous investigations (see e.g. [76, 132, 207, 215, 220, 229]), and we attempt to quantify the fourth aspect in a continuous manner in this study. Future work can use the present findings and modeling framework to study the role of positional heterogeneity in short-term plasticity (STP) protocols other than paired-pulse stimulation, such as high-frequency AP-stimulation (HFS).

Positional heterogeneity in the calyx of Held — or rather, its resulting heterogeneity in $p_{vr}$ — has previously been implicated in STP [127]: using a minimal number of 2 distinct vesicle positions (for readily and reluctantly releasable vesicles), each with an effective expression for $p_{vr}([Ca^{2+}])$, Trommershäuser and colleagues concluded heterogeneity to be required for supporting activity during steady-state synaptic depression. Our modeling framework can be extended to include their biophysically modeled refill of the RRP, while their expressions for $p_{vr}([Ca^{2+}])$ could be replaced by a combination of heterogeneity in vesicle positioning and explicitly modeled release sensors (see chapter 4) [37, 76]. Such an extension of the present modeling framework would allow for incorporation of the recent finding that primed vesicles at the calyx of Held undergo a process of maturation after having been recruited to the RRP [230].

Furthermore, the contribution of more distant vesicles to evoked release — in particular, to facilitated and asynchronous release during HFS — could be studied. In our model, vesicle populations with a higher degree of positional heterogeneity (i.e. looser coupling) have a larger number of distant primed vesicles (fig. 6.2A). Their reluctance to be released by individual APs is potentially mitigated by the build-up of residual Ca$^{2+}$ during prolonged HFS [64] — depending on the Ca$^{2+}$-dynamics of the system and the Ca$^{2+}$-binding kinetics of an alternative sensor — thus affecting asynchronous release and/or facilitation. This method of facilitation would indeed correlate with the preference for loose coupling in synapses which specialise in presynaptic plasticity and in which reliability and speed of transmission is of less importance [77, 224, 231, 232] — as opposed to, for instance, the calyx of Held with its fast and reliable synaptic transmission [210].

The combined modeling approach of randomly positioning individual primed vesicles according to a particular distribution function and spatiotemporal sim-
ulations of Ca$^{2+}$- and Ca$^{2+}$ buffer-dynamics as presented in this study, together with a vesicle release model, can be highly instrumental in generating hypotheses on distance-controlled heterogeneity in $p_{v}$: an issue which currently cannot be directly addressed with experimental techniques. This approach can be extended to preparations other than the calyx of Held, provided reliable morphological and physiological data (e.g. on Ca$^{2+}$ buffers) are available.

**Acknowledgments**

We thank Victor Matveev, who kindly implemented (upon request) an option for saving the entire three-dimensional [Ca$^{2+}$]-field as a function of time in binary format in CalC version 7.4, and released this together with a Matlab script for extracting the binary data.
6.4 Materials and methods

The calyx of Held release model used in this study was taken from Walter et al., unpublished. All simulations were performed in custom-written software routines in Matlab 7.10.0 or R2010a (Mathworks). First, based on the user-specified value of the coupling strength parameter, the locations of all 1400 release sites were sampled using the acceptance-rejection method [223]. After importing the spatio-temporal simulation of calcium diffusion and buffering generated in CalC into Matlab, the calcium concentration was interpolated at all release sites. Subsequently, the steady-state condition for the system was calculated, which in turn was used as input for generating the initial state for the stochastic simulation. These simulations were run using the so-called direct method formulation of Gillespie’s stochastic simulation algorithm (SSA) [219]. The nature of our simulations allowed for parallel execution of (in principle) individual active zones — we chose to simultaneously simulate 70 AZs and repeated this 20 times in a parallel manner, if the system allowed for this (depending on the computer system used, simulations ran on 1–12 CPUs simultaneously). The resulting vesicular release rate was then convolved with an average mEPSC to obtain the EPSC. Each of these steps is explained in detail in the appendix below.
6.5 Appendix

