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Molecular mechanisms governing Ca^{2+} regulation of evoked and spontaneous release

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Abstract

The relationship between transmitter release evoked by action potentials and spontaneous release has fascinated neuroscientists for half a century, and separate biological roles for spontaneous release are emerging. Nevertheless, separate functions for spontaneous and Ca^{2+} - evoked release do not necessarily indicate different origins of these two manifestations of vesicular fusion. Here we review how Ca^{2+} regulates evoked and spontaneous release, emphasizing that Ca^{2+} can briefly increase vesicle fusion rates one- million fold above spontaneous rates. This high dynamic range suggests that docked and readily releasable pool (RRP) vesicles might be protected against spontaneous release while also being immediately available for ultrafast Ca^{2+} - evoked release. Molecular mechanisms for such release clamping of highly fusogenic RRP vesicles are increasingly investigated. Thus, we view spontaneous release as a consequence of the highly release competent state of a standing pool of RRP vesicles, which is molecularly fine-tuned to control spontaneous release.

Introduction

Neurotransmitter release at synapses is the main mechanism for fast information transfer between neurons. Release happens primarily when an action potential (AP) enters the nerve terminal and opens voltage-gated Ca^{2+} channels. Following AP activity, transmitter release can occur in either a fast phase, or in a slower phase [1, 2]. The latter, called asynchronous release, becomes more prominent upon the build-up of presynaptic residual Ca^{2+} following repetitive AP activity [3-8] (see ref. 9 for a recent review). In addition, release can also happen spontaneously in the absence of a presynaptic AP, creating so-called miniature excitatory (or inhibitory) synaptic currents, as originally discovered at the neuromuscular junction [10]. There is emerging evidence that spontaneous release can have functions for the maintenance of synapses and for dendritic protein translation [11-15]. Using optical measurements, evidence for

a separate origin of spontaneous release has been obtained in cultured CNS synapses [16, 17]. Nevertheless, other studies reached the conclusion that a common pool of vesicles drives spontaneous and evoked release [18-20].

In the present review, we contrast mechanisms of Ca^{2+} - evoked and spontaneous release at mammalian brain synapses. We will argue that, for a mechanistic understanding of spontaneous release, one needs to consider the properties of a standing pool of readily-releasable vesicles (RRP), the properties of which might explain many features of spontaneous release. We will first review studies of the Ca^{2+} requirements of vesicle fusion over a wide range of intracellular Ca^{2+} concentrations, relevant for fast, asynchronous and spontaneous release. We then review optical studies which have addressed whether spontaneous release is caused by vesicles of the same, or of a different pool [21]. Finally, we will discuss studies using genetic manipulation of presynaptic proteins, many of which have concomitant roles in the regulation of both spontaneous and evoked release. We conclude that a sizeable fraction of spontaneous release is a consequence of the standing pool of RRP vesicles, which must be made available for ultrafast Ca^{2+} - driven release, and is in addition fine-tuned to allow controllable rates of spontaneous release.

The Ca^{2+} regulation of vesicle fusion

To understand the Ca^{2+} regulation of transmitter release, it is imperative to consider how vesicles are prepared for fast, AP-driven membrane fusion. Classical studies at neuromuscular preparations [22], and later studies at CNS synapses [23, 24] and at secretory cells [25], have established that AP-evoked release draws from a functionally defined RRP. Electron microscopy shows that presynaptic active zones of glutamatergic synapses in the CNS typically have 3 - 10 docked vesicles [26-30], most of which could belong to the RRP [27, 30-32]

(Fig. 1a). The existence of a "standing" pool of RRP vesicles is a prerequisite for speed and synchronicity of release, since vesicles are arrested in a highly fusogenic state, and only require a Ca^{2+} rise to be released synchronously, but with quite low release probability of ~ 0.1 [33, 34].

The calyx of Held is a large glutamatergic excitatory synapse at which direct presynaptic (and postsynaptic) patch-clamp recordings can be made [35, 36]. Although this presynaptic nerve terminal harbors hundreds of active zones (~ 500 - 600; ref. 37; Fig. 1b), the ultrastructure [29, 30, 37] and the function of individual active zones [38] is thought to be overall similar to that of small excitatory synapses. The accessibility of the calyx terminal has allowed presynaptic voltage-clamp [39-41] and the application of Ca^{2+} uncaging techniques to a presynaptic nerve terminal [33, 42]; see also ref. 43, 44 for previous reviews). Ca^{2+} uncaging stimuli at the calyx synapse release a pool of vesicles that tightly corresponds to the RRP which can be released by depolarizations [33, 42, 45, 46]. These studies have revealed the following:

