## Kinetic models for synaptic vesicle release

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CHAPTER 7

# General discussion

The general aim of this thesis was to study the role of the energy barrier for synaptic vesicle fusion in the regulation of synaptic efficacy, using experimental approaches and kinetic modeling, and to investigate the contribution of various presynaptic proteins to this process. Furthermore, we modeled the effect of positional heterogeneity of primed vesicles at the active zone on AP-induced release.

In **chapter 2** we tested whether synaptic vesicle fusion is controlled in a supralinear way by changes in the activation energy for vesicle fusion, as predicted by the Arrhenius equation. We presented a novel method for simultaneously fitting RRP size and release rate constant from hypertonic sucrose (HS) responses — which revealed the predicted supralinearity for application of PDBu and removal of Complexin. Furthermore, we used the Arrhenius equation to supply the allosteric model for synchronous vesicle release [76] with a new theoretical interpretation. In **chapter 3** we studied the role of the fusion energy barrier in Synaptotagmin-1 (Syt-1)-dependent clamping of spontaneous release. We found an increased activation energy (and a smaller RRP size) in Syt-1-deficient synapses, prompting us to conclude that Syt-1 does not directly inhibit the fusion reaction itself, but probably reduces spontaneous release by inhibition of some other $Ca^{2+}$ sensor(s) for synaptic vesicle fusion. In **chapter 4** we used the concept of fusion rate modulation through changes in the activation energy for vesicle fusion to construct a two-sensor model as an extension of the allosteric model for $Ca^{2+}$-evoked release, in order to capture the $Ca^{2+}$-sensitivity of various Syt-mutants in the calyx of Held. The transient effect of high-frequency stimulation (HFS) on the fusion energy barrier was quantified in **chapter 5**, where the correlation between the potentiation of both release willingness and vesicular release probability ($p_{vr}$) during PTP was studied, in WT and Syt-1-deficient neurons. We reported both release willingness and $p_{vr}$ to be increased after PTP, in a Syt-1-independent manner, but found no direct correlation between the potentiation of both quantities. Finally, in **chapter 6** we used experimental data to constrain and quantify the degree of heterogeneity in vesicle positioning at the calyx of Held active zone, and studied its effect on AP-induced release.

## 7.1 Fast modulation of synaptic efficacy via the fusion energy barrier

### 7.1.1 Supralinear control of synaptic vesicle fusion

According to our findings [187], the fusion reaction rate can be changed in an exponential manner via an additive effect on the fusion energy barrier by various

presynaptic proteins, creating potentially a large dynamic range for regulation of release. This is a powerful way to regulate synaptic efficacy compared to, for instance, the $Ca^{2+}$-dependent increase of the RRP refill rate during prolonged HFS [47, 48], which affects the release rate linearly.

We showed that such a supralinear control mechanism is actually employed by the synapse in the cases of PDBu/DAG (presumably via the C1 domain of Munc13 [87]), Complexin (Cpx) and $Ca^{2+}$ (via the allosteric model of Syt [76], see discussion below) [187]. We therefore argue that other presynaptic factors that are known to affect the fusion energy barrier (or the release willingness) such as cAMP, Syntaxin, SNAP-25, the C2B domain of Munc13, the metabotropic $GABA_B$ receptor and Munc18 [45,84,110,112,150,161,187,234] most likely also modulate fusion in a supralinear manner, although this remains to be investigated. The prediction is that if these factors affect the fusion energy barrier independently, as we showed for PDBu and sucrose [187], they will amplify each other's effects on fusion in an supralinear manner. This might explain how application of PDBu or genetic perturbation of Syntaxin or Cpx can change the $Ca^{2+}$-sensitivity of vesicle release, without direct interaction with the $Ca^{2+}$ sensor for synchronous release, Syt [76, 163] (see also discussion below). Likewise, opposite effects on the fusion energy barrier might cancel each other out, suggesting for instance that application of PDBu or a mild HS-stimulus (e.g. 0.1M) might rescue the Cpx TKO phenotype [109], turning facilitation into depression during HFS.