6.5.1 Derivation of probability density function with coupling parameter \( \lambda \)

Purely randomly distributing vesicles over a circular active zone of radius \( r_{\text{max}} \) with the \( \text{Ca}^{2+} \) channel cluster at the centre will render the cumulative density function (cdf) of finding a vesicle at distance \( r \) from the cluster to be proportional to the area of this circle. As the aim of this study is to investigate the effect of heterogeneity in distance between vesicles and a \( \text{Ca}^{2+} \) channel cluster on evoked vesicle release, we multiply this area term by an exponential distribution. This extra term enables us to control the average value \( r_{\text{mean}} \) of the distance via a parameter \( \lambda \), which can be interpreted as a coupling strength between vesicle and \( \text{Ca}^{2+} \) channel cluster:

\[
f_{\text{guess}}(r) = 2\pi(r - r_0) \cdot \lambda e^{-\lambda(r - r_0)}, \tag{6.3}
\]

which is defined for \( r_0 \leq r \leq r_{\text{max}} \). Here \( f_{\text{guess}} \) is the ansatz we choose as a probability density function (pdf), while \( r_0 \) and \( r_{\text{max}} \) are respectively the minimal and maximal value of \( r \). Integration by parts of this function yields an expression for the unnormalised cdf:

\[
F_{\text{unnorm}}(r) = \int_{r_0}^{r} f_{\text{guess}}(r')dr' \\
= \int_{r_0}^{r} 2\pi(r' - r_0) \cdot \lambda e^{-\lambda(r' - r_0)}dr' \\
\equiv \int_{r_0}^{r} u \cdot \frac{dv}{dr'}dr' \\
= u \cdot v - \int_{r_0}^{r} \frac{du}{dr'} \cdot vdr' \\
= 2\pi(r - r_0) \cdot e^{-\lambda(r - r_0)} - \int_{r_0}^{r} 2\pi \cdot e^{-\lambda(r' - r_0)}dr' \\
= 2\pi \cdot (-(r - r_0) \cdot e^{-\lambda(r - r_0)} - \frac{1}{\lambda} \cdot (e^{-\lambda(r - r_0)} - 1)) \\
= 2\pi \cdot (-e^{-\lambda(r - r_0)} \cdot (r - r_0 + \frac{1}{\lambda}) + \frac{1}{\lambda})
\]

The proper cdf is then found by normalising this expression to its value at \( r_0 = r_{\text{max}} \):
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\[ F(r) \equiv \frac{F_{\text{unnorm}}(r)}{F_{\text{unnorm}}(r = r_{\text{max}})} = \frac{1}{\lambda} - e^{-\lambda(r-r_0)} \cdot (r - r_0 + \frac{1}{\lambda}) \cdot \left( \frac{1}{\lambda} - e^{-\lambda(r_{\text{max}}-r_0)} \cdot (r_{\text{max}} - r_0 + \frac{1}{\lambda}) \right) \text{(defined for } r_0 \leq r \leq r_{\text{max}}), \]

so that \( F(r_{\text{max}}) = 1 \). The proper pdf (eqn. 6.1) follows by taking the derivative of this function with respect to the distance \( r \):

\[ f(r) \equiv \frac{\text{d}F(r)}{\text{d}r} = \frac{(r - r_0) \cdot \lambda e^{-\lambda(r-r_0)}}{\frac{1}{\lambda} - e^{-\lambda(r_{\text{max}}-r_0)} \cdot (r_{\text{max}} - r_0 + \frac{1}{\lambda})} \text{(defined for } r_0 \leq r \leq r_{\text{max}}) \]

The maximum value of this function (eqn. 6.2) can be seen to occur at

\[ \frac{\text{d}f(r)}{\text{d}r} = 0 \rightarrow r_{\text{peak}} = r_0 + \frac{1}{\lambda} \]

For very small values of the coupling parameter (\( \lambda \rightarrow 0 \)), the pdf should be determined purely by the area of the active zone, and thus feature a constant multiplying the quantity \((r - r_0)\), as in the above equation 6.3. Let \( \lambda \rightarrow 0 \) in such a way that \( \frac{1}{\lambda} \gg r_{\text{max}} \) (and \( \lambda(r_{\text{max}} - r_0) \ll 1 \):

\[ f(r) \approx \frac{(r - r_0) \cdot \lambda e^{-\lambda(r-r_0)}}{\frac{1}{\lambda} \cdot (1 - e^{-\lambda(r_{\text{max}}-r_0)})} = \frac{\lambda^2(r - r_0)e^{-\lambda(r-r_0)}}{1 - e^{-\lambda(r_{\text{max}}-r_0)}} \approx \frac{\lambda^2(r - r_0) - \lambda^3(r - r_0)^2}{\lambda(r_{\text{max}} - r_0)} \approx \frac{\lambda(r - r_0)}{r_{\text{max}} - r_0} \]

\[ = C \cdot (r - r_0) \]

where \( C \) is a constant. Indeed, for negligible coupling strength (\( \lambda \rightarrow 0 \)), the vesicle positioning depends only on the radius of the circular AZ. The median \( r \)-value \( m \) of the pdf is the one for which the integral \( F(r) \) of \( f(r) \) is equal to \( 1/2 \):

\[ F(r) = \frac{(r - r_0)^2}{(r_{\text{max}} - r_0)^2} = \frac{1}{2} \text{ for } r_0 \leq r \leq r_{\text{max}} \]