• Ca^{2+} steps in the range of 1 - 8 µM induce release highly supra-linearly, and at higher intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the slope was shallower due to the beginning saturation of the Ca^{2+} sensor (Fig. 1c) [33, 42]. Double-logarithmic dose-response curves between release rates and $[Ca^{2+}]_i$ step amplitudes showed maximal slope value of ~ 4, similar to the classical estimate obtained by varying the extracellular $[Ca^{2+}]$ at the neuromuscular junction [47]. This number is a lower bound estimate of the number of Ca^{2+} ions controlling vesicle fusion. Indeed, a kinetic model with five Ca^{2+} binding sites was necessary to describe the observed high slope values [33, 42]. The Ca^{2+} sensor models predicted that during an AP, the "local" $[Ca^{2+}]_i$ signal at an average RRP vesicles transiently rises to 10 - 25 µM amplitude [33, 42], or up to 50 µM in mice [48] and rats [49] to cause fast, AP-driven release.

• Studies investigating the Ca^{2+} regulation of release below a micromolar, using weak flashes and buffered Ca^{2+} infusions into the calyx nerve terminal, found lower slope values (Fig. 1c, open symbols; ref. 50). At Ca^{2+} concentrations approaching the resting Ca^{2+} of ~ 50 nM, the measured release became gradually independent of Ca^{2+} (ref. 8, 50 - 52).

• Genetic deletion of Synaptotagmin-2 (Syt2), a C2 domain Ca^{2+} binding protein homologous to Syt1 found at forebrain synapses [53], led to the disappearance of the steeply Ca^{2+} - dependent part of release [8, 51, 52] . Thus it is clear that Syt2 is an essential part of the highly-supralinear Ca^{2+} sensor at the calyx of Held synapse.

Viewed together, release as investigated at this model synapse occurs in three regimes: > 1 µM $[Ca^{2+}]_i$, a high-cooperativity Ca^{2+} sensor such as Syt2 triggers release. Below 1 µM, a slow yet molecularly unidentified sensor pre-dominates, and both sensors seem to act on the same pool of RRP vesicles [8, 51]. Finally, at resting $[Ca^{2+}]_i$ and below, release is spontaneous, and largely Ca^{2+} - independent.

Since spontaneous release, release triggered with low Ca^{2+} cooperativity, and increasingly fast release with high Ca^{2+} cooperativity lie on a continuous dose-response curve, a simple interpretation is that all three manifestations of vesicular release are supported by RRP vesicles. Two kinetic models have been developed to explain the Ca^{2+} sensitivity of vesicle fusion over a wide range of Ca^{2+} (Fig. 1c), the "allosteric model" [50], and the "2 Ca^{2+} sensor model" [8, 51]. Both models have implemented low rates of fusion from non- Ca^{2+} - occupied states of the vesicle - Ca^{2+} sensor complex; they therefore both assume that spontaneous release is driven by RRP vesicles.

A high dynamic range of Ca^{2+} - evoked over spontaneous release

The Ca^{2+} dose-response curves of transmitter release (Fig. 1c) also illustrate the large dynamic range between spontaneous release, and the release rates reached transiently during an AP. Considering the number of active zones at a calyx (~ 500 - 600; ref. 37, 38), a spontaneous release frequency of 1 Hz corresponds to an average release rate of ~ 0.002 Hz per active zone; equaling one release event every 10 minutes (note however that spontaneous release can be heterogeneous between active zones [54-57] as discussed below). On the other hand, with Ca^{2+} steps to 30 - 50 µM the entire RRP is released with submillisecond kinetics with rates of > 2000 ves / ms [42, 46, 49]. These values are 6 orders of magnitude higher than the spontaneous release rate (Fig. 1c). One key feature enabling the high dynamic range is clearly the high-cooperativity Ca^{2+} sensor, which translates rises in $[Ca^{2+}]_i$ above ~ 0.5 µM highly-nonlinearly into release. Intermediate states of the dose-response curves might be visited physiologically in a few cases. For example, during repetitive AP activity, residual $[Ca^{2+}]_i$ can build up to a few hundred nanomolar and cause asynchronous late release [3-9]. Also, presynaptic G-protein coupled receptors can decrease the open probability of presynaptic Ca^{2+} channels [40], thus decreasing local $[Ca^{2+}]_i$ and transmitter release probability. The high dynamic range of evoked over spontaneous release also reflects the extreme synchronization of fast release during an AP -- at this speed, the release machinery cannot maintain high rates for times longer than a few millisecond because of the onset of RRP depletion [33, 46].

