

# Roles and Sources of Calcium in Synaptic Exocytosis



Zhao-Wen Wang, Sadaf Riaz, and Longgang Niu

**Abstract** Calcium ions ( $\text{Ca}^{2+}$ ) play a critical role in triggering neurotransmitter release. The rate of release is directly related to the concentration of  $\text{Ca}^{2+}$  at the presynaptic site, with a supralinear relationship. There are two main sources of  $\text{Ca}^{2+}$  that trigger synaptic vesicle fusion: influx through voltage-gated  $\text{Ca}^{2+}$  channels in the plasma membrane and release from the endoplasmic reticulum via ryanodine receptors. This chapter will cover the sources of  $\text{Ca}^{2+}$  at the presynaptic nerve terminal, the relationship between neurotransmitter release rate and  $\text{Ca}^{2+}$  concentration, and the mechanisms that achieve the necessary  $\text{Ca}^{2+}$  concentrations for triggering synaptic exocytosis at the presynaptic site.

**Keywords** calcium · exocytosis · neurotransmitter release · calcium channel · ryanodine receptor · mitochondrion

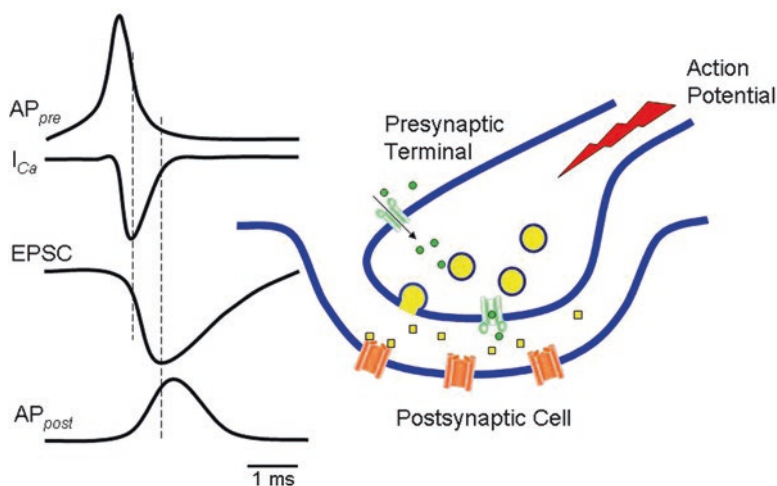
## 1 Introduction

Calcium ions ( $\text{Ca}^{2+}$ ) are involved in various biological functions. One of the most well-known functions is their role in triggering neurotransmitter release at the presynaptic nerve terminal. Over a century ago, Locke discovered that the transmission between nerves and muscles is significantly affected by the presence of  $\text{Ca}^{2+}$  in the surrounding medium [1]. Later research conducted by many others demonstrated that extracellular  $\text{Ca}^{2+}$  is crucial for evoked neurotransmitter release and that its concentration influences the amplitude of end-plate potentials recorded from muscle cells [2–5].

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Neurotransmitters can be released spontaneously or in response to action potentials. In general, spontaneous release occurs when individual synaptic vesicles undergo exocytosis, while action potential-evoked release reflects synchronized exocytosis of multiple synaptic vesicles. The role of  $\text{Ca}^{2+}$  in action potential-evoked release is well established. When an action potential reaches the nerve terminal, depolarization opens voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs) in the plasma membrane, resulting in an influx of  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  can also originate from the endoplasmic reticulum (ER) through a coupling between VGCCs in the plasma membrane and ryanodine receptors (RyRs) in the ER membrane [6, 7].  $\text{Ca}^{2+}$  then binds to  $\text{Ca}^{2+}$ -sensing proteins to trigger synaptic exocytosis (see Chapter “[Calcium Sensors of Neurotransmitter Release](#)”). The released neurotransmitters act on specific postsynaptic receptors, resulting in either excitatory or inhibitory currents in the postsynaptic cell, depending on the types of neurotransmitter and postsynaptic receptor, as well as physiological or experimental conditions. The temporal relationships among the presynaptic action potential, presynaptic  $\text{Ca}^{2+}$  current, and excitatory postsynaptic current (EPSC) are depicted in Fig. 1.  $\text{Ca}^{2+}$  release from the ER is not included in this figure because its kinetics are not well defined. As illustrated in the figure,  $\text{Ca}^{2+}$  influx begins around the peak of the action potential and ends before the nerve terminal is fully repolarized. Neurotransmitter release, as reflected by the postsynaptic currents, occurs with a further delay.



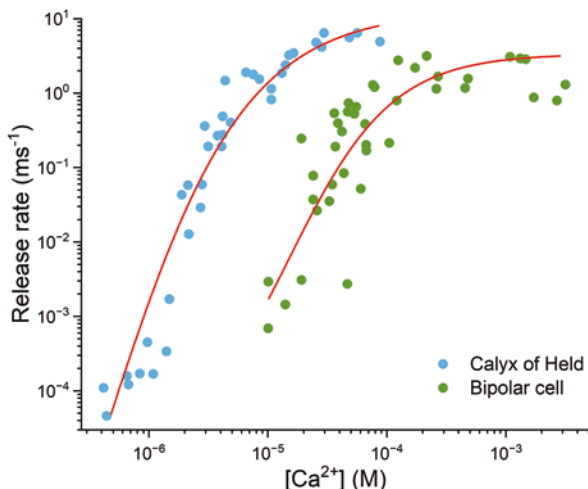
**Fig. 1** Pre- and postsynaptic events in response to a presynaptic action potential. Depolarization of the presynaptic terminal by the action potential ( $\text{AP}_{\text{pre}}$ ) causes  $\text{Ca}^{2+}$  influx ( $I_{\text{Ca}}$ ) through voltage-gated  $\text{Ca}^{2+}$  channels in the plasma membrane.  $\text{Ca}^{2+}$  triggers the release of a neurotransmitter, which causes excitatory postsynaptic current (EPSC) by activating ionotropic postsynaptic receptors. An action potential may occur in the postsynaptic cell ( $\text{AP}_{\text{post}}$ ) if the cell is depolarized beyond a threshold by the EPSC. (This schematic figure is based on published data [194, 195])