### 7.1.2 Contribution of fusion energy barrier manipulation to STP

As a higher fusion rate will result in a larger fraction of the RRP being released per AP, it seems plausible that a lowered fusion energy barrier corresponds with an increased $p_{vr}$, which is defined by the RRP fraction that is released during a single AP stimulation. We indeed found both to co-occur during PTP (chapter 5), but they did not correlate significantly. The HFS-induced reduction of the fusion energy barrier was concluded to be Syt-1-independent, since we found a similar reduction in Syt-1 KO cells. A candidate effector for this activity-dependent energy barrier reduction might be Munc13, which was implicated in STP and found to affect the $Ca^{2+}$-sensitivity of release [44,45,87]. Activation of the Munc13 C1 domain has been shown to lower the fusion energy barrier [87], while $Ca^{2+}$-bound calmodulin (CaM) binding to Munc13 potentiates the refilling rate of the RRP [44], and $Ca^{2+}$ binding to the Munc13 C2B domain regulates STP — potentially by lowering the fusion energy barrier [45]. Together, these adjacent signaling motifs have been proposed to let Munc13 function as a 'computational unit for synaptic transmission' [45]. Whether or not this computation

(release modulation) occurs via the fusion energy barrier could be explored by studying the amount of PTP and co-occurring change of the fusion energy barrier in Munc13 DKO neurons expressing particular Munc13 mutants (e.g. the $Ca^{2+}$-insensitive Munc13 DN mutant in [45]).

Priming, on the contrary, does not play a major role during PTP in hippocampal synapses in our hands: the average RRP recovery (measured by 0.5M HS) was only 70% 5 seconds after HFS, as opposed to a 21% increase of the release rate constant and a 32% potentiation of $p_{vr}$ (chapter 5). These findings corroborate the results of a previous study in excitatory hippocampal autapses, where the impact of priming during PTP was found to be negligible, while $p_{vr}$ was potentiated and the fusion energy barrier suggested to be lowered (inferred from the onset delay of HS responses) in a residual $Ca^{2+}$-dependent manner [55, 56]. In contrast, recent studies in the calyx of Held reported that the main factor determining PTP is the enhanced size of the RRP after HFS, and found this parameter to be controlled by $Ca^{2+}$-dependent isoforms of protein kinase C (PKC$\alpha/\beta$) [204, 205]. Possible explanations for these contradictory findings are that the hippocampal synapse and the calyx of Held employ different mechanisms for PTP, or that the experimental results were interpreted differently. Compared to HS-stimulation, back-extrapolation of the cumulative release resulting from HFS is known to underestimate the RRP, due to a subset of primed vesicles with low $p_{vr}$ that are reluctant to fuse during HFS but not during HS-stimulation [64] (see chapter 1). A temporary increase of the release willingness of these 'reluctant' vesicles by activation of PKC$\alpha/\beta$ after HFS could explain the increase in release during the second train stimulus in WT neurons [204].

### 7.1.3 Interpretation of hypertonic sucrose responses in terms of priming and release willingness

Priming and release willingness are most likely influenced by the same molecular processes, such as SNARE complex formation and modulation [144]. Therefore, to identify the exact role of presynaptic proteins in these processes it is important to be able to discriminate between molecular effects on priming and release willingness. As described in chapter 2, our method can make this distinction by fitting synaptic responses to maximal and submaximal HS concentrations.

In some cases, the use of a single concentration of HS to assess RRP size has led to confusing results in different preparations. For instance, a recent study in continental cultures concluded Cpx to be essential for vesicle priming, based on a reduced amount of release found during stimulation with 0.5M HS after knock-down (KD) of Cpx in these neurons [163]. On the contrary, in autaptic cultures deletion of Cpx was reported not to affect RRP size, but to change the