In cultured hippocampal synapses, spontaneous- and Ca^{2+} -evoked release has also been quantified in more detail. Ca^{2+} uncaging in the axons of autaptic cultures also showed a steep slope between release rate and $[Ca^{2+}]_i$ [58]. While AP-evoked release has a roughly similar release probability of ~ 0.1 at the calyx [33] and at hippocampal synapses [34], spontaneous

release seems higher at hippocampal synapses than at the calyx. Using optical methods, an average rate of spontaneous events of 0.01 Hz was found per synapse [54]. This estimate agrees with electrophysiological measurements from hippocampal synapses in autaptic cultures (0.01 Hz per active zone [59, 60]). Although direct comparison between the two preparations should be taken with caution, the values indicate that spontaneous release rate per active zone could be ~ 5 -times higher at excitatory hippocampal synapses than at the calyx.

Distinguishing between Ca^{2+} - dependent and Ca^{2+} - independent spontaneous release

Spontaneous release has been classically defined as release occurring in the absence of AP activity, and is therefore usually measured in the presence of Na^{+} channel blockers like tetrodotoxin. Nevertheless, the absence of APs does not necessarily imply the absence of Ca^{2+} signals. Indeed, earlier studies have shown that at some synapses, minis can depend on intracellular Ca^{2+} stores [61, 62]. More recently, the spontaneous opening of voltage-gated Ca^{2+} channels, especially of R-type channels, was shown to contribute a significant fraction of spontaneous release in excitatory hippocampal synapses (ref. 63; but see ref. 64 who did not find a role for Ca^{2+} channels at excitatory cortical synapses). A contribution of spontaneous Ca^{2+} channel openings to minis has also been described for inhibitory synapses [65, 66]. Therefore, it is important to subdivide "spontaneous release" into two categories:

• Ca^{2+}-independent spontaneous release, which occurs in the absence of a Ca^{2+} stimulus for release; thus, at a constant resting $[Ca^{2+}]$ in the terminal (30 - 50 nM; ref. 67). The studies at the calyx synapse have shown that at resting $[Ca^{2+}]$, the chance to find all Ca^{2+} ion binding sites of the high Ca^{2+} cooperativity sensor fully occupied, is vanishingly low [33, 42, 50]. Furthermore, infusing 10 mM BAPTA into the wild-type calyx did not reduce spontaneous release rates [52]. Therefore, spontaneous release at the calyx represents a basal form of

spontaneous release independent of Ca^{2+}, and is likely caused by spontaneous energy fluctuations leading to the overcoming of the energy barrier for vesicle fusion.

• Ca^{2+} - driven spontaneous release, which is caused, for example, by spontaneous opening of voltage-gated Ca^{2+} channels [63, 65, 66] or store-operated Ca^{2+} - release [61, 62]. In this case, local $[Ca^{2+}]_i$ transients around a micromolar are likely reached at some vesicles [63], which might lead to the activation of the slow release sensor [52], or of Synaptotagmin - like Ca^{2+} sensors [68]. From the viewpoint of the release machinery, the two forms of spontaneous release are quite different -- one represents truly "spontaneous" release, whereas the other is caused by Ca^{2+} activation of the vesicle fusion machinery, albeit in the absence of AP stimulation.

Two of the recent studies have modelled how spontaneous Ca^{2+} channel openings cause spontaneous release [63, 66]. Both studies assumed that vesicles that support spontaneous release have intracellular Ca^{2+} sensitivities as measured at the calyx synapse (Fig. 1c; ref. 50, 51). One of the studies found that in excitatory hippocampal synapses where several Ca^{2+} channels drive fast fusion during an AP ("domain overlap"), the spontaneous opening of a Ca^{2+} channel drives release with a small probability ($p \sim 0.01$ for R-type channels, or one release event for every hundredth opening [63]). This at first hand counterintuitive result is expected for a domain overlap geometry, since in this arrangement only the pooling of multiple Ca^{2+} channel microdomains (~ 5 - 15) is capable to cause release efficiently during an AP [69-71]. Therefore, spontaneous openings of single Ca^{2+} channels, will produce much smaller local $[Ca^{2+}]_i$ signals (~ 5 - 15-fold smaller), allowing only a vanishingly small spontaneous fusion probability because of the highly non-linear relation between release and $[Ca^{2+}]_i$. The finding that Ca^{2+} - dependent spontaneous release can be quantitatively explained by assuming Ca^{2+} sensitivities of release as found at the calyx synapse [63, 66], is consistent with the view that Ca^{2+}-driven

spontaneous release is carried by RRP vesicles. Ca^{2+}- dependent spontaneous release might also explain part of the higher spontaneous rates observed at hippocampal synapses as compared to the calyx (see above).