## 2 Neurotransmitter Release Rate Is Supralinearly Related to $\text{Ca}^{2+}$ Concentration

The rate of neurotransmitter release is quantitatively related to the concentration of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ). Dodge and Rahamimoff [4] demonstrated that at the frog neuromuscular junction (NMJ), the rate of neurotransmitter release increases with elevated extracellular  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_o$ ). However, the relationship is not linear. When  $[\text{Ca}^{2+}]_o$  is relatively low, a small change in concentration results in a large change in neurotransmitter release, as reflected by the amplitude of end-plate potentials. Plotting the results in double-logarithmic coordinates yields a straight line with a slope of approximately 4, leading to the conclusion that the rate of neurotransmitter release is proportional to  $[\text{Ca}^{2+}]_o$  raised to the fourth power (Release rate  $\propto [\text{Ca}^{2+}]_o^4$ ).

The work of Dodge and Rahamimoff showed the quantitative relationship between neurotransmitter release rate and  $[\text{Ca}^{2+}]_o$ . However, the  $\text{Ca}^{2+}$  sensor of the synaptic release machinery is located inside the presynaptic nerve terminal. Thus, intracellular rather than extracellular  $\text{Ca}^{2+}$  triggers neurotransmitter release. What would be the relationship between neurotransmitter release rate and cytoplasmic  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_i$ )? This relationship has been analyzed with a technique called  $\text{Ca}^{2+}$ -uncaging. In this technique, a photolysable  $\text{Ca}^{2+}$ -chelator such as DM-nitrophen is introduced into the presynaptic nerve terminal through a whole-cell patch-clamp glass pipette.  $\text{Ca}^{2+}$  is released from the photolysable  $\text{Ca}^{2+}$ -chelator upon flash of an ultraviolet light, resulting in a rapid and uniform increase of  $[\text{Ca}^{2+}]_i$  at the presynaptic nerve terminal. The level of  $[\text{Ca}^{2+}]_i$  at the presynaptic nerve terminal may be controlled by varying the light intensity and measured by imaging with a low-affinity fluorescent  $\text{Ca}^{2+}$  indicator. The rate of neurotransmitter release is evaluated by measuring either membrane capacitance of the presynaptic terminal, which increases when the plasma membrane area enlarges with synaptic vesicle fusion, or the amplitude of excitatory postsynaptic potentials or currents, which reflect postsynaptic responses to the released neurotransmitter. This  $\text{Ca}^{2+}$ -uncaging technique is apparently suitable for analyzing the relationship between release rate and  $[\text{Ca}^{2+}]_i$  because it triggers release of the same pool of synaptic vesicles as do action potentials [8]. Analyses of several nerve terminals, including the goldfish retinal bipolar cell synaptic terminal [9] (Fig. 2), crayfish motor neuron terminal [10], and rat calyx of Held presynaptic terminal [8, 11] (Fig. 2), have revealed a non-linear dependence of the release rate on  $[\text{Ca}^{2+}]_i$  with a slope of approximately 3–4 in plots with double-logarithmic coordinates, which resembles the relationship between the release rate and  $[\text{Ca}^{2+}]_o$  [4].

The reason why neurotransmitter release rate is supralinearly related to  $[\text{Ca}^{2+}]_i$  is not entirely clear. Several mechanisms have been suggested to explain the apparent  $\text{Ca}^{2+}$  cooperativity. One possible explanation is that the  $\text{Ca}^{2+}$  binding properties of synaptotagmin could be responsible for the cooperativity [12]. A minimal kinetic model, based on flash-photolysis data from the calyx of Held synapse, indicates that five identical  $\text{Ca}^{2+}$ -binding steps are required before synaptic vesicle fusion [8, 11]. The total number of  $\text{Ca}^{2+}$  binding sites in synaptotagmin I matches the number of

