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Review

The Nanophysiology of Fast Transmitter Release

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Action potentials invading the presynaptic terminal trigger discharge of docked synaptic vesicles (SVs) by opening voltage-dependent calcium channels (CaVs) and admitting calcium ions (Ca^{2+}), which diffuse to, and activate, SV sensors. At most synapses, SV sensors and CaVs are sufficiently close that release is gated by individual CaV Ca^{2+} nanodomains centered on the channel mouth. Other synapses gate SV release with extensive Ca^{2+} microdomains summed from many, more distant CaVs. We review the experimental preparations, theories, and methods that provided principles of release nanophysiology and highlight expansion of the field into synaptic diversity and modifications of release gating for specific synaptic demands. Specializations in domain gating may adapt the terminal for roles in development, transmission of rapid impulse frequencies, and modulation of synaptic strength.

Early Studies on the Transmitter Release Mechanism

The seminal experiment demonstrating that transmitter release from presynaptic terminals requires both simultaneous nerve terminal depolarization and the presence of extracellular Ca^{2+} [1] was the lifetime favorite (personal communication) of Sir Bernard Katz (Nobel Laureate in Physiology and Medicine, 1970). This key observation, together with the findings that depolarization opens presynaptic **CaVs** (see Glossary) [2] admitting Ca^{2+} into the nerve terminal cytoplasm [3] and that intracellular Ca^{2+} can gate transmitter release [4] by the fusion of SVs [5] with the surface membrane, formed the basis of the 'calcium hypothesis' of transmitter release gating. The essential link between influx of Ca^{2+} through the channel and its subsequent binding to the **SV sensor** was not discussed in Katz's monograph and is the subject of this review. Several previous reviews [6–9] provide additional perspectives on this subject.

Exemplar Presynaptic Experimental Preparations

Because the vast majority of presynaptic terminals are small and inaccessible, progress in this field has relied heavily on a relatively few exemplar model synapses and the application of remarkably innovative experimental assay methods.

Squid Giant Synapse (SGS)

The SGS (Figure 1A) was the first synapse at which it was possible to record directly from the presynaptic terminal [10]. Application of a two- or three-electrode voltage clamp (Figure 1A, lower panel) [2,11] heralded the modern era in synaptic research, relating presynaptic inward Ca^{2+} current to transmitter output. Three findings at the SGS were of particular significance with respect to release gating. First, a minimum latency of 0.2 ms between Ca^{2+} influx through the CaVs and transmitter release [12] implied that at least some CaVs must lie within ~100 nm from the SV sensors. Second, the demonstration that, typically, transmitter release was maximal during the repolarization phase of the action potential [13] permitted calculation of realistic Ca^{2+} influx rates; and third, the finding by the Charlton group that fast- but not slow-binding

Trends

Single domain gating appears to predominate where transmission fidelity is paramount [73]. Possible reasons include: minimal delay due to the virtually instantaneous access of Ca^{2+} to the SV sensor; protection against docked-SV depletion and transmission failure; a metabolic advantage by minimizing Ca^{2+} clearance; and avoiding detrimental effects of high cytoplasmic Ca^{2+} [8].

Overlapping domain gating may predominate where the amplitude of transmitter release is more important than the frequency of impulses [76] or where release is subject to modulation of presynaptic CaVs. While rod synapses contradict this general idea, since they exhibit an exquisite dynamic range while relying on single domain gating [68], it is possible that this idea holds for neurons.

Single domain-type synapses may pass through a pre-maturity overlapping domain stage, raising the possibility that amplitude coding is important during synapse formation [60].

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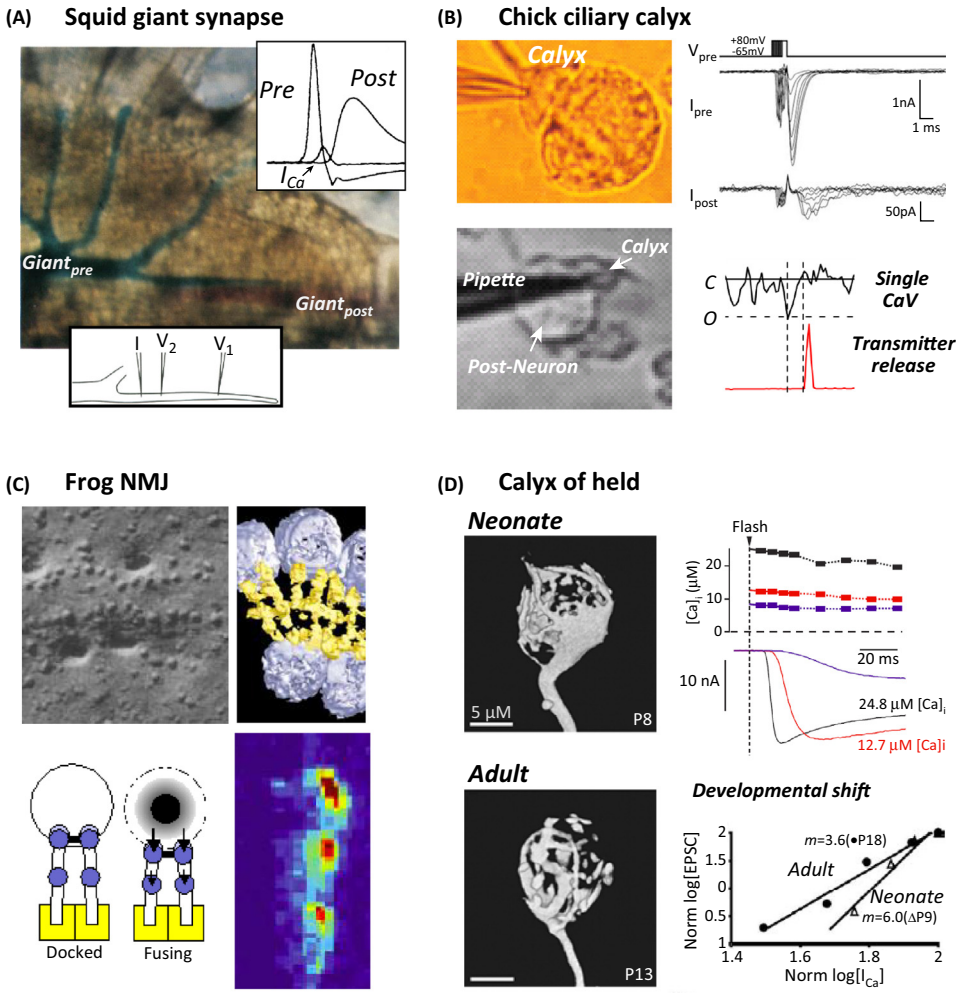


