New Insights Into Molecular Players Involved in Neurotransmitter Release

The strength of a synapse can profoundly influence network function. How this strength is set at the molecular level is a key question in neuroscience. Here, we review a simple model of neurotransmission that serves as a convenient framework to discuss recent studies on RIM and synaptotagmin.

The Basics of Synaptic Transmission

Most neurons in the mammalian central nervous system are connected by chemical synapses. These specialized structures do not simply transmit a sequence of action potentials (APs) from one cell to the next but can act as filters to modify signals (1). For example, connections that are strong (like the climbing fiber synapse of the cerebellum) reliably transmit individual, isolated APs yet depress in response to trains where the same stimuli are delivered at high frequency. On the other hand, synapses that are initially weak (like the parallel fiber synapse of the cerebellum) tend to facilitate, giving a larger response per stimulus if APs are delivered in bursts. These examples illustrate how strong synapses can function as low-pass filters and weak synapses can operate as high-pass filters, influencing how information flows through neural circuits (1). In addition to these relatively rapid effects (on the order of seconds), activity-dependent presynaptic changes can occur on longer timescales (hours or longer) and lead to enduring modifications of network properties (35). Thus what determines a synapse’s strength at the molecular level is a key question that has important implications for circuit function. Here, we review a simple model of neurotransmitter release that serves as a framework to discuss potential influences on synaptic strength. We then discuss recent examples from work on molecules that control the efficacy of synapses.

Before addressing neurotransmitter release, it is worth briefly reviewing the anatomy of the structure involved, as determined from three-dimensional reconstructions based on electron microscopy. Although there is considerable variety among synapses, small (~1-μm diameter) synapses of the central nervous system typically have on the order of a hundred vesicles, of which only a small subset (~5%) are in direct contact with the presynaptic membrane (49). This subset of vesicles, referred to as the docked pool, contacts the membrane in a region known as the active zone in direct apposition across the synaptic cleft with a specialized area on the postsynaptic cell, known as the postsynaptic density (55).

When an AP arrives at a presynaptic terminal, the depolarization leads to Ca\(^{2+}\) channel opening. Calcium ions rush into the terminal down their electrochemical gradient and bind to a Ca\(^{2+}\) sensor (5), widely thought to be synaptotagmin on the synaptic vesicle membrane (10, 31, 44, 61). Through a series of steps that takes <1 ms and is not fully understood, this binding leads to SNARE-dependent membrane fusion of the vesicle with the membrane and release of neurotransmitter into the synaptic cleft (11). Neurotransmitter quickly diffuses across the cleft and binds to postsynaptic ionotopic receptors that cause rapid conductance changes and/or metabotropic receptors that lead to slower effects mediated by G-protein-coupled receptors in the postsynaptic cell.

A Framework to Study Neurotransmitter Release

A simple model used as a framework to study this process posits that, as a result of a single AP in the presynaptic neuron, a response (Q) is elicited in the postsynaptic cell (54):

$$Q = n \cdot P_v \cdot q$$  \hspace{1cm} (1)

where $n$ is the number of primed vesicles, i.e., vesicles immediately available for fusion (also known as the readily releasable pool or RRP). These vesicles have undergone all biochemical steps except for the final Ca\(^{2+}\)-dependent fusion step. $P_v$ is the probability that each of those vesicles has of fusing with the membrane in response to one AP. Lastly, $q$ is the size of the postsynaptic response to a single vesicle fusion event (also known as quantal size).

Two important assumptions implicit in this model are that all primed vesicles have the same fusion probability and that they behave independently. Even if these assumptions do not hold in every case, the model is still a very useful way to think about synaptic transmission. Note that the model makes no restrictions on the possibility of more than one vesicle fusing at once (multivesicular release) at a given synapse. For some time,
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there was a debate regarding whether multivesicular release could take place at synapses of the mammalian central nervous system. However, there is now convincing evidence that this can happen (2–4, 43), so it does not seem warranted to modify the model to restrict the possibility of multivesicular release occurring. Finally, a very important point regards how this model connects to the typically measured parameter $P_r$ (neurotransmitter release probability). $P_r$ is the probability that a synapse will not fail (14), i.e., that a presynaptic AP will elicit a response in the postsynaptic cell. This will be equivalent to the probability that at least one vesicle fuses in response to one AP. Given the implicit binomial distribution underlying the model:

$$P_r = 1 - (1 - P_v)^n$$  \hspace{1cm} (2)

With this framework in mind, it is worth exploring how $n$, $P_v$, and q can be influenced to determine synaptic strength.