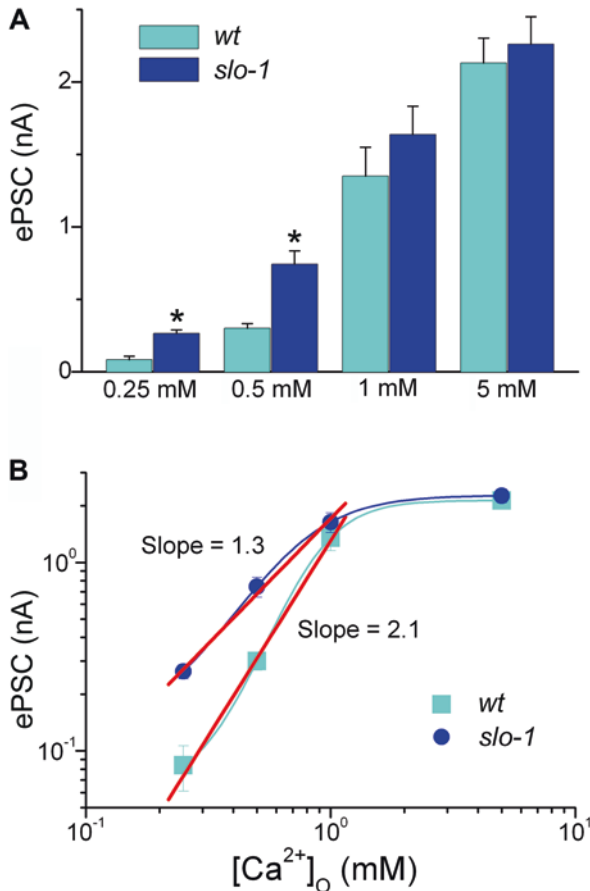


**Fig. 2** Relationship between  $Ca^{2+}$  concentration and neurotransmitter release rate in the calyx of Held and goldfish retinal bipolar cell presynaptic terminals. The graph shows that the rate of neurotransmitter release is proportional to the fourth power of  $Ca^{2+}$  concentration and that the sensitivity to  $Ca^{2+}$  differs by approximately one order of magnitude between the two synapses. (The graph was generated from published figs [9, 11] using the Digitizer tool of Origin Pro software (OriginLab Corporation))

$Ca^{2+}$  binding steps in this proposed  $Ca^{2+}$  binding kinetic model [12]. Additionally,  $Ca^{2+}$  cooperativity for the fast synchronous release component is eliminated in *Drosophila* synaptotagmin null or C2B domain-deletion mutants [13, 14]. Another explanation is that the apparent  $Ca^{2+}$  cooperativity may reflect the mean number of SNARE complexes that mediate a vesicle fusion, since SNARE complexes can form oligomers of 3–4 [15]. Another possibility is that SNARE proteins might be responsible for  $Ca^{2+}$  cooperativity, as mutations of either syntaxin 1A or synaptobrevin reduces  $Ca^{2+}$  cooperativity at the *Drosophila* NMJ [16]. It is also possible that the overlapping of  $Ca^{2+}$  micro- or nanodomains at the active zone might contribute to the  $Ca^{2+}$  cooperativity [17], or that the saturation of cytoplasmic  $Ca^{2+}$  buffer(s) might contribute to the supralinearity [18]. Moreover, the function of presynaptic BK channels might affect the apparent  $Ca^{2+}$  cooperativity, as seen in analyses of neuromuscular transmission in wild-type and *slo-1* (BK channel) mutants of *Caenorhabditis elegans*. In the *slo-1* loss-of-function (*lf*) mutant, the apparent  $Ca^{2+}$  cooperativity is decreased because SLO-1 dysfunction increases neurotransmitter release at low but not high  $[Ca^{2+}]_o$  [19]. These proposed models suggest that the molecular basis of  $Ca^{2+}$  cooperativity is still poorly understood and may be attributable to more than one mechanism.

The relationship between  $[Ca^{2+}]_i$  and neurotransmitter release rate can vary between different synapses, as illustrated by several examples. At some synapses, such as the squid giant synapse [17] and chick ciliary ganglion synapse [20], the apparent  $Ca^{2+}$  cooperativity is approximately 1, indicating a linear relationship between  $[Ca^{2+}]_i$  and release rate. At other synapses, including the excitatory synapse

between sensory afferent fibers and motoneurons in rat lumbar spinal cord [21] and the synapse between mossy fiber boutons and granule cells in rat cerebellum [22], the apparent  $\text{Ca}^{2+}$  cooperativity ranges from 1.2 to 1.6. In other cases, such as the rat calyx of Held synapse, activation of protein kinase C can change the apparent  $\text{Ca}^{2+}$  cooperativity from  $\sim 4$  of the control level to  $\sim 3$  [23]. The apparent  $\text{Ca}^{2+}$  cooperativity derived from evoked postsynaptic currents at the *C. elegans* NMJ is 2.1, which is reduced to 1.3 when the *slo-1* BK channel is mutated (Fig. 3) [19]. Therefore, the relationship between  $[\text{Ca}^{2+}]_i$  and neurotransmitter release rate can be complex and vary between different synapses and between different experimental conditions.



**Fig. 3** The apparent  $\text{Ca}^{2+}$  cooperativity is decreased in *C. elegans slo-1* (BK channel) mutants. (a) The amplitude of evoked postsynaptic currents (ePSC) was significantly increased at the *C. elegans* neuromuscular junction in a *slo-1* null mutant at 250 or 500  $\mu\text{M}$  but not 1 or 5 mM  $[\text{Ca}^{2+}]_o$ . The asterisk indicates a statistically significant difference compared with the wild-type (WT). This figure was adapted from reference [19]. (b) The same data as in (a) but plotted using logarithmic coordinates, showing that the apparent  $\text{Ca}^{2+}$  cooperativity, as indicated by the slope factor from a linear fit, was decreased in the *slo-1* mutant. These findings suggest that SLO-1 plays a role in the regulation of synaptic transmission