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The value of \( m \) in the absence of coupling approaches

\[
m = 289 \text{ nm (} \lambda \to 0) ,
\]

which can be appreciated by plotting the cdf derived from the pdf \( f(r) = C \cdot (r - r_0) \), with \( C \) an arbitrary constant, \( r_0 = 20 \text{nm} \) and \( r_{\text{max}} = 400 \text{ nm} \) (suppl. fig. 6.1B).

### 6.5.2 Using the acceptance-rejection sampling method for sampling vesicle locations

In order to sample random vesicle positions from the above pdf (equation 6.1), we follow the approach as given in [223]. The general idea is to randomly generate a candidate position on the interval \([r_0, r_{\text{max}}]\), and then to test if this vesicle position is acceptable (or likely), given the shape of the pdf at hand. For instance, a pdf corresponding to the strongly coupled situation is not expected to have a lot of vesicles lying far away, so vesicles close to \( r_0 \) are more likely to be accepted. Once the position of the first release site has been accepted, the algorithm moves on to the second one, and so on, until a position for all 1400 release sites has been sampled from the pdf \( f(r) \) (eqn. 6.1).

First, we generate a candidate random position \( r_{\text{rand}} \) by drawing a random number \( u_1 \) from the uniform distribution \( U(0,1) \) on \([0,1]\):

\[
r_{\text{rand}} = r_0 + u_1 \cdot (r_{\text{max}} - r_0)
\]

Suppose now that there is a function \( h(r) \) and a constant \( c \) such that

\[
c \cdot h(r) \geq f(r) \quad (\forall r \in [r_0, r_{\text{max}}])
\]

In other words, \( c \cdot h(r) \) ‘covers’ the pdf \( f(r) \) along the entire domain \([r_0, r_{\text{max}}]\). If we simply take \( h(r) \) equal to the uniform distribution, the value of \( c \) can be found by determining the maximal value \( f(r_{\text{peak}}) \) of the pdf, which occurs at \( r_{\text{peak}} = r_0 + 1/\lambda \) (eqn. 6.2). This way, the condition for acceptance of a randomly generated position \( r_{\text{rand}} \) becomes

\[
f(r_{\text{peak}}) \cdot u_2 \leq f(r_{\text{rand}}),
\]

where \( u_2 \) is another randomly drawn number from the uniform distribution \( U(0,1) \). In words, this means that we draw a vertical line at position \( r_{\text{rand}} \),
and we uniformly sample along this line, accepting the position $r_{\text{rand}}$ only if the value of $f(r_{\text{peak}}) \cdot u_2$ is equal to or less than the pdf-value $f(r_{\text{rand}})$ at this position. Vesicles at position $r_{\text{rand}} = r_{\text{peak}}$ are therefore always accepted, and positions close to $r_{\text{peak}}$ are very likely to be accepted.

Rejection of $r_{\text{rand}}$ means sampling a new $r_{\text{rand}}$ and a new $u_2$, and checking the above acceptance criterion again for these new values. Accepted positions are stored, and the algorithm keeps repeating the above procedure until all 1400 release sites are provided with a position — the distribution of which will resemble the continuous pdf (e.g. in the way the distribution in fig. 6.2C1 resembles the red curve in fig. 6.2A).

### 6.5.3 Modeling AP-evoked $[\text{Ca}^{2+}]$ transients

For spatio-temporal simulation of $\text{Ca}^{2+}$ diffusion and buffering, we used the ‘calcium calculator’ tool (CalC [220]) and assumed the diffusion of $\text{Ca}^{2+}$ and buffers not to be restricted by the presence of vesicles at the AZ. Meinrenken and colleagues concluded that the release of most vesicles at the AZ was likely governed by a cluster of $\sim 12 \text{ Ca}^{2+}$ channels, which together produce a $[\text{Ca}^{2+}]$ transient that is approximately Gaussian at distances larger than $\sim 30$ nm from the cluster centre [215]. Therefore, we modeled the $\text{Ca}^{2+}$-influx per CCC as a Gaussian wave coming from a point source. This Gaussian had an amplitude of 1.34 pA per AZ and a full-width-at-half-maximum (FWHM) of 0.46 ms, resulting in a charge influx of 0.64 fC per AP per AZ. The charge increase due to $\text{Ca}^{2+}$-influx per simulated AZ (with a volume $0.8 \times 0.8 \times 0.4 \, \mu\text{m} = 0.256 \, \mu\text{m}^3$) corresponds to an increase of unbuffered $\text{Ca}^{2+}$ by