Several factors can influence the number of primed vesicles. In a simplified model, $n$ will be set by the interplay of rates of docking (and undocking), priming (and unpriming), and possibly (see below) spontaneous fusion (FIGURE 1A):

$$N_{\text{total}} = \frac{k_{\text{docking}}}{k_{\text{undocking}}} N_{\text{docked, unpaired}} + \frac{k_{\text{priming}}}{k_{\text{unpriming}}} n$$

where $N_{\text{total}}$ is the total number of synaptic vesicles in a nerve terminal; $N_{\text{docked, unprimed}}$ is the number of vesicles in contact with the presynaptic membrane that are not fusion competent; $k_i$ is the rate constants for process $i$, where $i$ can be docking, undocking, priming, unpriming, and spontaneous vesicle fusion.

In this scenario, the rate constants of each step will influence $n$, but so will any change in the total number of vesicles in the terminal merely due to mass action. For example, anything that increased $N_{\text{total}}$ would raise $n$ without controlling docking or priming processes per se. Additionally, even in the absence of stimulation, there is a low rate of neurotransmitter release due to spontaneous fusion of synaptic vesicles with the membrane. Although there is some debate as to whether these vesicles are in the primed pool to begin with (19, 27, 28, 66), if they were, any change in the spontaneous fusion (or mini) rate could lead to a reduction in steady-state $n$. An alternative proposal for the control of $n$ posits that fusion can only take place at certain “slots” or “sites” (41). In this scenario, control of the number of the sites is key. Thus the important variables would be the abundance of molecules that make up the slots and the rate constants of assembly and destruction of the sites (FIGURE 1Aii). These two scenarios are not incompatible but rather should be considered extremes in a continuum of possibilities. The first implicitly assumes that the molecules necessary for fusion (presumably the site constituents) are in abundance and are not rate limiting. Conversely, the second assumes that construction of sites is much slower than the docking and priming reactions such that, once a site is formed, it captures a primed vesicle quickly. If the rates of the various processes outlined above are comparable, $n$ will be set by a complex interplay between the number of vesicles, the number of sites, and the rates of docking and priming within those sites. Which extreme of the model is a better representation of what actually occurs during priming is currently unknown. However, the fact that synaptic vesicle fusion and docking are localized to a specialized region of the plasma membrane (the active zone) supports the concept of slots, although what is rate limiting in the process of docking and priming a vesicle at those sites is unclear.

An interesting question is whether docking is the morphological equivalent of priming or if any additional biochemical steps are needed to make a vesicle fusion competent. An illustrative example of how thinking on this point has evolved is munc13, the mammalian homolog of a Caenorhabditis elegans gene that causes severe paralysis when mutated (unc13) and contains domains that can bind phorbol esters and $Ca^{2+}$ (9). Knocking out both isoforms expressed at hippocampal slice cultures using high-pressure freezing and electron tomography, circumventing potential aldehyde fixation artifacts (58). Contrary to the previous findings, this study concluded that the docked vesicle pool was almost completely eliminated in the absence of munc13 and proposed that docking is the morphological correlate of priming or if any additional priming steps needed to take place before achieving fusion competence. This issue was revisited in a recent study on hippocampal slice cultures using high-pressure freezing and electron tomography, circumventing potential aldehyde fixation artifacts (58). Contrary to the previous findings, this study concluded that the docked vesicle pool was almost completely eliminated in the absence of munc13 and proposed that docking is the morphological correlate of priming or if any additional priming steps needed to take place before achieving fusion competence. This issue was revisited in a recent study on hippocampal slice cultures using high-pressure freezing and electron tomography, circumventing potential aldehyde fixation artifacts (58).
high-pressure freezing and electron tomography is needed to shed more light on whether docked vesicles are the morphological equivalent of the readily releasable pool. If there are additional biochemical steps necessary for fusion competence after vesicle docking, eliminating molecules critical only for those steps would lead to smaller \( n \)-assayed physiologically (see below)—yet no changes