Another divalent cation that has been implicated in controlling neurotransmitter release under experimental conditions is  $Mg^{2+}$ . However, unlike  $Ca^{2+}$ ,  $Mg^{2+}$  inhibits release. An increase in  $[Mg^{2+}]$  reduces the  $Ca^{2+}$  sensitivity of neurotransmitter release [4]. It has been suggested that  $Mg^{2+}$  antagonizes the function of  $Ca^{2+}$  through competitive effects [21, 24], although the exact mechanism is unclear. One possibility is that  $Mg^{2+}$  blocks  $Ca^{2+}$  entry through membrane  $Ca^{2+}$  channels [25, 26]. In addition,  $Mg^{2+}$  has been shown to activate the BK channel when applied to the cytoplasmic side in inside-out membrane patches [27–29]. Because the BK channel is an important negative regulator of neurotransmitter release [30, 31] (also see Chapter “Regulation of Neurotransmitter Release by  $K^+$  Channels”), an increase in  $[Mg^{2+}]$  could potentially downregulate neurotransmitter release via the BK channel. However, it should be noted that a relatively high  $[Mg^{2+}]_i$  is needed to activate the BK channel [28, 29], making it questionable whether  $[Mg^{2+}]_i$  can reach sufficiently high levels to regulate the function of presynaptic BK channels in neurons.

### 3 $Ca^{2+}$ Concentrations Required for Neurotransmitter Release Vary from Synapse to Synapse

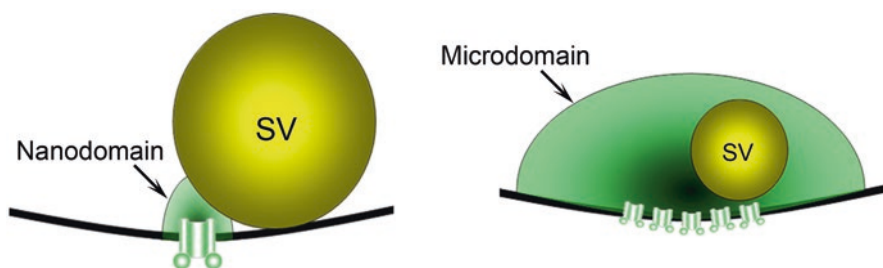
The fast phase of action potential-induced neurotransmitter release requires relatively high concentrations of  $Ca^{2+}$ . For example, at the squid giant synapse terminal, studies on the effects of intraterminally injected  $Ca^{2+}$  chelators with different affinities suggest that several hundred micromolar  $[Ca^{2+}]$  may be necessary to trigger neurotransmitter release [32]. Similarly, at the goldfish retinal bipolar neuron synaptic terminal,  $Ca^{2+}$ -uncaging experiments indicate that a minimum of  $\sim 10 \mu M$   $[Ca^{2+}]$  is required to initiate neurotransmitter release, and  $\sim 200 \mu M$   $[Ca^{2+}]$  is needed to achieve release at half maximal rate [9]. In permeabilized synaptosomes prepared from rat cerebral cortex, glutamate release has a threshold of  $\sim 50 \mu M$   $[Ca^{2+}]$ , with half-maximal and maximal release occurring at 200–300  $\mu M$  and 1 mM  $[Ca^{2+}]$ , respectively [33]. These findings suggest that action potential-evoked neurotransmitter release at these synapses may require hundreds of micromolar  $[Ca^{2+}]$ . However, at the calyx of Held glutamatergic terminal,  $Ca^{2+}$ -uncaging experiments suggest that 10–25  $\mu M$   $[Ca^{2+}]$  is sufficient to induce the peak release rate [34] or to mimic the release caused by action potentials [8, 11]. Thus, the concentration of  $Ca^{2+}$  required for fast neurotransmitter release can vary as much as one order of magnitude among different synapses (Fig. 2).

### 4 $Ca^{2+}$ Forms High Concentration Domains at the Presynaptic Terminal

The increase in presynaptic  $[Ca^{2+}]_i$  following an action potential is typically small compared to the concentration required for fast neurotransmitter release, as evidenced by studies at the squid giant synapse, where presynaptic  $[Ca^{2+}]_i$  increases by

5 nM from a resting level of approximately 50–100 nM [17]. Therefore, the spatially averaged  $[Ca^{2+}]_i$  following an action potential is much lower than that required for fast neurotransmitter release. Mathematical modeling from the 1980s suggests that  $[Ca^{2+}]_i$  does not change uniformly in response to action potentials but forms hemispheric high-concentration domains (<50 nm in radius) around the open  $Ca^{2+}$  channels [35–37]. These domains, resulting from the entry of  $Ca^{2+}$  through single channels, are known as  $Ca^{2+}$  nanodomains [35, 38] (Fig. 4) and typically peak within 1 ms of channel opening, with  $[Ca^{2+}]_i$  as high as a few hundred micromolar at the center of the domain but dropping rapidly with distance from the channel [37, 38]. Clustering of  $Ca^{2+}$  channels can lead to the formation of larger  $Ca^{2+}$  microdomains [35, 38] (Fig. 4). However, the terms “nanodomain” and “microdomain” are not always consistently defined. For example, Neher and colleagues use the term “nanodomain” to refer to elevated  $[Ca^{2+}]_i$  in the immediate vicinity (10–100 nm) of a few  $Ca^{2+}$  channels, and “microdomain” to refer to elevated  $[Ca^{2+}]_i$  within 1  $\mu$ m of active zones [39].