Origin of spontaneous release from a separate vesicle pool?

Our considerations so far suggest that Ca^{2+} - dependent and Ca^{2+} - independent spontaneous release are simply carried by RRP vesicles. Nevertheless, many studies in the past decade have found evidence for a separate origin of spontaneous and evoked release. This idea was first inspired by observations of SNARE protein mutations in mice and drosophila, which affected evoked release more strongly than spontaneous release [72-75]. For example, deletion of the canonical v-SNARE Synaptobrevin-2 (Syb2) in mice reduced spontaneous release and release evoked by hypertonic sucrose application to about 10% of control, whereas AP-evoked release was affected more strongly, with only ~1% of control remaining [73]. Such observations are often taken as evidence in favor of a separate origin of those minis that remain in the absence of Syb2 (~ 10%). However, an alternative explanation is that the remaining release-competent vesicles have a disturbed Ca^{2+} sensitivity or a disturbed coupling to Ca^{2+} channels, which would explain the stronger effects of Syb2 deletion on Ca^{2+} - evoked release. Indeed, Syb2 cleavage by Tetanus-toxin affects fast release evoked by depolarization more strongly than Ca^{2+} uncaging-evoked release, which suggested that Syb2 cleavage leads to a mis-localization of RRP vesicles with respect to Ca^{2+} channels [76].

A second complication for the interpretation of the Syb2 KO data is possible redundancy with other paralogs like Synaptobrevin-1 (Syb1). Syb1 is expressed in a subpopulation of hippocampal neurons [77], and might be the main isoform at mature hindbrain synapses[78 79]. Syb1 can rescue the transmitter release deficits found in Syb2 KO mice, and genetic

inactivation of Syb1 leads to a severe loss of the hypertonicity-evoked release that remains in the Syb2 KO [77] -- thus, Syb1 might compensate for part of the Syb2 loss. On the other hand, recent work on Vti1a, a non-canonical SNARE protein, is in favor of the interpretation of the Syb2 KO data. In cultured hippocampal synapses, shRNA - mediated knockdown (KD) of Vti1a decreased spontaneous release by about two-thirds; KD of Vti1a also further suppressed the reduced mini-frequency remaining in Syb2 KO mice [80]. Another study showed that the glycoprotein reelin increases spontaneous release without affecting evoked release; this modulation persisted in Syb2 KO neurons but was abolished by KD of the alternative vesicular SNARE, VAMP7 [81]. These studies suggest selective roles of Vti1a in spontaneous release, and of VAMP7 in the modulation of spontaneous release by reelin [81], and should be further validated following genetic removal of the proteins.

Several studies have used optical approaches of vesicle recycling in cultured hippocampal synapses, to further investigate whether spontaneous release originates from the same, or a separate pool of vesicles [21]. Styryl dyes like FM1-43, FM2-10 and others are taken up during an exocytosis - endocytosis cycle [82, 83], and staining of vesicle pools with FM dyes is usually obtained by repeated electrical stimulation. However, the dyes are also taken up in the presence of TTX presumably caused by spontaneous release. An early study with cultured cortical neurons and FM1-43 staining found that within a population of synapses with high spontaneous activity, spontaneous- and evoked release probability were correlated [84]. A study using cultured hippocampal neurons and FM2-10 found that fluorescence loaded after spontaneous release was de-stained less efficiently by subsequent AP stimulation, and vice-versa, which suggested that there was some separation between vesicle pools for spontaneous and Ca^{2+} - evoked release [16, 85]. Nevertheless, subsequent studies using similar approaches, or expanded two-color styryl dye measurements reached the opposite conclusion [18, 20]. Studies

with a more recent optical tool for vesicle dye uptake, biosyn (based on biotinylated Syb2; ref. 17), have also produced divergent views favoring a separate [17], or common origin [19] of vesicles for spontaneous and for Ca^{2+} evoked release. Therefore, experiments from staining vesicle pools with styryl dyes and similar imaging approaches have produced controversial interpretations. Differences in the optical analysis methods and background subtraction routines [18], as well as different styryl dyes and their employed concentrations [20, 21] are discussed as reasons for the discrepancies.