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<th>Key presynaptic control variables</th>
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<tr>
<td><strong>i</strong></td>
<td>Number of vesicles</td>
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| **B**                            |                   |
| **i**                            | Abundance and properties of Ca\(^{2+}\) channels |
| **ii**                           | Vesicle proximity to Ca\(^{2+}\) channels |
| • Local Ca\(^{2+}\)              |                   |
| **iii**                          | Abundance and properties of Ca\(^{2+}\) buffers |
| **iv**                           | Abundance and properties of SNARE complexes |
| • Fusogenicity                   |                   |

| **C**                            |                   |
| **i**                            | Neurotransmitter concentration: Transporter abundance and properties |
| **ii**                           | Size of vesicles |
| **iii**                          | Fusion mode: Full fusion or kiss and run |
in the number of docked vesicles assayed ultrastructurally. On the contrary, if docking is equivalent to priming, we would expect a tight correlation of effects on \( n \) and the number of docked vesicles over a wide variety of molecular interventions. Based on results from docking of synaptic vesicles in \( C. \) elegans or of dense core vesicles in chromaffin cells, particularly interesting candidates to revisit in mammalian synapses with high-pressure freezing are syntaxin, SNAP-25, synaptotagmin, and munc18 (12, 13, 22). Below, we discuss recent experiments reducing the levels of the protein RIM at the calyx Held (23), which causes a decrease in the primed pool size (assayed physiologically) that correlates well with a corresponding reduction in the number of docked vesicles (assayed by electron microscopy after chemical fixation). These recent experiments further support the idea that docking is the morphological equivalent of priming.

When considering how the value of \( P_v \) can be modulated, the main points to examine are local \( \text{Ca}^{2+} \) (its concentration at the vesicle) and vesicle fusogenicity (FIGURE 1B). \( P_v \) is steeply dependent on the \( \text{Ca}^{2+} \) concentration at synaptotagmin molecules on primed synaptic vesicles (7, 10, 52, 61), so control of that local concentration will have a strong effect on synaptic strength (53). Local levels of \( \text{Ca}^{2+} \) will be influenced both by how much \( \text{Ca}^{2+} \) comes into the terminal in the first place and by how much the concentration decays between the source (\( \text{Ca}^{2+} \) channels on the presynaptic membrane) and primed vesicles. The amount of \( \text{Ca}^{2+} \) influx will be set by the intrinsic characteristics and abundance of \( \text{Ca}^{2+} \) channels (FIGURE 1B(i)), the extra- to intracellular electrochemical \( \text{Ca}^{2+} \) gradient, and the shape of the AP waveform. The concentration decay to the vesicle will depend on the average distance between vesicles and \( \text{Ca}^{2+} \) channels (referred to as the coupling between them; FIGURE 1B(ii)) and will also be shaped by any molecules between the channels and vesicles that can bind \( \text{Ca}^{2+} \) (FIGURE 1B(iii)). Depending on their concentrations, mobility, binding, and unbinding rate constants, these \( \text{Ca}^{2+} \) buffers can potentially influence the local concentration of \( \text{Ca}^{2+} \) at primed vesicles (56). The second major determinant of \( P_v \) is primed vesicle fusogenicity (FIGURE 1B(iv)). This will be a combination of how intrinsically fusion-willing a primed vesicle is, which can be estimated from spontaneous release rates under conditions of very low \( \text{Ca}^{2+} \), and how the likelihood of fusing with the membrane increases as calcium ions bind to synaptotagmin molecules on the vesicle (34). Fusion willingness might depend on the exact state of the \( \text{trans}-\)SNARE complexes that are necessary for fusion. Another potential influence would be the abundance of SNARE complexes on a primed vesicle (39), which will be set by the combined local concentrations of the SNARE complex constituents: syntaxin, SNAP25 (on the presynaptic membrane), and synaptobrevin (on the synaptic vesicle). Thus molecules such as complexin, tomosyn, or munc18 that can interact with the assembled SNARE complex or its constituents could potentially alter the likelihood that a vesicle will fuse by affecting the abundance or conformation of \( \text{trans}-\)SNARE complexes (67). Additionally, the details of how \( \text{Ca}^{2+} \) sensors bind \( \text{Ca}^{2+} \) and how those changes increase the likelihood of vesicle fusion will be critical in setting vesicle fusogenicity (34, 61).