The concentration of  $Ca^{2+}$  in  $Ca^{2+}$  microdomains has been assessed by analyzing “hot spots,” which are putative  $Ca^{2+}$  microdomains, in  $Ca^{2+}$  imaging experiments. At the presynaptic terminal of the squid giant synapse,  $[Ca^{2+}]_i$  in the  $Ca^{2+}$  microdomain is 200–300  $\mu$ M [40], which is consistent with estimates made by analyzing the inhibitory effects of different  $Ca^{2+}$ -chelating agents on neurotransmitter release [32]. In contrast, at the presynaptic terminal of goldfish retinal bipolar cells, the  $Ca^{2+}$  microdomain has an average concentration of  $\sim$ 2  $\mu$ M and a peak concentration of  $\sim$ 7  $\mu$ M at its center [41]. In addition to  $Ca^{2+}$  imaging techniques, presynaptic BK channel activity has been used to measure local  $[Ca^{2+}]_i$  resulting from  $Ca^{2+}$  entry through VGCCs. This approach takes advantage of the  $Ca^{2+}$ -dependent property of the BK channel and the physical colocalization of the BK channel with VGCCs at the presynaptic terminal. Analyses have shown that local  $[Ca^{2+}]_i$  can exceed 100  $\mu$ M at the presynaptic terminal of cultured *Xenopus* NMJ preparation [42]. The  $Ca^{2+}$  concentrations determined using the  $Ca^{2+}$  imaging and BK channel sensor approaches are consistent with those required to trigger fast neurotransmitter release at these synapses.



**Fig. 4**  $Ca^{2+}$  accumulates at the inner mouth of voltage-sensitive  $Ca^{2+}$  channels to form  $Ca^{2+}$  nanodomains and microdomains. A  $Ca^{2+}$  nanodomain results from the opening of one  $Ca^{2+}$  channel, whereas a  $Ca^{2+}$  microdomain results from the opening of a cluster of  $Ca^{2+}$  channels [35, 196]



The properties of  $\text{Ca}^{2+}$  microdomains at presynaptic terminals have been analyzed using various imaging techniques. At the presynaptic terminal of the squid giant synapse, *n*-aequorin-J imaging reveals that a stable set of quantum emission domains (QEDs) develop in response to sustained 10 Hz stimulation. These QEDs are 0.25 to 0.6  $\mu\text{m}^2$  in size and have an average lifetime of 200 ms [40]. However, the measured lifetime of QEDs is longer than expected for the transient  $\text{Ca}^{2+}$  signal in the microdomain. This is likely due to technical limitations [40].

At the presynaptic terminal of a cultured frog NMJ preparation, low-affinity  $\text{Ca}^{2+}$  indicator Oregon Green 488 confocal imaging shows that action potentials induce spot-like fluorescent transients. The fluorescent spot peaks within  $\sim 1$  ms, decays with one rapid ( $\tau_1 = 1.7$  ms) and two slow components ( $\tau_2 = 16$  ms,  $\tau_3 = 78$  ms), and is 0.6–3.0  $\mu\text{m}$  in full width at maximum [43]. Total internal reflection fluorescence microscopy (TIRFM) has also been adapted to measure  $\text{Ca}^{2+}$  signals in  $\text{Ca}^{2+}$  nano- or microdomains. This technique provides excellent spatial and temporal resolutions as fluorescent excitation is restricted to a thin ( $\sim 100$  nm) layer at the refractive boundary between the microscope cover glass and the cell [44]. Using TIRFM, fluorescent “hot spots” are observed near the plasma membrane at the presynaptic terminal of goldfish bipolar neurons in response to membrane depolarization [45]. The fluorescent hot spot has two components, including a fast component that rises and declines abruptly with membrane depolarization and repolarization, and a slow component that rises steadily during depolarization and declines rather slowly after repolarization. Interestingly, the slow component is also observed outside of the fluorescent hot spots, suggesting that it likely reflects global cytoplasmic [ $\text{Ca}^{2+}$ ] changes that are also observed using standard fluorescence microscopy [46–48]. Another study using TIRFM shows that depolarization generates  $\text{Ca}^{2+}$  microdomains that appear within 20–40 ms and disappear within 20–40 ms in goldfish retinal bipolar cells [41].

The distance between  $\text{Ca}^{2+}$  nano- or microdomains and the  $\text{Ca}^{2+}$  sensor for synaptic vesicle exocytosis is a topic of interest in neuroscience research. Electron microscopy tomography of frog NMJs has revealed that VGCCs are located only 10–20 nm away from synaptic vesicles [49]. Mathematical modeling of the calyx of Held presynaptic site has suggested that the distance between synaptic vesicles and clusters of VGCCs ranges from 30 to 300 nm, with an average of about 100 nm [50].

To estimate the distance between  $\text{Ca}^{2+}$  channels and the  $\text{Ca}^{2+}$  sensor for exocytosis, researchers often compare the inhibitory effects of the  $\text{Ca}^{2+}$ -chelating agents BAPTA and EGTA on neurotransmitter release. Although BAPTA and EGTA have similar equilibrium affinities for  $\text{Ca}^{2+}$ , BAPTA binds  $\text{Ca}^{2+}$  several hundred times faster than EGTA because EGTA, but not BAPTA, is protonated at physiological pH, and the bound protons must dissociate from EGTA prior to  $\text{Ca}^{2+}$  binding [51]. If the  $\text{Ca}^{2+}$  domain is hundreds of nanometers away from the  $\text{Ca}^{2+}$  sensor, both BAPTA and EGTA would inhibit neurotransmitter release. However, if the  $\text{Ca}^{2+}$  domain is tens of nanometers away from the  $\text{Ca}^{2+}$  sensor, only BAPTA would inhibit release [52].