Molecular mechanisms for a high dynamic range

A large body of work exists on the molecular mechanisms of SNARE-mediated vesicle fusion, which has been addressed at various model systems ranging from yeast to mouse (for reviews, see refs. [86-90]). Together, these studies have identified proteins involved in vesicle docking and priming (SNARE proteins themselves, and Munc13), as well as Ca^{2+} sensors (Synaptotagmins) and other SNARE-protein binding proteins like Complexins which are involved in defining the highly fusogenic state of RRP vesicles (Fig. 2a). We will now review studies which showed that proteins involved in regulating the final steps of vesicle fusion often concomitantly regulate spontaneous release.

Proteins which increase both spontaneous and evoked release. Several studies have used genetic - and other molecular manipulations at the calyx synapse to investigate the function of presynaptic proteins for transmitter release over a wide range of $[Ca^{2+}]_i$ (Fig. 2b). It had been known that phorbol esters, which substitute the phospholipase-C product diacyl- glycerol (DAG), increase spontaneous and evoked transmitter release at synapses [91, 92]. This effect is mediated, in part, by binding of DAG to Munc13-1 [93, 94], as well as by activation of protein kinase-C and subsequent phosphorylation of Munc18-1 [95, 96]. Ca^{2+} uncaging studies at the

calyx showed that phorbol esters increase the efficiency of Ca^{2+} steps in inducing release, without a change in the pool size [50, 97], nor a major increase in presynaptic Ca^{2+} current, which showed that phorbol esters directly regulate the vesicle fusion willingness, or its Ca^{2+} sensitivity [50, 97, 98]. The potentiation of spontaneous release was continuous with the potentiation of release evoked by Ca^{2+} uncaging, such that the dose-response curve had an offset at low Ca^{2+} and became more shallow in the steep range after phorbol ester application (Fig. 2b, red line [50]). These findings could be accounted for by changing a single rate constant of fusion willingness in the allosteric model [50]; these studies also showed that RRP vesicles can be a substrate for second messenger regulation [94]. However, it also became apparent that there was a certain segregation of the two molecular pathways since Munc13-1 seemed more relevant for the potentiation of spontaneous release [94], whereas PKC phosphorylation of Munc18-1 was more relevant for AP - evoked release [96].

The conclusion that phorbol esters increase the fusogenicity, or fusion willingness of RRP vesicles was also derived in a study of hippocampal synapses. In this preparation vesicle fusogenicity can be directly assessed through the use of hypertonic release stimulation, which triggers the release of the entire RRP in the absence of Ca^{2+} [23]. Activation of Munc13-1 by phorbol ester did not affect pool size, but led to a potentiation of vesicle fusion willingness), which in turn increased spontaneous and Ca^{2+} triggered release in a similar fashion [99]. These experiments again support the idea that spontaneous and Ca^{2+} - evoked release are different manifestations of a common underlying pool of RRP vesicles.

The SNARE proteins constitute the central machinery of membrane fusion, and the primed state probably corresponds to a partially assembled SNARE complex, arrested in a meta-stable state [90]. The t-SNARE Syntaxin exists in two conformations -- closed and open -- and

must be brought to the open state before SNARE complex assembly can proceed [100]. A mutation forcing Syntaxin1B into its open state was engineered into mice, which resulted in increased release probability but fewer RRP vesicles at hippocampal synapses [101]. The mutation increased the amount of SNARE complex formation [102], and the speed of Ca^{2+} evoked release was increased. Measurements of the intracellular Ca^{2+} dose-response curve of release at the calyx synapse showed a gradual shift towards the left together with an increased mini frequency (Fig. 2b, dashed red line [102]), reminiscent but not identical to the phorbol ester effects. In another study, the levels of Syntaxin expression were experimentally manipulated by various methods, and this changed the rates of both evoked- and spontaneous release in a manner which correlated with the expression level of Syntaxin [60]. Taken together, second messenger modulation of Munc13 and Munc18 by DAG and phosphorylation, and an increased rate of SNARE protein complex formation by manipulating Syntaxin all have positive effects on both spontaneous - and evoked release.

Proteins with opposite roles for spontaneous and evoked release. The two synaptic proteins Synaptotagmin and Complexin enable synchronous and efficient Ca^{2+} triggered release [34, 103], but in addition play an important role in regulating spontaneous release. Deletion of Syt1/2 leads to an enhanced spontaneous release in nearly all preparations and species studied [52, 68, 104-107], and this effect has been interpreted as a "clamping" function of Syt1/2 under unstimulated conditions [52, 104]. The clamping role for Syt1 was not found in single neuron autaptic cultures [103, 108]; the difference with mass cultures or brain slices might be caused by a non-cell autonomous effect arising from the presence of both glutamatergic and GABAergic neurons in the same neuronal micronetwork [109-111].