For \( q \), there can be both pre- and postsynaptic influences. On the presynaptic side, the amount of neurotransmitter per vesicle might vary with the number or activity of neurotransmitter vesicular transporters present (FIGURE 1C(i)) and the vesicle size (FIGURE 1C(ii)). Additionally, there is a controversy regarding “kiss and run,” i.e., events that do not lead to full fusion and collapse of the vesicle (16, 21, 24, 73). If these events involve a small pore that opens only briefly or flickers, they could lead to less neurotransmitter release into the cleft (FIGURE 1C(iii)). Finally, although we focus on presynaptic properties in this review, it is worth keeping in mind that the abundance and specific properties of postsynaptic receptors, together with their orientation relative to release sites, can profoundly influence the size of the postsynaptic response.

So far, we have discussed what happens when a single AP invades the nerve terminal. However, neurons can burst, firing many APs at frequencies

![FIGURE 1](https://www.physiologyonline.org/physiology/2012/02/18/physiologyonline.org figure1.png)
up to 100 Hz and higher. Under those circumstances, synaptic transmission will be influenced by factors beyond those considered above (18, 68). The size of the primed pool of vesicles at any given moment will be determined by the balance of vesicle fusion and priming rates. Under conditions of sustained activity, not only vesicle fusion but also priming rates are Ca\(^{2+}\) dependent (17, 42, 59, 65). Therefore, Ca\(^{2+}\) channel inactivation (69) or facilitation (29, 38), exhaustion of extracellular Ca\(^{2+}\) in the synaptic cleft (8, 46), saturation of intracellular Ca\(^{2+}\) buffers (6), and the rates of Ca\(^{2+}\) clearance (57) during a burst of APs can all shape the postsynaptic response. Additionally, if there are slots for release, these might require a clearance time before they become available once more (26, 41). Finally, on the postsynaptic side, receptors may saturate, desensitize (68), or even diffuse in and out of the postsynaptic density (25) during a burst. These effects can complicate attempts to use postsynaptic measurements as linear indicators of presynaptic activity.

**Model Systems and Methods to Study Synaptic Transmission**

Given this panoply of potential influences on the neurotransmitter release process, to convincingly ascribe the functions of a molecule to any one of the processes mentioned above requires considerable effort. Typical experiments involve genetic manipulation of neurons to eliminate or replace certain molecules or increase their concentration. If there are pharmacological tools available to interfere with a molecule’s function, these can also be used, ideally coupled with experiments in the absence of the molecule to test for any off-target effects. There are many possible techniques and preparations to estimate \(n\), \(P_v\), and \(q\), with a few general considerations applicable to any of them.

Methods to measure \(n\) tend to rely on a strong, fast stimulus that causes exocytosis of all primed vesicles before there is time for significant replenishment of that pool of vesicles through priming processes. Under those conditions, the size of the response will be equivalent to \(n\). In cases where there is significant replenishment during the stimulus, a model of the vesicle priming process is needed to correct the estimate appropriately. To measure \(P_v\), the size of the response to one AP is simply divided by \(n\). This places a minimum requirement on the sensitivity of any method used to determine \(P_v\), since it must be possible to measure the response to a single AP precisely. Finally, to estimate \(q\), it is necessary to determine the postsynaptic response to a single vesicle fusion event or quanta. A usual approach is to use the average response to spontaneous, low-frequency events that happen in the absence of stimulation and that are presumed to correspond to single vesicles fusing.