There are two primary methods for determining whether the release of synaptic vesicles is regulated by  $\text{Ca}^{2+}$  nano- or microdomains. The first method involves

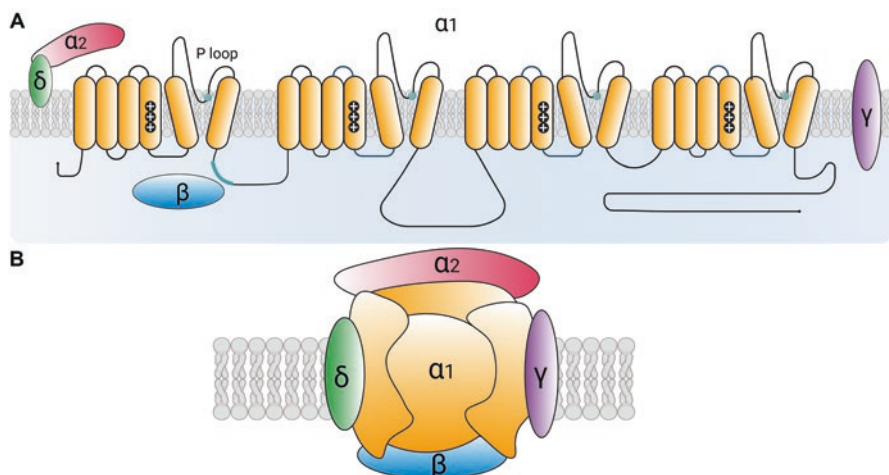


analyzing the effects of BAPTA and EGTA on neurotransmitter release [35]. If only BAPTA can inhibit the release, it is likely that the release is controlled by  $\text{Ca}^{2+}$  nanodomains. If both BAPTA and EGTA can inhibit the release, it is likely that the release is controlled by  $\text{Ca}^{2+}$  microdomains. This analysis has suggested that the release at squid giant synapse [53], goldfish retinal bipolar synapse [54, 55], and mature calyx of Held synapses [56] is controlled by  $\text{Ca}^{2+}$  nanodomains. In contrast, the release at immature calyx of Held synapses [56, 57], cortical pyramidal neurons [58, 59], and cerebellar parallel fiber-Purkinje cell synapse [60] is controlled by  $\text{Ca}^{2+}$  microdomains.

The second approach involves analyzing the relationship between the release rate and the number of functional  $\text{Ca}^{2+}$  channels or  $\text{Ca}^{2+}$  influx [38, 61]. If there is a linear relationship between the two, it suggests that the release is triggered by  $\text{Ca}^{2+}$  nanodomains. If there is a supralinear relationship, it suggests that the release is triggered by  $\text{Ca}^{2+}$  microdomains. Using this method, it has been suggested that the release at the squid giant synapse and chick ciliary synapse is mediated by  $\text{Ca}^{2+}$  nanodomains, while the release at the calyx of Held synapse is triggered by  $\text{Ca}^{2+}$  microdomains [61]. Growing evidence suggests that nanodomain coupling may be more prevalent than microdomain coupling in mammalian central neurons [52, 62–64]. Nanodomain coupling can improve the accuracy of synaptic transmission [52, 62]. For more information on nanodomain coupling, two excellent review articles are available [38, 52].

## 5 Voltage-Gated $\text{Ca}^{2+}$ Channels Are the Primary Source of $\text{Ca}^{2+}$ for Neurotransmitter Release

Neuronal VGCCs generally consist of four subunits:  $\alpha_1$ ,  $\beta$ ,  $\alpha_2$ , and  $\delta$  [65]. The  $\alpha_2$  and  $\delta$  subunits are also collectively called the  $\alpha_2\delta$  subunit because they are formed from cleavage of a single translational product. The  $\alpha_1$  subunit is a large (190–250 kDa) transmembrane protein consisting of four repeat domains. The membrane topology of each repeat domain resembles that of the  $\alpha$  subunit of a typical voltage-gated  $\text{K}^+$  channel, with six membrane-spanning segments (S1–S6), a P (pore) loop between S5 and S6, cytoplasmic amino and carboxyl terminals, and positively charged residues in the S4 segment. The  $\delta$  subunit is also a membrane-associated protein with a single membrane-spanning domain. The  $\beta$  and  $\alpha_2$  subunits have no integral membrane spanning domains. The  $\beta$  subunit interacts with the intracellular loop between the first and second repeat domain of the  $\alpha_1$  subunit, whereas the  $\alpha_2$  subunit associates with the  $\delta$  subunit on the extracellular side through a disulfide linkage. In skeletal muscle and some other tissues, VGCCs may include an additional  $\gamma$  subunit, which is an integral membrane protein with four membrane-spanning domains. The structures of  $\text{Ca}_v1.1$ ,  $\text{Ca}_v2.2$ , and  $\text{Ca}_v3.1$  channels have been determined by single-particle cryo-electron microscopy (cryo-EM) [66–71], which provide major insights into the molecular architecture underlying their biophysical properties. A schematic diagram of one  $\alpha_1$  with various auxiliary subunits is shown in Fig. 5.