For Complexin, a role in clamping of spontaneous release is clearly documented in invertebrates [14, 112-114]. In mammalian neurons, however, loss of Complexin leads to *reduced* spontaneous release along with a reduction of vesicle fusogenicity in autaptic neurons [115]; both forms of release were also reduced in auditory brain slices [116]. In hippocampal mass cultures, KD of Complexin led to an upregulation of spontaneous transmitter release, in support of a clamping hypothesis of Complexin [117, 118]. A recent study showed that the increased spontaneous release following Complexin deletion in this preparation is in part mediated by a compensatory increase in Complexin 3 expression [119]. Taken together, the Complexin phenotypes between the invertebrate- and mammalian synapses show some differences regarding the penetrance of the unclamping phenotype. This may reflect distinct functional roles of spontaneous and evoked release at invertebrate neuromuscular junctions and mammalian central synapses.

Studies of Ca^{2+} dependent release over a wide range of [Ca^{2+}] at the calyx of Held have clearly demonstrated the role of Synaptotagmin1/2 as the main Ca^{2+} sensor for the steeply Ca^{2+} dependent part of release [51, 52]. The calyx synapse as well as other hindbrain neurons express Synaptotagmin-2 (Syt2) [107], a close homologue of Synaptotagmin (Syt1) which is not detectable by immunohistochemistry at the calyx [51]. In Syt2 KO mice, release lost its steeply Ca^{2+} dependent component, and only a shallow Ca^{2+} dependent release, with slope values of 2 (ref. 51), or of 1 in more recent studies [8, 52] remained (Fig. 2b, blue line). The remaining release in Syt2 KO mice must be mediated by a secondary Ca^{2+} sensor with low Ca^{2+} cooperativity, which was not yet identified at the calyx synapse. Evidence for Doc2 proteins (ref. 120, 121; but see ref. 122) and Synaptotagmin-7 (ref. 123, 124) as slow Ca^{2+} sensors have been obtained at other synapses. Interestingly, release below ~ 1 µM [Ca^{2+}] was stronger in the Syt2 KO mice as compared to control mice (see Fig. 2b; ref. 52), indicating that loss of

Syt2 unmasked the action of a secondary Ca^{2+} sensor. Genetic deletion of Syt2 at the calyx also leads to a strongly increased mEPSC frequency (~ 20 - 40 fold [52, 107]); however, the spontaneous release in Syt2 KO mice was sensitive to BAPTA (unlike spontaneous release at the wild-type calyx[52]; see above), and thus likely depends on the coupling between the slow sensor and an unknown intracellular Ca^{2+} release pathway [52]. Thus, part of the unclamped spontaneous release might represent an additional gain of function effect observed in the Syt2 KO mice (see also ref. 68 for unclamping effects of the Syt1 KO in cultured cortical synapses). Therefore, the clamping deficits apparent in Syt1/2 mutant synapses are not necessarily equal to a clamping role found in *in-vitro* fusion assays, where adding Syt1 reduced Ca^{2+} *independent* SNARE mediated membrane fusion rates [125]. At synapses, the clamping function of Syt1/2 depended in part on an intact poly-basic site of the C2B domain [52, 126], a site which is implicated in the SNARE binding properties of Syt1 (ref. 127). More work into the mechanisms of the clamping functions is needed, but it is interesting to note that independent clamping and release-promoting roles of Synaptotagmins and Complexins should allow these proteins to contribute to a high dynamic range of synapses.

Heterogeneous contributions of individual synapses to spontaneous and evoked release

Two recent studies have developed quantal resolution optical imaging at the drosophila neuromuscular synapses, to investigate whether neighboring active zones at a giant synapse contribute equally, or differentially to spontaneous and evoked release [56, 57]. Spontaneous release frequency was not correlated with evoked release probability [56, 57]; in one study, subsets of active zones at the extreme ends of the spectrum supported spontaneous - or Ca^{2+} evoked release almost selectively [57], at least within the limited observation period of the experiments. These findings indicate that individual active zones have highly variable contributions to spontaneous and evoked release, possibly calling for separate pools [56, 57].