vesicles' release willingness instead [109]. These differences can be reconciled in view of the relation between release rate constant and depleted RRP fraction discussed in chapter 2, which shows that the fraction of RRP depleted by a HS-stimulus depends on the release rate reached during stimulation, and that only beyond a certain release rate the complete RRP is depleted [187]. A 0.5M HS-stimulus appears to release vesicles with a considerably slower rate (time-to-peak ∼3s [163]) in continental cultures than in autapses (time-to-peak <1s [109]), implying that it increases the release willingness to a lesser extent in continental cultures compared to autapses. This is probably caused by a smaller sucrose concentration gradient due to a slower application system (pico-spritzer) used in these studies or a less confined dendritic tree compared to autapses. Therefore, the partial RRP depletion after 0.5M HS in continental cultures of Cpx KD neurons (interpreted by the authors as a priming defect), could be well explained by a reduction of release willingness upon Cpx deletion (as in autapses), which cannot be increased sufficiently by the weaker 0.5M HS in continental cultures to achieve complete depletion of the RRP.

In another example, 0.5M HS was also unable to completely deplete the RRP in autapses with reduced levels of the SNARE protein Syntaxin1 [144] — generated by endogenous hypomorphic expression of the isoform Syntaxin-1B combined with RNAi-mediated KD — most likely due to the severe effect of this manipulation on release willingness [110, 187]. Potentiating the release willingness by application of HS concentrations higher than 0.5M led to a depletion of the full RRP in this study [144].

## 7.2 Regulation of fusion by Synaptotagmin

### 7.2.1 A new interpretation of the allosteric model for synaptic vesicle release

We have shown in chapter 2 that the allosteric model of Lou et al. [76] for the $Ca^{2+}$-sensitivity of (synchronous) vesicle fusion can be well explained in terms of additive effects of the $Ca^{2+}$ sensor on the fusion activation energy when binding $Ca^{2+}$ ions [187]. This interpretation of the model by Lou et al. yields several predictions. It suggests that the factor f — describing the increase of the release rate constant upon binding of one $Ca^{2+}$ ion — is a property of the $Ca^{2+}$ sensor (and its interaction with the plasma membrane), while this is not necessarily the case for the release willingness parameter $l_+$, which might be modulated by factors independent of the $Ca^{2+}$ sensor. Indeed, $l_+$ (which is supralinearly affected by PDBu [76, 187]) could still be potentiated by PDBu application in Syt-1 KO neurons (chapter 3). This implies that the apparent $Ca^{2+}$-sensitivity of release could be changed by independent factors affecting

the fusion energy barrier via $l_+$, which has been suggested for PDBu, cAMP and SNAP-25 [76, 112, 234], and which could also hold true for Syntaxin [110]. Cleavage of SNAP-25 by neurotoxin BonT/A leads to a strong reduction in $Ca^{2+}$-sensitivity of release, whereas an increased mEPSC frequency and $Ca^{2+}$-sensitivity are observed in both PDBu-stimulated neurons as well as in neurons expressing only the 'open' conformation of Syntaxin1B. Furthermore, this new interpretation of the allosteric model provides an explanation for the functional cooperation of Syt-1 and Cpx [163]: by acting on the fusion energy barrier via $l_+$, Cpx is capable of controlling AP-evoked and spontaneous release (removal of Cpx reduces both forms of exocytosis), in addition to HS-induced release [109, 187].

Based on our observation that removal of Syt-1 does not lower the fusion energy barrier, we concluded that Syt-1 itself does not represent this barrier (chapter 3). Hence, the factor f most likely represents an active lowering of the fusion energy barrier, instead of a passive lowering by a (slight) dislodgment of Syt-1 from a fusion-preventing position in the release machinery [176]. Therefore, neurons expressing mutated copies of Syt-1 mimicking $Ca^{2+}$-binding or increasing the $Ca^{2+}$ affinity should result in a lower fusion energy barrier under resting conditions, leading to an increased mEPSC frequency. A higher frequency of spontaneous release was indeed observed in Syt-1 mutants with a higher $Ca^{2+}$ affinity [140].