Figure 1. Exemplar Presynaptic Terminals. Experimental investigation directly on the presynaptic terminal is difficult mostly because of small size, inaccessibility, or heterogeneity and hence much of what we know about the general principles of synaptic transmission physiology derives from exemplar experimental models. (A) Squid giant synapse (SGS). The giant synapse is embedded in the stellate ganglion of the squid where a presynaptic giant axon from the optic ganglion synapses onto a giant axon in each of the mantle nerve bundles. The contact from the presynaptic giant onto the postsynaptic giant axon in the last mantle bundle is termed the SGS (see lower inset) [10]. In the figure the presynaptic giant axon has been filled with dye [11]. Ca^{2+} gating of transmitter release was analyzed by voltage clamp of the presynaptic terminal using two or three (as shown, lower inset) sharp electrodes while also recording from the post-synaptic neuron. Right inset: The presynaptic calcium current (I_{Ca}) evoked by an action potential-simulated voltage command (Pre) triggers transmitter release detected as an excitatory postsynaptic current (Post) [13]. (B) Chick ciliary ganglion calyx synapse (chick CC). The presynaptic terminal at calyx-type synapses envelopes the postsynaptic neuron with a sheet-like or reticulated membrane process [79]. The chick CC was isolated either attached to (upper-left panel) [80,81] or partially removed from (lower-left panel) [16] the postsynaptic neuron. Double whole-cell patch clamp recording (upper-right panel) demonstrated a near-single-power relationship between the presynaptic I_{Ca} (I_{pre}) and the postsy-naptic current (I_{post}), favoring single domain gated release (Box 1) [15]. Cell-attached patch recording of single CaV activity was conducted on partially isolated calyces [16] while monitoring transmitter release (lower-right panel). The finding that transmitter release is linked to individual Ca^{2+} current flickers argued that fusion of a synaptic vesicle (SV) was gated by the opening of a single presynaptic voltage-dependent calcium channel (CaV) [17]. O, open; C, closed single-channel current level. (C) Frog neuromuscular junction. Freeze-fracture replicas of the transmitter-release face of actively secreting terminals exhibit two pairs of large particle rows bordered by SV fusion profiles (top-left panel) [82]. A putative scaffold linking the SVs to the particles has been imaged by electron microscopy tomography [83] and evidence suggests that the structure contracts during exocytosis [64] (top-right and bottom-left panels, respectively). Action potentials in the nerve terminal evoke discrete Ca^{2+} plumes, as detected by fluorescent dye, corresponding to the opening of individual CaVs (lower-right panel) [25]. (D) Neonatal calyx of Held (nCoH) and adult CoH. The rodent CoH undergoes increased

Glossary

Microdomain: a high-concentration plume of ions in the cytoplasm resulting from the overlap of nanodomains from a cluster of open membrane channels. The size and concentration profile of microdomains can be predicted to be variable as they are affected by the number of channels in the cluster, their spatial relationship, and their individual open–closed fluctuations.

Nanodomain: a high-concentration plume of ions in the cytoplasm resulting from the opening of a single ion channel. The nanodomain forms and collapses virtually instantaneously on channel opening and closing and can be predicted to be relatively consistent in profile for given cell conditions.

Nanophysiology: a new term coined to include processes operating at submicron distances where function must ultimately be related to constraints on individual molecules.

Overlapping domain gating: activation of the SV sensor by Ca^{2+} entering through more than one surface membrane CaV.

Ribbon synapse: a transmitter release site common in sensory cells characterized by an electron-dense structure that abuts the SV fusion region.

Single domain gating: activation of the SV sensor by Ca^{2+} entering through a single surface membrane CaV.

SV sensor: the molecular apparatus associated with the docked SV responsible for binding multiple Ca^{2+} ions and thence gating SV fusion. The SV sensor includes synaptotoagmin-1 but may require other ion-binding or function-translating molecules.

Voltage-dependent calcium channel (CaV): there are three families of CaVs: CaV1, CaV2, and CaV3. Of these, CaV2.1 and CaV2.2 are the principle types involved in gating SV fusion at fast-transmitting synapses but CaV2.3 can also play a role. However, CaV1-family channels are preferred at sensory receptor ribbon-type synapses and CaV3 channels can also gate release at a few synaptic contacts.

intracellular Ca^{2+} scavengers block release [14] strengthened the argument that the SV sensors are located close to the CaV pores.

Chick Ciliary Calyx (CC)

Applying the patch clamp method to a calyx-type synapse isolated from the chick ciliary ganglion (Figure 1B, upper-left panel) provided the first opportunity to explore presynaptic Ca^{2+} currents [80,81,86] in vertebrates. Later these were correlated with transmitter release (Figure 1B, upper-right panel) [15]. The chick CC came into its own as an experimental preparation with the application of the cell attached patch clamp recording method to the exposed presynaptic transmitter-release face (Figure 1B, lower-left panel) and the recording of single release site-associated CaVs [16]. The addition of a luminescent assay for transmitter release to this method permitted the direct correlation of single presynaptic CaV channel activity with SV fusion and discharge (Figure 1B, lower-right panel) [17].

Frog Neuromuscular Junction (NMJ)

Nanometer-resolution observations on the active zone of the frog NMJ have provided key structural details of resting and actively secreting synapses. Belt-like active zones exhibit parallel pairs of transmembrane particles that are believed to include CaVs [18–20]; SV fusion profiles were observed abutting these particle rows [19,21] (see [8]) (Figure 1C, upper-left panel). Electron microscopy (EM) tomography revealed an associated submembrane scaffold (Figure 1C, upper-right panel) while a quantitative analysis of particle distributions in the freeze-fracture replicas argues that this scaffold contracts during fusion (Figure 1C, lower-left panel). With respect to release gating, the frog NMJ has had two important resurgences, the first associated with selective and irreversible toxin block of the CaVs [22,23] and, more recently, the imaging of Ca^{2+} entry through individual channels (Figure 1C, lower-right panel) [24,25].

The Neonatal Rodent Calyx of Held (nCoH)

The nCoH (Figure 1D, upper panels) in the auditory pathway of newborn rodents, another calyx-type synapse, stimulated a new wave of transmitter release research because of the relative ease of simultaneous pre- and postsynaptic patch clamp recording [26,27]. Key discoveries included new perspectives on presynaptic input–output relations [7] and exploration of the SV sensor binding affinity (Figure 1D, upper-right panel) and kinetics [28,29].