Nevertheless, it should be considered that some important presynaptic factors support spontaneous, and Ca^{2+} evoked release in opposing ways, without the necessity to postulate separate vesicle pools (Synaptotagmins and Complexins; see above). Thus, one could assume that active zones mainly involved in spontaneous release might combine, first, a low effective Ca^{2+} sensitivity of release; second, an unclamped release apparatus (thus, low copy numbers of Syt1/2 - and/or Complexin proteins), and third, a low number of Ca^{2+} channels, or spatially not-well coupled channels. On the other hand, active zones which mainly support Ca^{2+} evoked release likely have a high dynamic range, possibly mediated by high numbers of proteins like Syt1/2, Complexin, and Bruchpilot [128]; the latter protein was found to have a clamping role at the drosophila synapse [57], in addition a to its role in active zone assembly and localization of presynaptic Ca^{2+} channels [128]. Additional factors, like the number of docked vesicles, modulation of the spontaneous fusion willingness by DAG (see above) and other second messengers [129], and the type of Ca^{2+} channels and their spontaneous opening rate might further increase the variability in the contribution of spontaneous versus evoked release across individual active zones. Nevertheless, the large degree of heterogeneity revealed by quantal resolution Ca^{2+} imaging is intriguing [56, 57], and merits future studies to address the molecular rationale, and the functional meaning of active zones which differentially support spontaneous and evoked release.

Conclusion

We have reviewed evidence for a large dynamic range between Ca^{2+} - evoked release, and spontaneous release, as well as studies of the molecular basis of vesicle fusion conducted at various CNS synapses. These studies showed that Ca^{2+} evoked - and spontaneous release are often concomitantly regulated, in either opposing or equal directions, when perturbing proteins controlling the final steps of vesicle fusion. The simplest explanation of the Ca^{2+} -

dose -response curves of transmitter release obtained at the calyx synapse is that Ca^{2+} evoked - and spontaneous release is carried by RRP vesicles, but with strikingly different rates [50, 51]. Other studies showed that low rates of background release can be caused by the spontaneous, stochastic opening of Ca^{2+} channels [63, 64, 66], and these results are again consistent with the view that RRP vesicles can carry spontaneous release. Thus, spontaneous release from a standing pool of RRP vesicles is not a leak or an accident of nature [130, 131], but it is highly regulated by various presynaptic proteins, by second messengers, and by local Ca^{2+} sources. Indeed, in our view it seems contradictory to equip synapses with a high density of docked vesicles, without making use of this reservoir for low rates of biologically relevant [11-15] spontaneous release. Thus, independent functions for spontaneous release might be subserved by a finely regulated spontaneous fusion of RRP vesicles, without the necessity to postulate a separate origin of vesicles underlying spontaneous release.

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Figure legends

Figure 1: Ca^{2+} sensitivity of transmitter release over a wide range of $[Ca^{2+}]_i$ measured at a large CNS synapse.

a Scheme of the readily-releasable pool (RRP) of vesicles which is thought to underlie Ca^{2+} - evoked release at synapses.

b Schematic representation of the calyx of Held synapse as a model for investigating presynaptic function.

c The Ca^{2+} - dose response curve of release as measured at the calyx of Held synapse (reproduced, with permission, from ref. 50). Note the double-logarithmic scales, and the range of a steep slope (~ 3 - 4) between ~ 1 and 8 μM $[Ca^{2+}]_i$. An about one-millionfold dynamic range is apparent between release evoked by relevant local $[Ca^{2+}]_i$ (20 - 50 μM; corresponding release rate ~ 1000 ves./ms), and spontaneous release at the resting $[Ca^{2+}]_i$ (~ 0.5 Hz).

Figure 2. Molecular mechanisms of a high dynamic range of transmitter release

a, Scheme of a docked, and readily - releasable vesicle with partially assembled SNARE complex, and associated proteins.

b, The Ca^{2+} sensitivity of release over a wide range of intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, for different molecular perturbations at the calyx of Held synapse. Black curve, control data in wild-type synapses [50, 51]; red curve, after phorbol ester treatment in wild-type synapses [50]; blue curve, in Syt2 KO mice [51, 52]; red dashed line, in a Syntaxin1B "open form" knock-in (KI) mouse [102]. Note that in all cases, molecular perturbation of the release machinery led to concomitant changes in the lowermost part of the dose - response curve indicating changes in spontaneous release rate, as well as changes of Ca^{2+} - evoked release at high $[Ca^{2+}]_i$.

c, Scheme of the energy diagram of a primed vesicle in the RRP before fusion. An energy barrier separating the primed vesicle from the fused state is normally lowered upon Ca^{2+} binding to the sensor, but the energy barrier can also be overcome spontaneously with a low rate.