### 7.2.2 Clamping release: who competes for what?

Multiple studies have suggested that Syt-1 clamps spontaneous and asynchronous release by suppressing an alternative $Ca^{2+}$ sensor [37, 147, 163]. Competition for a common substrate essential for release could be a mechanism by which clamping occurs [147, 176]: if a large fraction of this substrate is 'occupied' by Syt-1, the release kinetics of the asynchronous sensor will (appear to) be altered. One candidate substrate would be the SNARE complex, to which the C2 domains of $Ca^{2+}$ sensors bind in a $Ca^{2+}$-dependent manner [176]. As the 'zippering up' of SNARE complexes drives fusion [29], capturing a large share of these complexes is a potent way to outcompete other $Ca^{2+}$ sensors [176]. Alternatively, Syt-1 could clamp spontaneous and asynchronous release by occupying the majority of PIP2 molecules, a lipid occurring in enriched patches in the plasma membrane [176]. The abundance of PIP2 strongly affects the $Ca^{2+}$-sensitivity of Syt-1 [235], as well as the activity of other C2 domain-containing proteins, such as CAPS and Munc13 [45, 236]. Such a scenario is compatible with our model presented in chapter 4, since multiple $Ca^{2+}$ sensors can act simultaneously, but with altered $Ca^{2+}$-binding kinetics (depending on the amount of PIP2 'captured' by a sensor). Apart from competition for PIP2 binding, another potential cause of an apparent change in $Ca^{2+}$-binding kinetics could be the shielding of the asynchronous sensor by Syt-1 from $Ca^{2+}$ ions in the synaptic terminal — either

by physically blocking its C2 domain(s), or by capturing a substantial fraction of the inflowing $Ca^{2+}$ ions before they reach the other sensor. As the increase in volume-averaged $Ca^{2+}$ concentration per AP-stimulus equals a few tens of free $Ca^{2+}$ ions per synaptic terminal (see [16, 229] and chapter 6), the latter might have a significant impact.

## 7.3 Towards a generic model for synaptic vesicle release

It is the consensus that all synapses in the central nervous system share the same molecular mechanisms and that different synaptic phenotypes are due to variation in the synaptic parameters between synapses. In this thesis we proposed a general principle by which synapses can efficiently modulate their efficacy via effects on the fusion energy barrier for fusion. In the following section we discuss several other issues that need to be addressed in order to arrive at a generic computational model for synaptic vesicle release.

### 7.3.1 Implications of additivity in the energy barrier domain: many proteins, one fusion pathway?

A number of studies report that mutants of certain presynaptic proteins affecting release willingness — and (thus) the fusion energy barrier — have a profound impact on vesicle release kinetics, both in resting neurons as well as during STP [87, 109, 110]. Based on our findings, we hypothesised that molecular changes involving various proteins (including the SNARE proteins, Cpx, Munc13, Munc18 and the asynchronous sensor), changes in their number or stoichiometry and their association with or dissociation of additional factors may all independently lower the fusion energy barrier [187]. An important corollary of having additive effects by these proteins on the fusion energy barrier is that they are expected to exert their release-modulating functions independently on the same release pathway. This notion opposes the release models proposed in [124,133,135], which postulate separate fusion pathways (for synchronous and asynchronous release), for a single vesicle state in the model. Future studies will need to clarify which factors determine the energetic state of a vesicle and to what extent each of these factors contributes to modulation of the fusion energy barrier height.