The best biophysical measurements of synaptic transmission currently available come from the calyx of Held, a large, cup-like excitatory synapse in the auditory pathway (47, 50). This giant synapse has hundreds of active zones and effectively operates as a parallel array of small synapses. It has become a valuable model system to study synaptic transmission because its large size makes it amenable to both pre- and postsynaptic whole-cell recording and Ca\(^{2+}\) uncaging experiments. In this synapse, \(n\) has been estimated as the size of the response to a step increase in Ca\(^{2+}\) concentration using uncaging, a constant current, or a burst of APs. At the moment, Ca\(^{2+}\) uncaging is the only tool that allows unambiguous separation of effects on local Ca\(^{2+}\) and vesicle fusogenicity by bypassing Ca\(^{2+}\) channels entirely and elevating Ca\(^{2+}\) with spatial uniformity to cause neurotransmitter release. The resulting measure of the intracellular Ca\(^{2+}\) sensitivity of neurotransmitter release can be used—in combination with modeling—to test whether an intervention changes vesicles’ basal fusion willingness or, alternatively, modifies how their likelihood of fusing scales with increasing Ca\(^{2+}\) (34). Critically, factors that affect fusogenicity can be assayed independently of whether the intervention of interest led to a modification of local Ca\(^{2+}\) levels after an AP. Historically, the main disadvantage of the calyx of Held preparation has been the difficulty in achieving molecular control of the system beyond nonlethal knockouts or peptide injections (but see below).

Perhaps the most widely used system for molecular studies of presynaptic release in mammalian synapses is primary culture of cortical or hippocampal rodent neurons, studied electrophysiologically. Cultures of neurons can be prepared easily from genetically modified mice and studied even in cases where the mutations are lethal beyond birth. Simple transfection protocols to knock down, overexpress, or replace proteins of interest can be used for detailed studies of structure-function relationships. In these cultures, which can be dissociated or autaptic (where a single neuron is grown on a microisland of glia and synapses onto itself), \(n\) is usually estimated as the response to application of a hypertonic solution containing 500 mosM of sucrose. This stimulus was shown to correlate with the size of the primed pool of vesicles as determined by depletion experiments using bursts of action potentials (45). However, the mechanism involved remains mysterious, and its correspondence with primed vesicles accessed with physiological stimuli is under debate (40, 60). Alternatively, bursts of 20–40 APs at 20–40 Hz have been used to deplete the pool. Unfortunately, there is typically substantial replenishment of primed
vesicles during this stimulus, and a large correction must be applied (40, 60). A downside of the primary culture system was, until very recently (10), the inability to perform Ca\textsuperscript{2+} uncaging experiments to directly probe the intracellular Ca\textsuperscript{2+} sensitivity of the fusion process. Additionally, any electrophysiological method will be affected by the complications inherent in using a postsynaptic measure to estimate presynaptic function.

A recent method developed by our laboratory (3) offers another potential way of studying \( P_v \) and \( n \) using a direct optical presynaptic readout based on the pH-sensitive GFP pHluorin (36) tagged to the lumen of the vesicular glutamate transporter (64). Synaptic vesicles are acidic (pH of \( \sim 5.6 \)), so upon fusing to the membrane and exchanging protons with the extracellular milieu there is an increase in pH to \( \sim 7.4 \). The pKa of the fluorescent reporter (\( \sim 7.1 \)) is such that it increases its fluorescence 20-fold upon that transition (48). Under appropriate imaging conditions, a response to a single AP can be detected with a temporal resolution of 10 ms (Ref. 3, see also Refs. 4, 20). The \( n \) can be determined by measuring the saturation of exocytosis.