**Fig. 5** Schematic diagrams showing the membrane topology and organization of voltage-gated  $\text{Ca}^{2+}$  channel (VGCC) subunits. **(a)** Membrane topology of  $\text{Ca}^{2+}$  channel subunits. The  $\alpha_1$  subunit consists of four repeat domains (I, II, III, and IV). Each repeat domain has six membrane-spanning segments (S1–S6) with a P (pore) loop between S5 and S6. The  $\beta$  subunit interacts with the  $\alpha_1$  subunit at the intracellular loop between the first and second repeating domains. The  $\delta$  subunit has a single membrane-spanning segment. The  $\alpha_2$  subunit associates with the  $\delta$  subunit through a disulfide linkage on the extracellular side. The  $\gamma$  subunit has four putative membrane-spanning segments. **(b)** Overall structure of a VGCC, which includes the  $\alpha_1$ ,  $\beta$ ,  $\delta$ , and  $\alpha_2$  subunits. (Created with [BioRender.com](https://www.biorender.com))

$\text{Ca}^{2+}$  channels are encoded by multiple genes and have diverse functional and pharmacological properties. Several methods have been used to classify  $\text{Ca}^{2+}$  channels. They are classified into high voltage-activated (HVA) and low voltage-activated (LVA) channels according to the degree of membrane depolarization needed for activation. LVA channels activate at a threshold of approximately  $-70$  mV, whereas HVA channels activate at a threshold of approximately  $-20$  mV [65].

$\text{Ca}^{2+}$  channels are classified into L-, N-, P/Q-, R-, and T-types according to biophysical/pharmacological properties and tissue distribution. The L-type channel was named for its relatively large single channel conductance and long open duration (slow inactivation). The T-type channel was named for its tiny conductance and transient opening. The N-type channel was named because it was first identified in neurons. The P-type was first described in Purkinje cells. The Q-type was named because it was blocked by the same toxin that blocks the P-type (omega-agatoxin IVA) but with a lower sensitivity to the toxin and distinct inactivation kinetics, and because the letter “Q” follows “P” in the English alphabet. P- and Q-type channels are often collectively called the P/Q-type. The R-type was named for being resistant to organic calcium channel antagonists available at the time [65, 72, 73].

With the molecular cloning of the different subunits of  $\text{Ca}^{2+}$  channels, a nomenclature system based on compositions of the  $\alpha_1$  subunit was introduced in 1994 [74]. The  $\alpha_1$  subunit of skeletal muscle  $\text{Ca}^{2+}$  channels was named as  $\alpha_{1S}$ , and subsequently

cloned  $\alpha_1$  subunits as  $\alpha_{1A}$  through  $\alpha_{1E}$ . However, this nomenclature system cannot conveniently accommodate newly identified  $Ca^{2+}$  channels and does not reflect the evolutionary relationship among the different  $\alpha_1$  subunits. As a result, a new nomenclature system based on amino acid sequence was adopted in 2000 [75].  $Ca^{2+}$  channels are named  $Ca_{v,x,y}$ , where Ca indicates the principal permeating ion, v indicates the principal physiological regulator (voltage), and x is a numerical identifier of the  $\alpha_1$  subunit subfamily, and y is a number indicating the order of discovery of the  $\alpha_1$  subunit within that subfamily. In this new nomenclature system, primary sequences of  $\alpha_1$  subunits are greater than 70% identical within the same subfamily, but less than 40% between different subfamilies. It is worth noting that the  $\alpha_1$  subunits of P- and Q-type channels are encoded by the same gene. The distinct biophysical and pharmacological properties of P- and Q-type channels are caused by different splice forms of the  $\alpha_1$  subunit and different  $\beta$  subunits [72]. Table 1 shows the relationships among the different nomenclatures, and commonly used blockers specific to the  $Ca^{2+}$  channels.

Many studies have been conducted to identify  $Ca^{2+}$  channels that trigger neurotransmitter release at presynaptic nerve terminals. They are generally identified by analyzing the effects of specific  $Ca^{2+}$  channel blockers on the amplitude of evoked postsynaptic currents or potentials, or on the amplitude or slope of field excitatory postsynaptic potentials. These analyses have revealed that  $Ca_{v,2.1}$  (P/Q--type) and  $Ca_{v,2.2}$  (N-type) channels play prominent roles in neurotransmitter release at many synapses, such as excitatory synapses in the hippocampus [76–79], inhibitory synapses in the cerebellum and spinal cord [78], and dopaminergic synapses in the striatum [80]. At some synapses, only one type of channel appears to be responsible for  $Ca^{2+}$  influx at the presynaptic terminal. For example, neurotransmitter

**Table 1** Classification of voltage-gated  $Ca^{2+}$  channels (VGCCs)

Activation voltage	Pharmacological and biophysical properties	$\alpha_1$ Subunit composition	$\alpha_1$ Subunit sequence	Most commonly used blockers
HVA	L-type	$\alpha 1S$	$Ca_v1.1$	Dihydropyridine antagonists
		$\alpha 1C$	$Ca_v1.2$	
		$\alpha 1D$	$Ca_v1.3$	
		$\alpha 1F$	$Ca_v1.4$	
	P/Q-type	$\alpha 1A$	$Ca_v2.1$	$\omega$ -Agatoxin IVA
N-type	$\alpha 1B$	$Ca_v2.2$	$\omega$ -Conotoxin GVIA	
R-type	$\alpha 1E$	$Ca_v2.3$	SNX-482	
LVA	T-type	$\alpha 1G$	$Ca_v3.1$	Mibefradil
		$\alpha 1H$	$Ca_v3.2$	
		$\alpha 1I$	$Ca_v3.3$	