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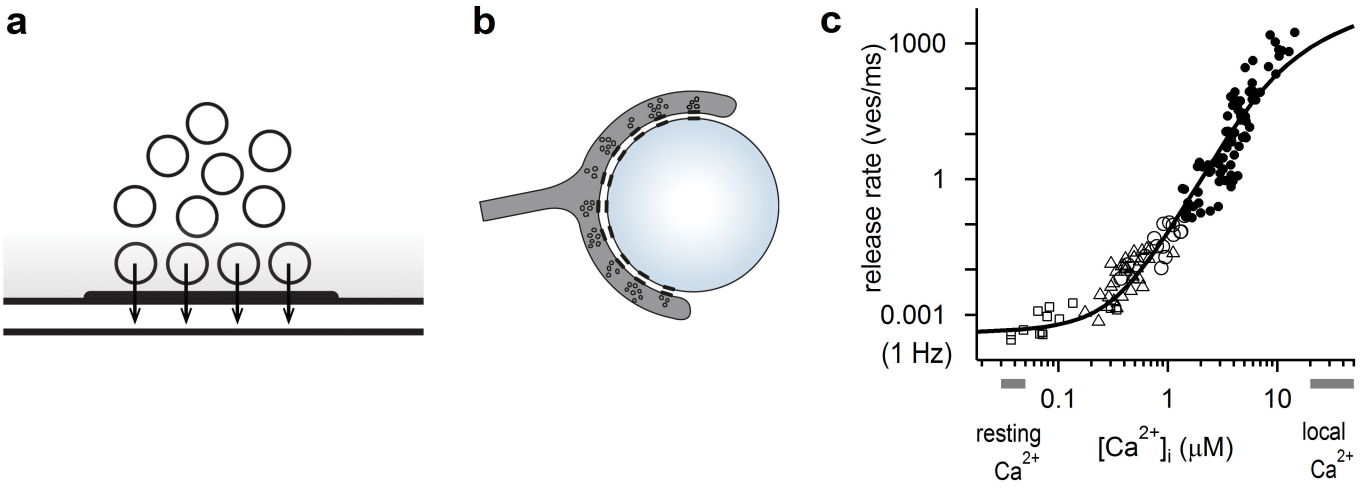

Figure 1

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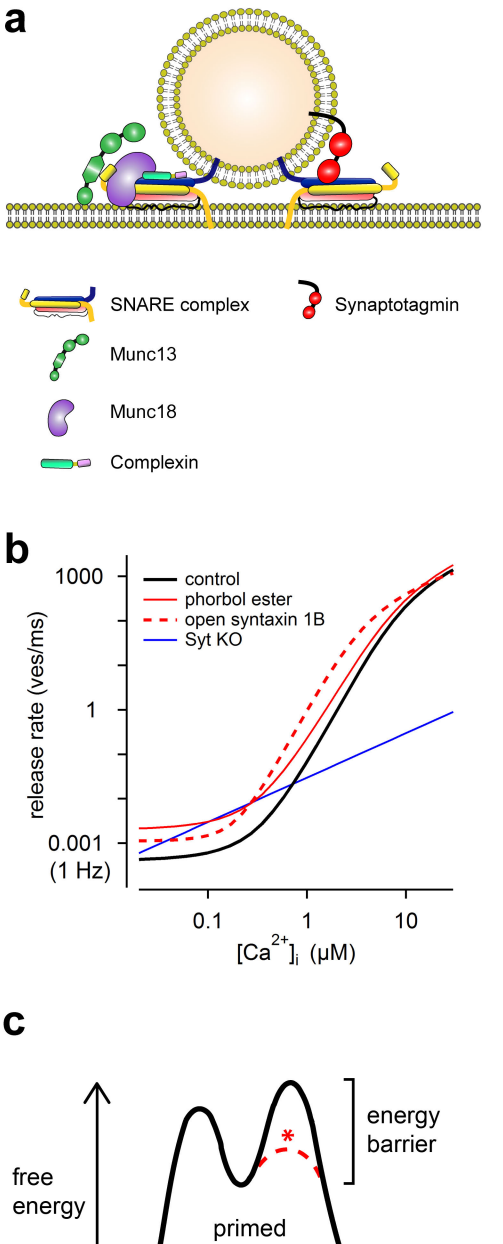

Figure 2