**FIGURE 2.** Optical method used to determine \( n \) and \( P_v \).

**A:** exocytosis is measured in response to a burst of 20 APs at 100 Hz in the presence of elevated (4 mM) extracellular Ca\textsuperscript{2+} (average of 12 synapses across 4 trials). After six APs, there is no further exocytosis, indicating depletion of a readily releasable pool of vesicles. Thus the amplitude of this plateau is a measure of \( n \). Note that the exocytosis rate drops to zero and thus there is no need to apply a correction for refilling of the RRP during the stimulus. Inset: response to one AP in 2 mM external Ca\textsuperscript{2+}; exocytosis on same scale. **B:** response to a single AP under standard Ca\textsuperscript{2+} conditions (2 mM) can be measured with excellent sensitivity (top: average of 20 trials, scaled up fourfold from inset in A). Dividing the response to one AP by \( n \) provides an estimate of \( P_v \). **C:** the same techniques can be combined with Ca\textsuperscript{2+} imaging to estimate \( P_v \) as a function of intracellular Ca\textsuperscript{2+} increases in response to 1 AP. Note the sigmoidal curve resulting from the Hill relation with a cooperativity coefficient of 3.4, in excellent agreement with equivalent experiments performed previously in the calyx of Held (51). **D:** using Eq. 2 and an estimate of the RRP size of four vesicles, we can predict the relation of \( P_v \) to increases in intracellular Ca\textsuperscript{2+} based on the measured relationship between the latter and \( P_v \).

Light blue shading in C and D indicates region where Ca\textsuperscript{2+} entry can be modulated from zero- to twofold of normal, with corresponding effects on \( P_v \) and \( P_e \). Note that the expected dependence of \( P_v \) on Ca\textsuperscript{2+} is steeper around the standard conditions region (2 mM extracellular Ca\textsuperscript{2+}). Data in A–C come from Ref. 3 and are used here with permission.
in response to a burst of APs at 100 Hz (FIGURE 2A). Conveniently, under these stimulation conditions, exocytosis completely exhausts the RRP before it can be repopulated with new vesicles. Therefore, there is no need to correct the estimate of primed pool size due to refilling, as is typically the case when using weaker stimulation protocols (40, 60). Subsequently, by comparing the response to one AP with the RRP size, we obtain $P_v$ (FIGURE 2B). Importantly, the saturation of exocytosis with 100-Hz stimulation is not the result of $Ca^{2+}$ channel inactivation and agrees well with an alternative method using single APs under conditions of very large $Ca^{2+}$ entry (3). Using this sensitive technique, we also studied how $P_v$ varied as a function of increases in intracellular $Ca^{2+}$ in response to a single AP (FIGURE 2C). The resulting sigmoidal relationship where standard $Ca^{2+}$ conditions are situated quite low on the curve highlights how small changes in $Ca^{2+}$ entry can lead to large effects on baseline $P_v$. These changes in $P_v$ will presumably cause even greater shifts in $P_v$ (using our estimate of $n = 4$ vesicles in Eq. 1; FIGURE 2D), illustrating how forms of modulation that regulate $Ca^{2+}$ entry even slightly can sharply modify synaptic efficacy. Regrettably, our method does not allow a study of $q$ and has less temporal resolution and sensitivity than electrophysiological methods. However, the lack of a postsynaptic component to the measurements can simplify the study of $P_v$ and $n$. Furthermore, in contrast to electrophysiological studies in culture, it is not affected by changes in the number of synaptic contacts since measurements come from averages—not sums—across synapses. Finally, because it provides information from individual small synapses in parallel, it can potentially allow studies of determinants of synaptic strength between synapses of the same axon (unpublished work from our laboratory).

In what remains, we discuss a few recent exciting studies where molecules involved in neurotransmitter release were analyzed in sufficient detail to determine their function in terms of the models and concepts presented above. The examples come mainly from the calyx of Held, where new tools have been developed that allow molecular control at this synapse.