The Adult Rodent CoH

The degree to which the findings at the nCoH could serve as a general model of synaptic transmission came into question when it became apparent that there is an abrupt change in release gating physiology during development (Figure 1D, lower-right panel) [30]. This functional switch coincided with the onset of hearing [31], increased fenestration of the calyx (Figure 1D, lower-left panel), and high-frequency discharges [32], suggesting that the nCoH was an intermediary stage in the development of the mature CoH.

Models of Ca^{2+} Transmitter Release Gating

The evolution in our understanding of transmitter release physiology could serve as a textbook example of the cycle between conceptual models and their experimental scrutiny [33]. We start here with a model in which the CaVs are distributed randomly across the presynaptic surface membrane and SV fusion is gated by the entering Ca^{2+} that fills the nerve terminal. This view, which we term the 'gross entry' model (Figure 2A), corresponds approximately to the state of the

fenestration during development from its neonatal (upper-left panel) to adult (lower-left panel) form [84]. The Ca^{2+}-binding properties of the SV sensor were characterized in the nCoH by flash-photolysis release of Ca^{2+} while recording the postsynaptic current to monitor transmitter release (upper-right panel) [29]. Release gating exhibits a developmental shift from primarily overlapping to primarily single domains, as demonstrated by a reduction in the slope of the CaV titration plot [30]. Modifications have been made to figure panels for presentation purposes.

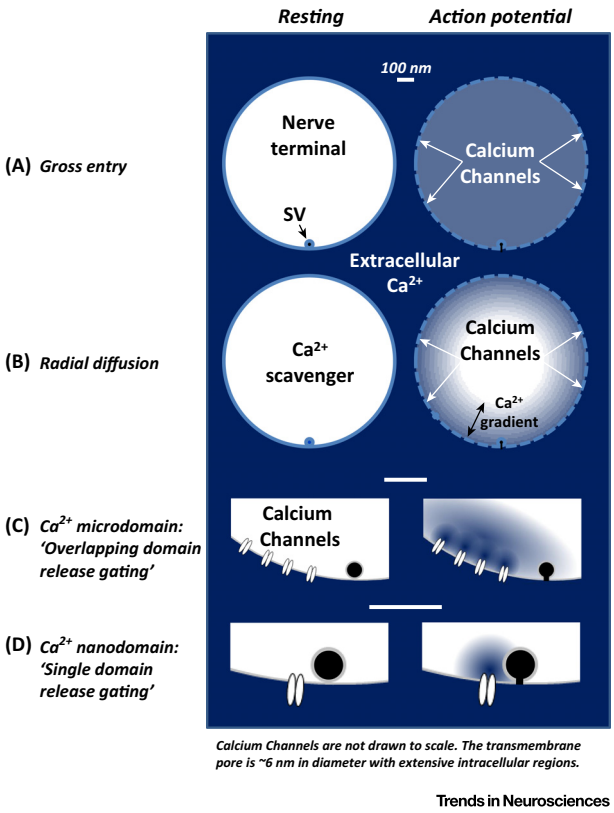


Figure 2. Models of Evoked Calcium Channel Gating of Synaptic Vesicle (SV) Fusion. (A) Gross entry. Voltage-dependent calcium channels (CaVs) are located randomly across the nerve terminal surface membrane in this basic model. Action potentials open the channels and the Ca^{2+} that enters equilibrates in the cytoplasm while binding to the SV sensor and triggering SV fusion. (B) Radial diffusion. Addition of a high-capacity and fast-binding cytoplasmic Ca^{2+} scavenger to the gross entry model results in a radial concentration gradient and a more rapid decline in free Ca^{2+} at release sites after the CaVs close. (C) Microdomain. Each open CaV generates a nanodomain of high Ca^{2+} concentration that declines steeply with distance from its pore. Overlap of many nanodomains from a cluster of open CaVs produces a Ca^{2+} 'microdomain' that is sufficiently large to reach and activate nearby SV sensors to gate fusion. (D) Single domain. The CaVs are located sufficiently close to the SV sensors that their individual Ca^{2+} nanodomains saturate the Ca^{2+}-binding sites to trigger SV fusion.

field at the time of Katz's monograph [5]. However, attempts to create a mathematical model of synaptic transmission based on work at the SGS led to the realization that that not only must there be an abrupt entry of Ca^{2+} to initiate transmitter release; the ion must then be removed rapidly for release to also terminate abruptly [34]. In the ensuing 'radial diffusion' model (Figure 2B), Ca^{2+} removal from the release site was achieved by linear diffusion of the ion toward the nerve terminal interior combined with its sequestration by cytoplasmic scavengers [34].

Microdomain Gating

An alternative to the radial diffusion model became necessary with the realization that although this model could account for Ca^{2+} dynamics during a single impulse, it could not do so for a high-frequency impulse train [35,36]. The suggestion that Ca^{2+} gradients might occur not only radially into the terminal but also laterally along the membrane surface first appeared in a mathematical model of release [12] and heralded the modern era of release site gating physiology.

The concept of the **microdomain** has its roots in the realization that a single open CaV generates a discrete plume of Ca^{2+} centered on its pore [37], now termed a **nanodomain**. This seminal concept served as the basis for the microdomain hypothesis (Figure 2C) in which the nanodomains from a tight cluster of individual CaVs overlapped and activated the SV sensors of nearby docked SVs (hence, **overlapping domain gating**) [36,38–40]. The microdomain concept in essence solved the issue of rapid Ca^{2+} clearance and survives as a model to explain release gating at least at some synapses [7,41,42].

Nanodomain Gating

Since the microdomain model could account for release physiology, a study using the newly discovered irreversible CaV2.2 blocker ω-conotoxin GVIA that proposed a far more intimate

release gating mechanism was met with some skepticism. Based on the principal of a parallel relationship between transmitter release and the number of active CaVs (Box 1), it was proposed that a single CaV could gate SV fusion if it was sufficiently close for its Ca^{2+} nanodomain to saturate all of the Ca^{2+}-binding sites on the SV sensor (Figures 2D and 3C) [23]. Some support for this '**single domain**' hypothesis was provided soon after at the SGS when the modeled Ca^{2+} current associated with action potential broadening during potassium channel block was related to transmitter output [43–45].