$$\Delta[\text{Ca}^{2+}] = \left( \frac{1}{2} \times \frac{0.64 \cdot 10^{-15} \, \text{C}}{9.65 \cdot 10^4 \, \text{C/mol}} \right) \cdot \frac{1}{0.256 \cdot 10^{-15} \, \text{L}} \approx 13 \, \mu\text{M}$$

Taking the action of buffers into account (see below), this results in a volume-average of 408 nM free $\text{Ca}^{2+}$. The size of the simulation volume ($0.256 \, \mu\text{m}^3$) was chosen such that the increase in volume-averaged $[\text{Ca}^{2+}]$ due to a single AP corresponds closely to the experimentally observed value of 379 nM [229]. This approach indirectly takes into account the $\text{Ca}^{2+}$ contributed by channels outside the AZ, under the assumption that these do not significantly alter the $[\text{Ca}^{2+}]$ transients [215].

We used a calcium diffusion coefficient $D_{\text{Ca}} = 220 \, \mu\text{m}^2/\text{s}$, while setting the resting $\text{Ca}^{2+}$ concentration $[\text{Ca}^{2+}]_{\text{basal}}$ to 50 nM and assuming a linear extrusion mechanism, which reduces the surplus of $\text{Ca}^{2+}$ via $\gamma \cdot ([\text{Ca}^{2+}] - [\text{Ca}^{2+}]_{\text{basal}})$ [215]. Furthermore, two $\text{Ca}^{2+}$ buffers were included: an endogenous fixed buffer (EFB) and ATP as an endogenous mobile buffer. For some simulations, varying concen-
trations of the mobile exogenous buffer BAPTA were added [215]. All simulation parameters can be found in the table below.

When CalC encounters a numerical instability during the calculations, the differential equation solver jumps back in time to re-calculate the solution, but does not overwrite the previously saved data (CalC v7.4). This results in duplicates in the simulations, which have to be removed when the data is imported into Matlab. For the purposes of this chapter, we instructed CalC to save the concentration field every simulated 20 µs, on a 51 × 51 × 51 node grid spanning the simulation volume. For simplicity, the Ca²⁺ sensor for each vesicle was assumed to be positioned at 20 nm (approximately half the diameter of a vesicle) above the plasma membrane. Subsequently, [Ca²⁺] was interpolated at each of the randomly generated locations \( r_{\text{rand}} \) (see above) — which can be identified with points along the x-axis (\( y = 0 \) nm) because of rotational symmetry of the system — at a height \( z = 20 \) nm, yielding the Ca²⁺ concentration over time for one release site. This process is used to create all the [Ca²⁺] transients for the 1400 release sites, as well as one for all the vesicles in the upstream (non-releasable) state (which are assumed to be at a fixed distance of 200 nm; Walter et al., unpublished), and the result is used to drive the stochastic model.

6.5.4 Stochastic simulation algorithm (SSA)

Many vesicle release models employ systems of coupled ordinary differential equations and real-valued variables in order to describe the system as a continuously and deterministically evolving one. However, synaptic transmission is known to be an inherently stochastic process (characterised e.g. by a ‘release probability’). Furthermore, the discreteness of the system (which can be approximated using real-valued variables when the number of all reactants is very large) becomes important when considering the fact that the number of molecules and vesicles involved is not many orders of magnitude larger than one. Therefore, we built our vesicle release model using the so-called direct method formulation of Gillespie’s stochastic simulation algorithm (SSA) [219].

The SSA makes use of propensity functions \( a_j(\vec{x}) \), which depend on the current state vector of the system \( \vec{x} \) and on some reaction constant \( c_j \), with \( j \) the index of the reaction channel. The quantity \( a_j(\vec{x})dt \), with \( dt \) an infinitesimal time interval, is equal to the probability that reaction \( j \) will occur within the time interval \([t,t+dt]\). For a unimolecular reaction of some substance, the propensity function is \( a_j(\vec{x}) = c_jx_j \), where \( x_j \) is the number of molecules of that substance in the system, while for bimolecular interactions of two different substances, \( a_j(\vec{x}) = c_jx_jx_k \). Unimolecular reactions are independent of reaction volume, whereas bimolecular interactions are inversely proportional to reaction volume. The latter is due to the fact that the reactants will interact with each other sooner in a smaller reaction volume (for a thorough explanation and references,
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see [219]). This is an important point, as we have two bimolecular reactions in our system (Walter et al., unpublished), and we want to reduce the reaction volume by ‘cutting up’ the system into 1400 separate active zones with 1 release site and (70000/1400 =) 50 vesicles each.