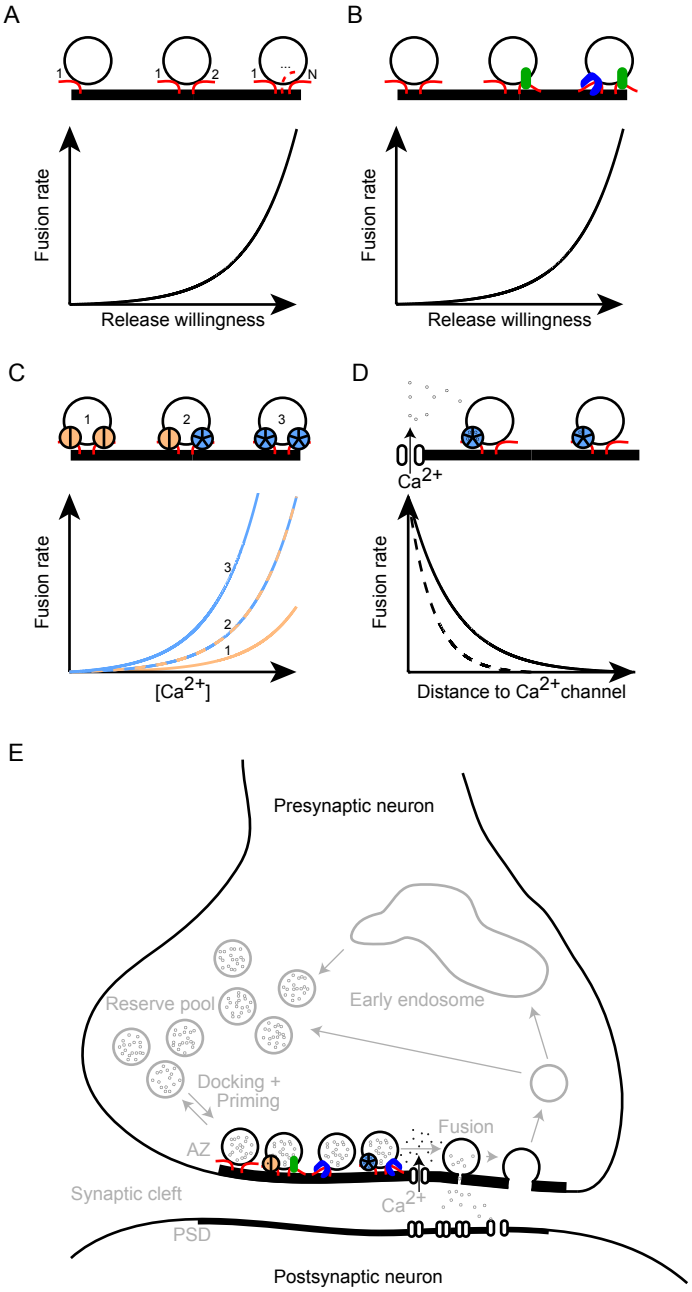
### 7.3.2 Release machinery-intrinsic heterogeneity within the RRP

Throughout this thesis, we assumed all primed vesicles to be equally affected by biochemical and genetic manipulations. A real RRP is most likely far from homogeneous, due to the plenitude of presynaptic factors and $Ca^{2+}$ sensors interacting with the release machinery (e.g. SNARE proteins, Cpx, Munc13 and Munc18; fig. 7.1B). Combined with the number of SNARE complexes per vesicle (fig. 7.1A), this will cause heterogeneity in release willingness. Also, a combinatorial model of n (fast or slow) $Ca^{2+}$ sensors acting on (the variable number of) N SNAREs provides a single vesicle with a potentially large number of $n^N$ fusion options [176] (chapter 4), leading to further deviations from our assumed homogeneity (fig. 7.1C).

### 7.3.3 Modeling processes upstream of fusion

During synaptic responses to brief stimuli (single AP and $Ca^{2+}$ uncaging) processes upstream of fusion play a minor role. When fitting prolonged HS stimuli, we assumed a constant priming rate [187]. However, more realistic models will require a description of activity-dependent vesicle replenishment. A number of studies on various assay systems have successfully modeled this in different ways [119–121, 123, 126–130] (see chapter 1); generally as a $[Ca^{2+}]_{global}$-dependent parameter drawing on an infinite reserve pool (upstream of the RRP and the fusion event) or boosting the recovery of refractory release sites (downstream) — the relative importance of both options is still a matter of debate [51]. A generic model could adapt these approaches to include a general (spatiotemporal) $[Ca^{2+}]$-dependence as opposed to a $[Ca^{2+}]_{global}$-dependent parameter and a more explicit description of the relevant presynaptic factors in-

**Figure 7.1** ***(facing page)*****:** *Various release-modulating components combine their individual contributions to affect the fusion rate in a complex manner.* (A) Heterogeneity in release willingness between primed vesicles, with higher release willingness values resulting in a higher fusion rate. The heterogeneity might be caused by the number (1…N) and degree of assembly of SNARE complexes (red; chapter 4). (B) Presynaptic proteins modulating the fusion machinery (green and blue), such as Complexin, Munc13 and Munc18, also affect release willingness (chapter 2). (C) The number, type and distribution of $Ca^{2+}$ sensors associated with the fusion machinery will affect the intrinsic $Ca^{2+}$-sensitivity of primed vesicles. (D) The further away a vesicle, the lower its fusion rate, due to the effects of $Ca^{2+}$ diffusion (solid line) and $Ca^{2+}$ buffering (dashed line). (E) Cartoon of the synaptic vesicle cycle (adapted from fig. 1.1), with an arbitrary combination of the components (A-D) inserted at their location of operation.