RIM, a Multi-functional Coordinator of Neurotransmitter Release

A recent set of papers (15, 23, 30) has probed with exquisite detail the functions of the RIMs, a set of proteins enriched at active zones, with multiple domains that allow them to interact directly with $Ca^{2+}$ channels, synaptic vesicles, the active zone machinery, and the priming protein munc13 (37). In a series of elegant experiments where the major isoforms of RIM were eliminated selectively at the calyx of Held by conditional knockout (23), a number of interesting results were obtained that can be placed in the framework presented previously (FIGURE 3). An 80% reduction in the response to a single AP was used as a starting point for a thorough dissection of the consequences of the absence of RIM. The reduction in synaptic strength was mostly attributed to an RRP size defect (75% reduction in $n$) and to a lesser extent to a reduction in $P_v$ (30% decrease). The $n$ was determined from two independent methods (depletion using AP bursts or $Ca^{2+}$ uncaging), giving very similar results. Furthermore, electron microscopy showed that a reduction in the number of docked vesicles (80%) could completely account for the drop in the number of primed vesicles, suggesting that at this synapse the former is the morphological equivalent of the latter. As regards the reduction in $P_v$, further experiments were carried out that showed a decrease in synaptic, but not somatic, $Ca^{2+}$ current density, a looser coupling between those channels and synaptic vesicles, and even a slightly lower intracellular $Ca^{2+}$ sensitivity of vesicles. Other tests excluded the possibility of effects on $Ca^{2+}$ channel inactivation, AP waveform, or postsynaptic receptors. An analogous study on hippocampal
cultures came to similar conclusions and added interesting molecular details to the picture by localizing the effects on P_v to RIM’s PDZ domain and those on n to the amino-terminus of the protein (30). Interestingly, the RIM PDZ domain was shown to interact directly with N and P/Q type Ca^{2+} channels. The same study also showed a reduction in the abundance of synaptic Ca^{2+} channels by immunostaining, consistent with the reduction in Ca^{2+} current density seen at the calyx. As regards the effect on n, another study showed that RIM inhibits the homodimerization of munc13 (15). Since homodimerized munc13 is unable to prime synaptic vesicles, this could explain why the absence of RIM leads to a priming defect. These recent studies on RIM highlight how one family of proteins, by virtue of interactions with many other players, can be involved in the regulation of several aspects of neurotransmitter release. Given its coordinated functions, relatively short half-life (~1 h; Ref. 71), and correlation with activity at the single synapse level (33), it will be interesting to see whether the level of RIM at a synapse operates as a dynamic, rate-limiting control variable that sets synaptic strength.

Synaptotagmin, Not Just a Ca^{2+} Sensor for Neurotransmitter Release

Another recent paper illustrates how even a molecule such as synaptotagmin, which has a well established role as the Ca^{2+} sensor for neurotransmitter release, can have other functions that become evident with newer techniques (72). A novel molecular approach for the calyx of Held preparation was developed by creating a recombinant adenovirus that can be injected perinatally to achieve overexpression of a protein of interest in this synapse. This system was used to study in more detail a previously characterized mutant of synaptotagmin that had normal Ca^{2+} binding and folding properties (70) yet could not rescue synaptic transmission when introduced into a null background, hinting at other functions of synaptotagmin. An ~75% reduction in the response to a single AP was due to a reduction in both n and P_v. The number of primed vesicles was reduced ~40%, whereas P_v suffered an ~45% reduction when the mutant variant of synaptotagmin was overexpressed. Furthermore, the effect on P_v was not due to a change in Ca^{2+} currents or fusogenicity of primed vesicles, leading to the conclusion that the coupling between channels and vesicles had been affected and depended on the mutated residues present in synaptotagmin. In addition to the effects on isolated action potentials, this conversion of the calyx of Held to a lower P_v state led to facilitation during bursts of APs, illustrating how changes in single AP release parameters can potentially influence circuit function through effects on short-term plasticity. This study is another example of how adding molecular control to a preparation where rigorous biophysical methods are available can lead to new insights.

Conclusions

The picture that emerges from these recent studies is a complex one, with molecules exercising several functions through their various domains whose effects can only be discerned through careful experimentation. The development of new techniques that bring increasing molecular control to the calyx of Held are exciting breakthroughs. A combination of adenovirus expression strategy with conditional knockouts in the calyx of Held, will allow even more sophisticated structure-function experiments to take place at what remains the central nervous system synapse most amenable to detailed biophysical studies (for an early example of the potential for this kind of study, see Ref. 32). On the other hand, in the autaptic neuronal culture system, the recent development of Ca^{2+} uncaging will expand the applicability of this powerful biophysical technique to a widely used preparation (10). Finally, our own methods promise to bring the study of P_v and n down to the level of individual small synapses (3), where it will be possible to explore the nature and basis synapse-to-synapse variation in P_v and n. The combination of these different experimental avenues of attack should lead to exciting discoveries that expand our knowledge of the molecules that act as presynaptic determinants of neurotransmitter release.

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