Following Gillespie’s description of the direct method version of the SSA, we generate a \((23 \times 1)\)-matrix \(A\) containing all propensities \(a_j(\vec{x})\) for each active zone \((1 \leq j \leq 23, \vec{x} \text{ consists of 12 states})\). The propensities of the bimolecular reactions are multiplied by 1400, to balance the effect of reducing the system size. After initialising the system at \(t_0\) in state \(\vec{x}_0\), \(A\) is computed at each time \(t\), which is used as input for a Monte Carlo procedure for determining the time to the next reaction \(\tau\) and the reaction channel \(j\) [219]. This reaction is then executed by evolving the system from time \(t\) to \(t + \tau\) and the state vector from \(\vec{x}\) to \(\vec{x} + \delta_j\), where \(\delta\) is a \((23 \times 12)\) stoichiometry matrix. Each row \(\delta_j \equiv (\delta_{1j}, \ldots, \delta_{12j})\) in this matrix contains a state change vector (consisting of values +1, 0, -1) which specifies for each reaction channel \(j\) which reactants (-1) are converted into which products (+1). To be precise, in practice we use the Kronecker product of the identity matrix with this stoichiometry matrix to create a \((1610 \times 840)\) stoichiometry matrix for the 70 AZs that are simulated simultaneously. This adapted stoichiometry matrix is used as input for the aforementioned Monte Carlo procedure in each of the parallel threads (see Materials and Methods). This procedure is continued until some stopping criterion has been met.

6.5.5  Generating an initial state for stochastic simulations

In order to simulate the system in a stochastic manner, an integer number of vesicles and release sites are needed. To keep simulations computationally tractable, the behaviour of the system as a whole was simulated as the sum of 1400 independent subsystems, containing 1 release site and 50 vesicles each. A simple round off to integer values of the steady-state sizes of all states in the model and subsequent proportional distribution over the subsystems would introduce a shift towards a non-steady-state distribution. In that case, one would require a certain period of ‘pre-equilibration’ before the start of an actual simulation to allow all states to stochastically approach their ‘steady state’ values, which would strongly increase the duration of a simulation (by an unpredictable amount of time).

Therefore we used a different approach, based on the continuous situation: after solving the (continuous) steady-state distribution of the model, a cumulative distribution of all vesicles states is constructed (i.e. excluding the release sites). Subsequently, a value \(x\) from the uniform distribution \(U(0,1)\) is drawn, for each of the 70000 vesicles. For each \(x\) that, according to the constructed cumulative distribution for vesicles, lies in the interval corresponding to a release site-occupying vesicle state, a site is marked as occupied. For instance,
if the interval $[0.9819, 0.9995]$ corresponds to the first primed state $R_{0\text{Ca}}$ (in the continuous situation, about 1.76% of all vesicles in the system are in this state), then a sampled $x$ lying within this interval will result in a release site being occupied by a vesicle in state $R_{0\text{Ca}}$. The same goes for the second primed state $R_{1\text{Ca}}$ with an interval $[0.9995, 0.9997]$ (or 0.015% of all vesicles), and so on. Typically, this results in $\sim 87$–93% of the release sites being occupied with the default parameter settings, nicely including the deterministic value of 89%. The remaining vesicles are divided among the non-primed states D, NR, and F according to their sampled value $x$ so that each subsystem contains 50 vesicles. State D, containing the bulk of all the vesicles, corresponds to the interval $[0, 0.9629]$ of the cumulative distribution. The validity of this approach can be appreciated by creating a histogram of the number of vesicles (or sites) per state after a large number of samples have been obtained. The central limit theorem states that the result should be normally distributed with a mean value approaching the steady state value of the continuous case for a large number of samples — which is indeed what we observe (suppl. fig. 6.2).
6.5.6 System parameters