Cell-attached patch clamp recording from the transmitter release face at the chick CC [16] was used to test the single domain gating hypothesis directly. The membrane patch was depolarized to the CaV threshold to trigger single calcium channel current fluctuations while SV discharge was monitored using a luminescent assay for transmitter discharge (Figure 1B, lower-right panel). It was found that transmitter release remained time-locked to the single-channel current even when the channels were opening one at a time. Based on this finding it was concluded that a single channel can gate the fusion of a SV [17]. Integration of the time-locked calcium current predicted that SV fusion required the influx of less than 200 Ca^{2+}. It was also concluded that single-channel release gating requires that the two entities – the CaV and the SV sensor – must be linked by a molecular tether [17]. The single domain gating concept was formalized by a mathematical model based on a combination of data from the SGS and chick CC [46].

Single versus Overlapping Domain Gating

The single domain hypothesis held sway for a few years until findings at the nCoH argued strongly in favor of microdomain-based release gating [7,47–49]. The location of the nCoH in the rodent brain stem, and hence its potential to serve as a putative general model of release gating in the mammalian CNS, gave additional weight to these reports and initiated a period of stimulating single versus overlapping domain debate. To provide the necessary context for

Box 1. The CaV Titration Domain Gating Test

The principle of this test is to vary the number of activated CaVs (hence, titrate) and correlate this with the number of released SVs [39,85]. Multiple Ca^{2+} ions (n) must bind to activate the SV sensor. For simplicity we use $n = 4$ in Box 1 and Box 2 but $n = 5$ in most recently published models [28,29,36]. Where CaVs and SVs are remote from each other, the SV sensor can be activated only by a pooled microdomain from many channels (Figure IA). With recruitment of CaVs (top to bottom panels), the microdomain amplitude increases monotonically and the ion binds to a larger fraction of the SV sensor binding sites. However, the probability that all four sites on the same SV sensor will be occupied shows a curved relationship (Figure IA,C) and is described by $[CaV]^N$. Hence, in our illustrated example $N = 4$. Thus, for pure microdomain gating the experimentally determined N value is equal to (but cannot be greater) than n. However, if each SV sensor is located within the nanodomain of an individual CaV (Figure IB), all four binding sites can be saturated when each channel opens. It follows, then, that as more channels are recruited there is a parallel, or linear, increase in the number of activated SV sensors (Figure IB,C) and $N = 1$. The simplest method to analyze CaV titration is to plot the quantity of transmitter released against the number of open channels using double-logarithmic coordinates (Figure ID). The slope of a fitted unbroken line is a direct measure of the N value.

There are two main CaV titration assay variants. In the first, CaV recruitment is controlled by voltage clamp (CaV-TV). The protocol with the fewest ambiguities (avoiding confounding effects due to changes in membrane potential and hence Ca^{2+} driving force) is to trigger release using a family of Ca^{2+} tail currents generated by pulses of varying duration that depolarize the membrane to beyond the Ca^{2+} reversal potential (Figure 1B in main text, top-right panel). The amplitude of these tail currents is linearly related to the number of channels recruited and can then be plotted directly against the quantity of transmitter release [15].

The alternative method (CaV-TB) is to trigger release with an action potential while gradually blocking the channels [23] using either an irreversible blocker such as ω-conotoxin GVIA (CaV2.2) or ω-agatoxin GIVA (CaV2.1) or a pore-blocking cation with a relatively slow binding off rate. This method serves as a useful Ca titration method for synapses with small or dispersed terminals that are inaccessible to direct voltage clamp analysis.

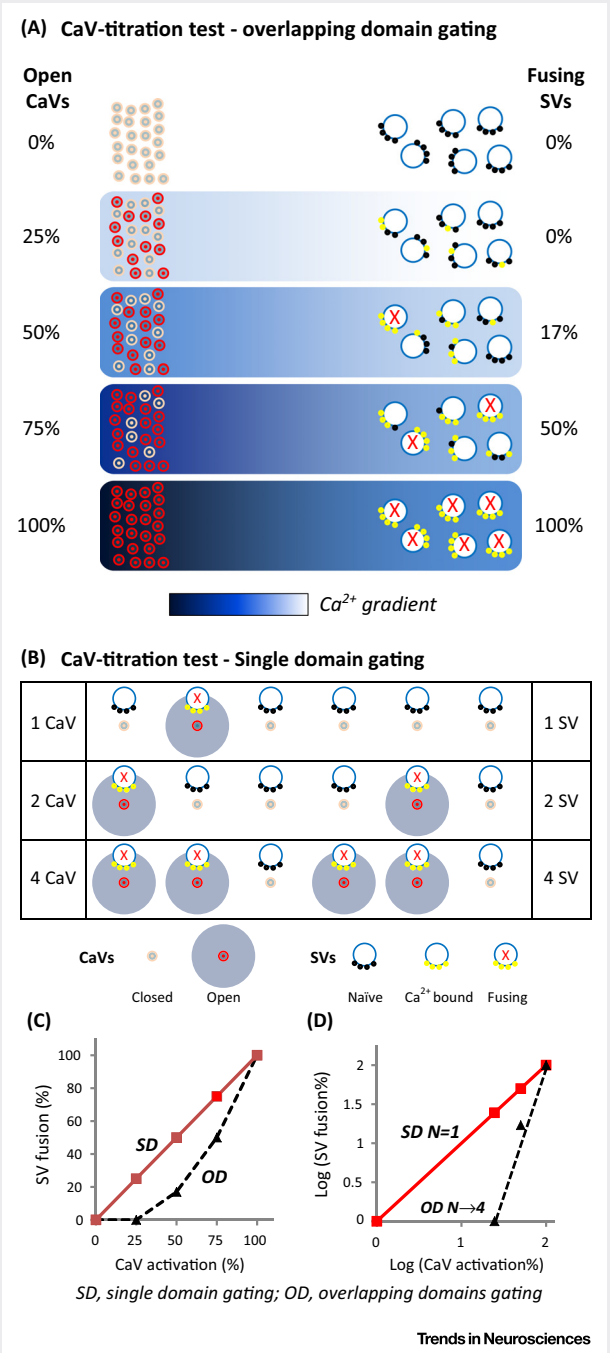


Figure I.

the issues involved we first cover the experimental strategies used to explore domain release gating mechanisms.