A
1
1
2
1
N
Fusion rate
Release willingness
B
Fusion rate
Release willingness
C
1
2
3
Fusion rate
3
2
1
[Ca2+]
D
Ca2+
Fusion rate
Distance to Ca2+channel
E
Presynaptic neuron
Reserve pool
Early endosome
Docking +
Priming
Fusion
AZ
Synaptic cleft
Ca2+
PSD
Postsynaptic neuron

volved (e.g. the binding of $Ca^{2+}$ to CaM, and the binding of $Ca^{2+}$-bound CaM to Munc13 [44, 49]).

### 7.3.4 From a 1D to a (3+1)D model

For the better part of this thesis, we applied and modeled homogeneous stimuli ($Ca^{2+}$ uncaging and HS) — with the exception of chapter 6 (AP). A generic model should in addition be able to describe the (high-frequency) AP-induced $[Ca^{2+}]$ transients in the synaptic terminal, which are spatially heterogeneous due to $Ca^{2+}$ buffering and diffusion and affect vesicles differently when positioned at different distances from the $Ca^{2+}$ source. The number of $Ca^{2+}$ channels per $Ca^{2+}$ channel cluster, the positioning/coupling of vesicles with respect to these clusters, and the effects of these parameters on STP have been subject of recent study in a number of assay systems [208, 209, 224, 232, 237]. Loose coupling was found and proposed as a framework for STP in hippocampal synapses [232], while tight coupling — possibly combined with a variable number of $Ca^{2+}$ channels per cluster [209] — was observed in the calyx of Held [208] (chapter 6). To change our 1D (time-dependent) models into a (3+1)D (time- and space-dependent) model of $[Ca^{2+}]$ and buffer dynamics, simulation packages such as CalC or MCell [220, 238, 239] could be used, combined with a model for vesicle positioning (see chapter 6 and e.g. [208, 209, 224, 232]; fig. 7.1D). Both CalC and MCell are publicly available tools to implement $Ca^{2+}$-influx, diffusion, buffering and binding to receptors. Where CalC produces a deterministic output using continuous partial differential equations, MCell uses Monte Carlo methods to incorporate stochasticity.

Other aspects pertinent to more realistic spatiotemporal $[Ca^{2+}]$ modeling could include the use of detailed ultrastructural knowledge about the complex geometry of the synaptic terminal (see e.g. [124, 208, 209]) and stochastic simulations of all presynaptic players involved in vesicle release (including individual $Ca^{2+}$ channels and $Ca^{2+}$ ions, see e.g. [16,124,240,241]), which could be achieved by using for instance the MCell or STEPS simulation packages [238, 239, 242]. Both MCell and STEPS allow for simulation of stochastic reaction-diffusion dynamics in complex 3D geometries.

### 7.3.5 Modeling short-term synaptic plasticity

Since STP is predominantly determined by the the complex interplay of all the aforementioned presynaptic mechanisms, their relative importance in shaping synaptic computation could be disentangled via simulations, using a model containing spatiotemporal $[Ca^{2+}]$ and buffer kinetics, $Ca^{2+}$-dependent control of

RRP-replenishment and release, activity-dependent modulation of release (willingness) and ultrastructural knowledge about the distribution of $Ca^{2+}$ channels and their location with respect to the $Ca^{2+}$ sensors [208, 209, 211, 237]. Such a generic model of the presynapse should be applicable to all CNS synapses when tuning the model parameters to correct values for each subtype. This will allow for studying a number of as yet poorly understood vesicular release phenomena (see e.g. [116, 125, 149, 203, 243]) and will be essential in understanding the role of presynaptic genes in healthy and cognitively dysfunctional brains.