The parameters used to generate the $[\text{Ca}^{2+}]$ transients in CalC, which are subsequently imported into Matlab to drive the vesicle release model, are as follows:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Unit</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Width</td>
<td>$800 \cdot 10^{-9}$</td>
<td>m</td>
<td>Dimensions of the simulated volume</td>
</tr>
<tr>
<td>Length</td>
<td>$800 \cdot 10^{-9}$</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>$400 \cdot 10^{-9}$</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_{\text{basal}}$</td>
<td>$50 \cdot 10^{-9}$</td>
<td>M</td>
<td>$[\text{Ca}^{2+}]$ under resting conditions [229]</td>
</tr>
<tr>
<td>$D_{\text{Ca}}$</td>
<td>220</td>
<td>$\mu\text{m}^2/\text{s}$</td>
<td>$\text{Ca}^{2+}$ diffusion coefficient [233]</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>400</td>
<td>1/s</td>
<td>Rate of linear $\text{Ca}^{2+}$ extrusion [215]</td>
</tr>
</tbody>
</table>

| $[\text{EFB}]_{\text{total}}$ | $5 \cdot 10^{-6}$ | M         | Endogenous fixed buffer concentration             |
| $k_{\text{on, EFB}}$         | 0.58 $\cdot 10^{-3}$ | M         | $[\text{ATP}]_{\text{total}}$ ATP concentration (mobile buffer) |
| $K_{\text{D, EFB}}$          | $2 \cdot 10^{-6}$ | M         | $\text{Ca}^{2+}$ dissociation constant of EFB     |
| $[\text{ATP}]_{\text{total}}$| Variable        | M         | ATP concentration (mobile buffer)                 |
| $k_{\text{on, ATP}}$         | $2 \cdot 10^{-9}$ | M         | $\text{Ca}^{2+}$ dissociation constant of ATP     |
| $K_{\text{D, ATP}}$          | $220 \cdot 10^{-9}$ | M         | Ca$^{2+}$ dissociation constant of BAPTA           |
| $D_{\text{BAPTA}}$           | 220            | $\mu\text{m}^2/\text{s}$ | BAPTA diffusion coefficient                       |
| $r_0$                  | $20 \cdot 10^{-9}$ | m         | See figure 6.1A                                   |
| $r_{\text{max}}$         | $400 \cdot 10^{-9}$ | m         | See figure 6.1A                                   |
| $z_{\text{sensor}}$       | $20 \cdot 10^{-9}$ | m         | Location (z-axis) of Ca$^{2+}$ sensor              |
| $r_{\text{NRP}}$          | $200 \cdot 10^{-9}$ | m         | Distance from CCC to NRP-vesicles                 |

To turn the vesicular release rates into an EPSC, the following mEPSC properties were used:

<table>
<thead>
<tr>
<th>mEPSC used for convolution</th>
<th>Value</th>
<th>Unit</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>$15 \cdot 10^{-12}$</td>
<td>A</td>
<td>In presence of CTZ and $\gamma$-DGG [222]</td>
</tr>
<tr>
<td>Rise time constant</td>
<td>$2 \cdot 10^{-6}$</td>
<td>s</td>
<td>From [221]</td>
</tr>
<tr>
<td>Decay time constant</td>
<td>$3 \cdot 10^{-3}$</td>
<td>s</td>
<td>From [221]</td>
</tr>
</tbody>
</table>
Supplemental figure 6.1: The coupling constant $\lambda$ strongly affects the vesicles’ positions. (A) The relationship between mean distance and coupling constant. The grey dashed line indicates the asymptote $r_{\text{mean}} = r_0$. (B) In the absence of channel-vesicle coupling ($\lambda \rightarrow 0 \, \text{nm}^{-1}$), the pdf becomes a linear function of the distance to $\text{Ca}^{2+}$ channels, so that the cdf is quadratic. The median $r$-value of the pdf is the one for which the cdf is equal to 0.5. In our setup, $r_{\text{median}} \sim 289 \, \text{nm}$.

Supplemental figure 6.2: The initial model states for stochastic simulations are sampled properly. To populate the model states in a stochastic simulation, we sample the initial state from a cumulative distribution based on the deterministic steady state. These histograms contain the initial populations of states $D$, $R_{0\text{Ca}}$ and Sites, resulting after taking 10000 samples. The red bar and number indicate the location and value of the mean $\mu$ of the sampled distribution. These mean values are identical or extremely close to the deterministic steady state values ($\mu_{D,SS} = 67400$, $\mu_{R_{0\text{Ca}},SS} = 1232$, $\mu_{\text{Sites},SS} = 154.5$; Walter et al., unpublished) and the distributions are normal (Kolmogorov-Smirnov test), illustrating the validity of our approach.