Experimental Tests

Two main approaches have been devised to distinguish between overlapping and single domain-based release site gating: titration of the number of activated CaVs (CaV titration) by

Box 2. The EGTA Block Domain Gating Test

To gate release, Ca^{2+} has to diffuse from the pore of the CaV to the SV sensor. The distance that Ca^{2+} can diffuse can be shortened by Ca^{2+} scavengers added to the cytoplasm. The domain gating test relies on the argument that the faster the scavenger Ca^{2+} capture rate, termed its 'on rate', the shorter the predicted ion diffusion distance [14]. Thus, BAPTA, a fast-on-rate scavenger, rapidly captures diffusing Ca^{2+} and inhibits release even when the channels are very close to the SV sensors. However, EGTA, with its much slower on rate requires more time to capture the ion and hence permits the ions to diffuse further. Thus, it inhibits release more effectively if the channels are located at some distance from the SV sensors. This is illustrated in Figure IA,B, where each pair of panels shows a large cluster of open CaVs located either distant from (top) or close to a field of docked SVs (for legend details, see Box 1 and Figure I in Box 1). The upper panel of each pair illustrates the concentration gradient of Ca^{2+} extending from the pooled nanodomains of the channel cluster microdomain. The lower panel is the same but illustrates the effect of cytoplasmic EGTA on the gradient and also transmitter release. While originally devised to test whether the Ca^{2+} that activates the SV sensor originates from a local or a distant source [14], the EGTA test has since been used to differentiate single and overlapping domain gating. A caution should be noted, however: the degree to which a slow-on-rate scavenger blocks release depends not only on its Ca^{2+} on rate but also on its concentration.

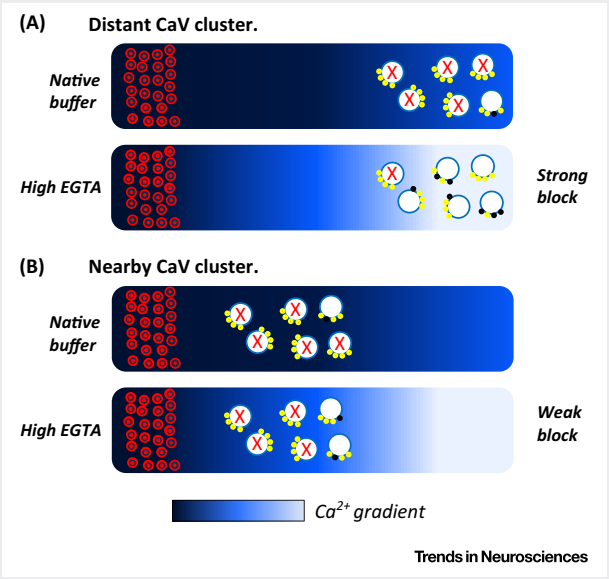


Figure I.

control of membrane voltage (CaV-TV) or by pharmacological block (CaV-TB) (Box 1) or the sensitivity of release to a Ca^{2+} scavenger with a slow capture rate – generally 10 mM EGTA (EGTA block; Box 2). Briefly, the shallower the relationship between the number of CaVs activated and the amount of transmitter release or the less sensitive transmission is to block by EGTA, the stronger the argument for release gating by single domains.

The nCoH and the Return of Nanodomain Release Gating

The demonstration of a steep CaV titration exponent and marked EGTA block at the nCoH argued in favor of overlapping and against single domain-based release [47]. Initially it was suggested that 60 or more channels must open to gate the fusion of each SV, but later this count was tempered to a microdomain of ten or more [7] and this hypothesis was supported by theoretical arguments arguing against the feasibility of nanodomain secretion [49].

The compelling evidence for overlapping domain-based secretion at the nCoH did not, however, result in a consensus in the field. The argument for single domain secretion at some synapses was reiterated in a review [9] and supportive evidence was obtained in a series of new reports. A study exploring the comparative role of CaV2.1 or CaV2.2 in release gating at the frog mitral

cell/amygdala neuron synapse reported a linear, not a cooperative, drop in transmitter release, implying single domain release [50]. Another report noted that action potentials triggered discrete and discernible single-channel activity at the frog NMJ (Figure 2D, lower-right panel) [25], a finding that was incompatible with extensive multichannel microdomains as predicted at the nCoH. Likewise, simultaneous pre- and postsynaptic patch clamp recording at the chick CC yielded a CaV titration exponent close to 1 [15] (Box 1 and Table 1), also favoring single domain-based release gating.

The CoH 'Developmental Shift'

A report that release gating exhibits considerably different characteristics in young adult rodents than in the neonate [30] sent shockwaves through the field. While overlapping domains could be confirmed in the nCoH, both the EGTA block and CaV titration tests demonstrated 'tightening' of the CaV–SV relationship in older animals (Figure 1D, lower-right panel). This was confirmed in several reports, albeit to varying degrees [51–53]. The switch in release physiology coincided with changes in other properties such as CaV types [54], action potential duration and release kinetics [55,56], and transmitter release probability [57]. The realization that these all correlated with the onset of hearing suggested that the nCoH represents a developmental stage of the fully formed calyx. Age-related 'release site tightening' has also been reported at hair cell synapses (Table 1) [58,59], raising the possibility that it represents a general step in fast-synapse development.

A recent study at the CoH correlated localization of individual CaVs in presynaptic release-face freeze-fracture replicas with assessment of domain activation using the EGTA block test [53]. It was concluded that CaVs exist in clusters of varying sizes that were larger and less compact in the adult than the neonate. While staining efficiency was likely to underestimate the total number, the more effective of two antibody batches identified around seven active-zone channels at the nCoH and ~12 at the CoH. Modeling studies based on both release probability and kinetics predicted that the SVs are <30 nm from the nearest CaV member of a cluster. The overall conclusion of the study was that fusion was gated at the edge of an overlapping domain generated by the channel cluster, termed the perimeter release model, and that the probability of gating by a single channel was very low. However, a recent modeling study argued that if the SV sensor is within the nanodomain of any individual CaV, release probability should be dominated by single-channel domains [60]. Further studies will be necessary to reconcile these interpretations.

Transmitter Release Gating and CaV Distribution

Exploration of release gating at the molecular level reveals interesting features and counterintuitive properties and differences for single and overlapping domain release gating. These include the surprisingly long reach of a single CaV domain, an 'overlap bonus' in release probability with multiple channels, and the impact of presynaptic CaV-open probability on the efficiency of release gating by nano- and microdomains.

The Long Reach of the Single Domain

It might be presumed that, because of the steeply declining Ca^{2+} concentration close to the pore, nanodomain gating requires the CaV to be very close, within ~20 nm of the SV sensor, to activate fusion. While the nanodomain profile of a presynaptic-type CaV2-family channel under physiological Ca^{2+} gradients [61] does exhibit a rapid decline close to the pore, there is still a significant concentration of the ion even 100 nm away (Figure 3A). Using published binding and activation properties of the SV sensor [28] (Figure 1D, upper panel), one can predict the release probability of a nearby SV. For example, the release probability of a SV located 30 nm from an open CaV is ~0.3 (Figure 3B) while one that is as far away as 50 nm is ~0.1% and hence still biologically significant [60,62].

Table 1. Comparative Biology of Domain Gating

Synapse	Species	Transmitter/ Transmission Type	SD or OD Gating[a]	Assay Method[b]	Refs
Neuron–Neuron Synapses					
Giant synapse	Squid	Glutamate/fast	SD	CaV-T[c]	[12]
				CaV-T[d]	[42]
CC	Chick	ACh/fast	SD	OM[e]	[17]
			SD	CaV-T-V	[15]
Mitral–amygdala	Frog	Glutamate/fast	SD	CaV-T-B	[9]
CoH[f]	Mouse	Glu/fast	SD > OD[g]	CaV-TV, EGTA-B	[30]
	Rat	Glu/fast	OD > SD[h]	CaV-TV	[51]
			OD >> SD[i]	EGTA-B	[52]
Retinal All-amacrine neuron	Mouse	Glycine	SD ~ OD[j]	EGTA-B	[54]
Hippocampal mossy fiber	Rat	Glutamate/slow, facilitating	OD[k]	CaV-TV, EGTA-B	[71]
Cortical inhibitory basket cell	Rat	GABA/fast	SD SD > OD	EGTA-B CaV-TB	[68] [69]
Cortical pyramidal tufted (facilitating)	Rat[l]	Glutamate/fast	OD > SD	EGTA-B	[66]
Cortical pyramidal bipolar (depressing)	Rat[l]	Glutamate/fast	SD > OD	EGTA-B	[66]
Cortical pyramidal	Mouse	Glutamate/fast	SD	EGTA-B	[67]
Hippocampal Schaffer collateral	Rat	Glutamate/fast	SD	CaV-TB[m]	[70]
Neuron–Effector Synapse					
NMJ	Frog	ACh/fast	SD	CaV-TB	[23]
			SD	OM[n]	[25]
			SD	CaV-TB	[82]
			SD	CaV-TB	[24]
Ribbon Synapses					
Auditory hair cell	Mouse	Glutamate/fast	SD	CaV-TV	[62]
			SD	CaV-TV	[55]
	Rat		SD	CaV-TV	[63]
	Bullfrog		SD[o]	CaV-TV	[83]
Retinal rod	Mouse	Glutamate/tonic depolarization	SD	CaV-T[p]	[8]
Spontaneous Quantal Transmitter Release[q]					
Hippocampal granule cell	Rat	Inhibitory/random	OD	EGTA-B	[72]
CoH	Rat	Excitatory/random	OD	OM	[73]

[a]SD, single domain gating; OD, overlapping domain gating.
[b]CaV-T, CaV titration; CaV-TV, voltage clamp variant; CaV-TB, channel block method (Box 1); EGTA-B, EGTA block method (Box 2); OM, other method.
[c]Hysteresis in the presynaptic–postsynaptic input–output relation data was prescient for SD gating.
[d]'Spike broadening' method, simulated calcium current.
[e]Cell-attached presynaptic transmitter-release face recording: correlation of single CaV channel openings with SV fusion.
[f]Mature calyx data only; synapse exhibits neonatal–mature release site tightening.
[g]Significant SD fraction.
[h]Small SD fraction.
[i]No significant SD (see discussion above).
[j]Synapse exhibits neonatal–mature release site tightening.
[k]Unusual SV sensor Ca^{2+} activation properties.
[l]13-day rat: possibly pre-release site tightening.
[m]Slow versus fast off rate divalent ion blockers.
[n]Single CaV nanodomain imaging (see discussion above).
[o]Data consistent with single domains but not stated.
[p]Linear dependence of release on CaV recruitment during tonic depolarizations.
[q]Included for comparison.

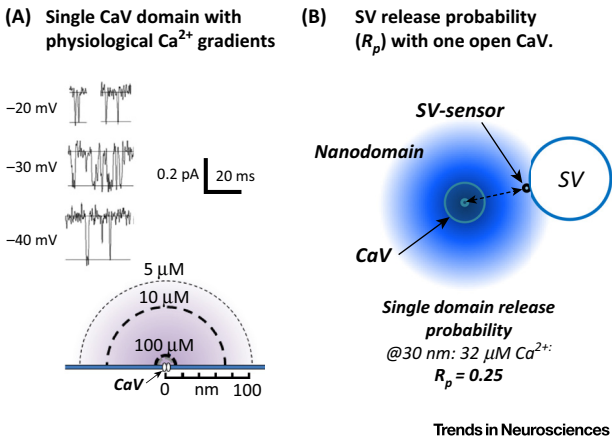


Figure 3. Essential Elements for the Modeling of Ca^{2+} Domain Transmitter Release Gating. (A) Quantitation of the single-channel domain. Upper panel: Examples of single voltage-dependent calcium channel (CaV) type 2.2 fluctuations recorded at the indicated membrane potentials with a physiological 2-mM extracellular Ca^{2+} concentration [61]. Lower panel: Single-channel domain modeled at 0.5 ms after channel opening based on the inward Ca^{2+} current amplitude at −65 mV (as for the repolarization phase of the action potential) with 50 μM of fast cytoplasmic Ca^{2+} buffer. The 5-, 10-, and 100-μM concentration domes are marked [61]. (B) Model of synaptic vesicle (SV) fusion gating with one open CaV. SV 'release probability' (R_P) calculated using the Ca^{2+} domain in (A) [61]. With the CaV located 30 nm away, the calculated Ca^{2+} concentration at the SV sensor is 32 μM and the R_P is 0.25 [60]. Modifications have been made to figures for presentation purposes.

CaV–SV Distance and the Release Gating Overlap Bonus

If there are multiple channels, the probability of gating SV fusion during an action potential will reflect two mechanisms: first the probability of gating by each channel single domain, plus an additional overlap bonus due to the summed Ca^{2+} from both channels and the steep relationship between cytoplasmic Ca^{2+} and SV sensor activation. The overlap bonus is small if the channels are very close to the SV sensor, as release is dominated by single domains, but becomes progressively larger as the channels are located further away [60]. However, note that the 'overlap bonus' will only occur if multiple channels are open at the same time and since the open probability of presynaptic CaVs is low (about 0.2 [53]) the overlap bonus will not be significant unless there is a large number of CaVs within range of the SV sensor [60].

Domain Gating and Ca^{2+} Entry

Ca^{2+} is maintained at very low concentrations in the cell to enable its use in various signaling pathways and also because high concentrations are toxic [63]. As discussed above, single domain-activated SV release requires the influx of only (or fewer than) ~200 Ca^{2+} ions, which corresponds to the inward current during a single flicker of a presynaptic-type CaV [8]. However, because of the diffusion distance, vastly more Ca^{2+} must enter for effective gating by remote channels. Thus, release gating using single domains in effect can guarantee that influx of the ion is held to a minimum. Not only does this maintain the signal-to-noise ratio for Ca^{2+} signaling pathways and avoid the toxic effects of high intracellular Ca^{2+} levels, it has the additional benefit of reducing the energetic demands necessary for ion extrusion [8,17].

Single Domain Gating and SV Tethering

The finding that a single CaV could gate SV fusion implied that the channel and SV must be linked by a tether [17]. The biochemical nature of this link is an area of current interest but is outside the scope of this review. However, evidence that the CaV–SV distance can shorten (Figure 1C, lower-left panel) [64] raises the intriguing possibility that changes in tether length, moving the SV sensor closer to or further from the channel pore, could serve as a mechanism to modulate synaptic strength.

Domain Gating and Synapse Types

Studies on the exemplar synapses detailed above led to the innovation and refinement of experimental methods to differentiate single and overlapping domain gating. Recently these methods and innovative variants have been adapted to evaluate domain gating at synapses with far less accessible presynaptic terminals. In effect, the field has moved into a new era of 'comparative biology' and 'functional adaptation', as illustrated in the following examples (see also Table 1).

Sensory Cell–Neuron Ribbon Synapses

Sensory cells, adapted for rapid and sustained release of transmitter onto a postsynaptic afferent nerve, typically utilize **ribbon synapses** that exhibit a specialized intracellular structure associated with SV transport. The role of these cells in sensation, the ribbon itself, and gating by CaV1 and not, as at classical neuronal synapses, CaV2-family calcium channels make ribbon synapses an interesting functional contrast to neuronal presynaptic terminals.

Auditory Hair Cell

Auditory hair cell synapses are capable of transmitting submillisecond-interval one-to-one action potential trains. At the inner hair cell synapse active zone, action potentials have been estimated to open fewer than ten of the ~80 available CaVs [65], a finding that is consistent with the virtually exclusively single domain gating as concluded in several recent studies (Figure 4A and Table 1) [59,65,66].

Retinal Rod

In contrast to the hair cell, release at the retinal rod ribbon synapse is activated by tonic depolarization. Nonetheless, SVs have been reported to be gated primarily by nanodomains [67], with the tight linkage providing the high gain and broad dynamic range required for the detection of faint or broadly varying light. Interestingly, single domain release gating persisted even for multivesicular release gated by strong depolarizations [67].

Neuron–Effector Endplate

The frog NMJ is discussed above, but the simplicity of the synapse combined with the structural homogeneity of the release sites has permitted a recent detailed structure–function correlation and the creation of a highly constrained mathematical model [24,68,69]. In addition to providing further support for single domain-based release gating at this synapse (Figure 4B), these studies predict a channel-to-SV-sensor distance of 23 nm [24,68]. These parameters have been used to predict that the SV sensor of a docked SV is located at the contact point of the vesicle with the surface membrane [60].

Neuron–Neuron Fast Synapses

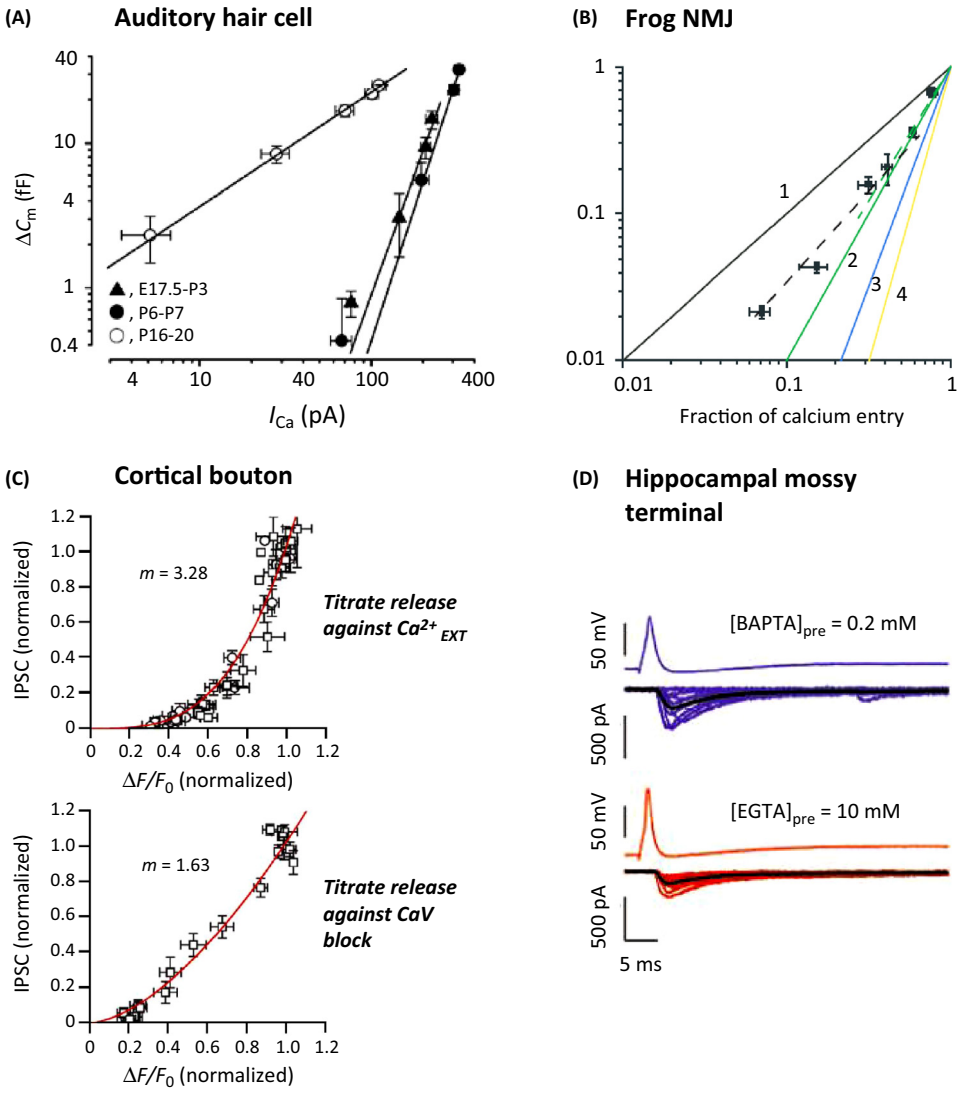
Although only a few of the vast range of neuron–neuron synapses have been explored with respect to domain gating, they have already provided intriguing evidence of diversity and specialization.

Retinal All-Amacrine Neuron

Transmitter release at the All-amacrine neuron–neuron synapse in the mammalian retina [58] is gated by CaV1-family channels, as in the sensory cell, but without a discernible release site ribbon. EGTA block was marked in young rats, consistent with gating by remote CaVs, but declined with development as the eye opened, similar to the nCoH–CoH switch (see above). Much reduced but still significant EGTA sensitivity in the adult suggests a mixture of single and overlapping domain gating [58].

Cortical Synapses

Application of the EGTA block test to pyramidal cell/target neuron synapses suggested heterogeneity in release gating: the facilitating contacts onto bi-tufted interneurons exhibited EGTA



Trends in Neurosciences

Figure 4. Domain Gating at Diverse Synapse Types. (A) Auditory hair cell. The hair cell serves as a presynaptic terminal at this synapse and is sufficiently large to permit direct whole-cell patch clamp analysis. Synaptic vesicle (SV) fusion was detected as an increase in surface membrane area, as monitored by capacitance. A steep voltage-dependent calcium channel (CaV) release relationship in young animals (filled symbols), indicative of microdomain-based gating, developed into a shallow, nanodomain-based one in the adult (open circles; see Box 1) [59]. (B) Frog neuromuscular junction. CaV activity was monitored by imaging Ca^{2+} influx through individual CaVs at high spatial and temporal resolution (Figure 1C, lower-right panel) while monitoring transmitter release by recording directly from the muscle fiber. The pharmacological block variant of the CaV titration method (Box 1) demonstrated a shallow, near-unitary slope (broken line; unbroken lines indicate predicted first-, second-, third-, and fourth-power relationships, as indicated), indicative of nanodomain-based release gating [24]. (D) Cortical inhibitory bouton synapses. The Ca^{2+} dependence of the SV sensor (n) was determined by titrating extracellular Ca^{2+} while monitoring action potential-triggered Ca^{2+} influx ($\Delta F/F0$) and the amplitude of the inhibitory postsynaptic current (upper panel). CaV titration analysis was conducted by gradually reducing the number of available CaV2.1 channels with low-concentration ω-agatoxin-IVA (lower panel). The shallow dependence of the transmitter release on CaV (m = 1.63) supports nanodomain-dominated transmitter release [73]. (D) Hippocampal mossy fiber synapse. Domain gating was explored using the EGTA block method. The top trace of each pair shows the presynaptic action potential (which remained constant with either treatment) while the bottom pair shows the excitatory postsynaptic current used to monitor transmitter release. Intracellular BAPTA, a fast-binding Ca^{2+} scavenger, was used as a positive control and caused a marked block (upper panel). A similar block was obtained with intracellular EGTA, a Ca^{2+} scavenger with a much slower binding rate (lower panel; see Box 2), arguing for microdomain-based release gating [75].

block, consistent with overlapping domains, whereas depressing contacts onto multipolar cells were resistant to the Ca^{2+} scavenger, favoring single domain gating [70]. The studies were, however, conducted on young rats, with the possibility of incomplete release site tightening in the former. Compelling evidence for nanodomain gating has been obtained at pyramidal–granule cell synapses with an estimated coupling distance of less than 30 nm [71].

Hippocampal Neurons

Basket cell inhibitory synapses in adult rats exhibited high resistance to EGTA block and low CaV block values (Figure 4C), arguing for very tight domains with the channel situated within 20 nm of the SV sensors [72,73]. Similar results were reported for the excitatory CA3–CA1 Shaffer collateral synapse based on CaV titration and Ca^{2+} imaging methods, suggesting that the SVs at this synapse are gated virtually exclusively by single CaVs [74].

Analysis of transmission at the hippocampal mossy fiber–CA3 synapse has provided the best evidence to date of overlapping domain release gating at mature synapses (Figure 4D), with the CaVs predicted to be >70 nm from the SV sensors [75]. This terminal has, however, several properties that distinguish it from the typical fast-transmitting synapse. These include relatively slow transmission, pronounced facilitation, and a curiously low SV sensor Ca^{2+} *n* value, leading the authors to suggest that it represents a presynaptic contact specialized for modulation [75].

Spontaneous Transmitter Release

Although outside the scope of this review, it is interesting to note that two reports conclude that at synapses where CaVs play a role in spontaneous SV fusion gating, they are located distant from the SV sensors [76,77].

Concluding Remarks

The study of transmitter release **nanophysiology** stands as a classic example of scientific discourse. Katz's calcium gating hypothesis [5] remains intact but the small step within it – the flight of Ca^{2+} from the CaV to the SV sensor – has resulted in several competing hypotheses. While there is compelling evidence that release can be gated by either Ca^{2+} nano- or microdomains, accumulating evidence from an increasing variety of fast-synapse preparations indicates that single domain gating is the norm. Single, as compared with overlapping, release gating has fundamentally different implications for the physiology and structural organization of the presynaptic transmitter release site. One requirement is that the SV must be located within range of the channel, presumably by a tether or anchor mechanism [17]. Recent evidence indicates that the channel can link directly to the SV independently of the surface membrane [78]. Identifying the molecular composition of this link is, perhaps, the most pressing next challenge for transmitter release biology (see Outstanding Questions).

Outstanding Questions

The following hypotheses may be worthy of further exploration.

Functional adaptation. Hypothesis: Presynaptic terminals that primarily code output frequency, such as faithful transmission of action potentials, favor single domain gating while synapses that code output amplitude, such frequency facilitation, favor overlapping domains. Thus, the former may be adapted primarily for accurate information transfer and the latter for variations in synaptic strength.

Tether length and SV release probability. Hypothesis: Synaptic strength can be modulated by alterations in the channel-to-vesicle tether length. Because of the steep concentration gradient of the nanodomain, the strength of single domain gating is strongly dependent on the distance between the channel pore and the SV sensor. Thus, SV release probability could be modulated both transiently and in the long term by factors that alter tether length (evidence for tether shortening has been reported; Figure 1C in main text, lower-left panel).

A CaV–SV molecular complex. Hypothesis: Synaptic vesicles are tethered to the calcium channel by a molecular link to ensure the close spacing required for single domain gating. Such a link is necessary to ensure that the SV sensor is located within the channel nanodomain.

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