This table was based on papers by Lacinova [65] and Catteralls et al. [72] VGCCs are classified into high voltage-activated (HVA) and low voltage-activated (LVA) channels according to the degree of membrane depolarization needed for activation, into L-, P/Q-, N-, R-, and T-type channels according to biophysical and pharmacological properties, and tissue distribution, into  $\alpha_{1S}$ ,  $\alpha_{1A-I}$  channels according to the  $\alpha_1$  subunit composition, and into  $Ca_v1.1-1.4$ ,  $Ca_v2.1-2.3$ , and  $Ca_v3.1-3.3$  according to the primary sequence of the  $\alpha_1$  subunit

release at mature NMJs is triggered by  $\text{Ca}^{2+}$  influx through  $\text{Ca}_v2.1$  alone [81–85]. At other synapses, such as the glutamatergic synapse between hippocampal CA1 and CA3 neurons, neurotransmitter release is triggered by  $\text{Ca}^{2+}$  influx through both  $\text{Ca}_v2.1$  and  $\text{Ca}_v2.2$  [76]. At still other synapses, such as the calyx of Held synapse,  $\text{Ca}_v2.3$  (R-type) as well as  $\text{Ca}_v2.1$  and  $\text{Ca}_v2.2$  channels contribute to the release [86]. Thus, members of the  $\text{Ca}_v2$  subfamily play important roles in triggering neurotransmitter release, but their relative contributions may vary from synapse to synapse.

$\text{Ca}_v1$  (L-type) and  $\text{Ca}_v3$  (T-type) channels are generally not involved in  $\text{Ca}^{2+}$  influx at the presynaptic nerve terminal, although some unusual examples have been reported. For instance, at the presynaptic terminal of rat retinal bipolar cells, both  $\text{Ca}_v1$  and  $\text{Ca}_v3$  channels contribute to neurotransmitter release [87]. At the presynaptic terminal of goldfish retinal bipolar cells,  $\text{Ca}_v1$  appears to be exclusively responsible for mediating neurotransmitter release [88]. It is still unclear why  $\text{Ca}_v2$  channels are more suited to control neurotransmitter release than  $\text{Ca}_v1$  and  $\text{Ca}_v3$  channels. One clue comes from analyses of the effects of exogenously introduced  $\alpha1$  subunits in the superior cervical ganglion (SCG). In SCG neurons, acetylcholine release is typically mediated by  $\text{Ca}_v2.2$ . However, when different  $\alpha1$  subunits were expressed in these neurons,  $\text{Ca}_v2.1$  and  $\text{Ca}_v2.3$  were localized to nerve terminals and could mediate synaptic transmission, whereas  $\text{Ca}_v1.2$  showed no presynaptic localization and no effect on synaptic transmission [89]. Similarly, *C. elegans* cholinergic motor neurons expresses both UNC-2 ( $\text{Ca}_v2$ ) and EGL-19( $\text{Ca}_v1$ ), which are located at the center and lateral areas of presynaptic sites, respectively [90]. Only UNC-2 is required for evoked neurotransmitter release although both channels contribute to minis [90, 91]. These results suggest that trafficking and localization to proper sites in nerve terminals may be a factor in determining whether a particular  $\text{Ca}^{2+}$  channel can contribute to neurotransmitter release.

The types of  $\text{Ca}^{2+}$  channels that facilitate neurotransmitter release at the presynaptic terminal are regulated during development and can change when the predominant channel is mutated. For instance, at thalamic and cerebellar inhibitory synapses, immature neurons predominantly use  $\text{Ca}_v2.2$  for neurotransmitter release, whereas mature neurons use  $\text{Ca}_v2.1$  [92]. At the rat calyx of Held synapse, neurotransmitter release is triggered by  $\text{Ca}^{2+}$  influx through  $\text{Ca}_v2.1$ ,  $\text{Ca}_v2.2$ , and  $\text{Ca}_v2.3$  during postnatal day 4–9 [86, 93]. However, contributions from  $\text{Ca}_v2.2$  and  $\text{Ca}_v2.3$  gradually decrease after postnatal day 7, and by postnatal day 10,  $\text{Ca}_v2.1$  almost exclusively mediates the release [93]. Wild-type mouse NMJ primarily relies on  $\text{Ca}_v2.1$  for neurotransmitter release [94]. However, in  $\text{Ca}_v2.1$  knockout mice,  $\text{Ca}_v2.2$  and  $\text{Ca}_v2.3$  are involved in mediating neurotransmitter release at the NMJ [94]. Similarly, at the NMJ of tottering mice, which carry a mutation in the  $\alpha1$  subunit of  $\text{Ca}_v2.1$ , the predominant  $\text{Ca}^{2+}$  channels that mediate neurotransmitter release are  $\text{Ca}_v2.2$  and/or  $\text{Ca}_v2.3$  [95, 96].

The developmental switch from  $\text{Ca}_v2.2$  to  $\text{Ca}_v2.1$  is potentially of physiological significance. In  $\text{Ca}_v2.1$  knockout mice, paired-pulse facilitation, which is typically observed in wild-type synapses, is often absent at the calyx of Held synapse [97] and NMJ [94]. Moreover, synaptic depression in response to high-frequency

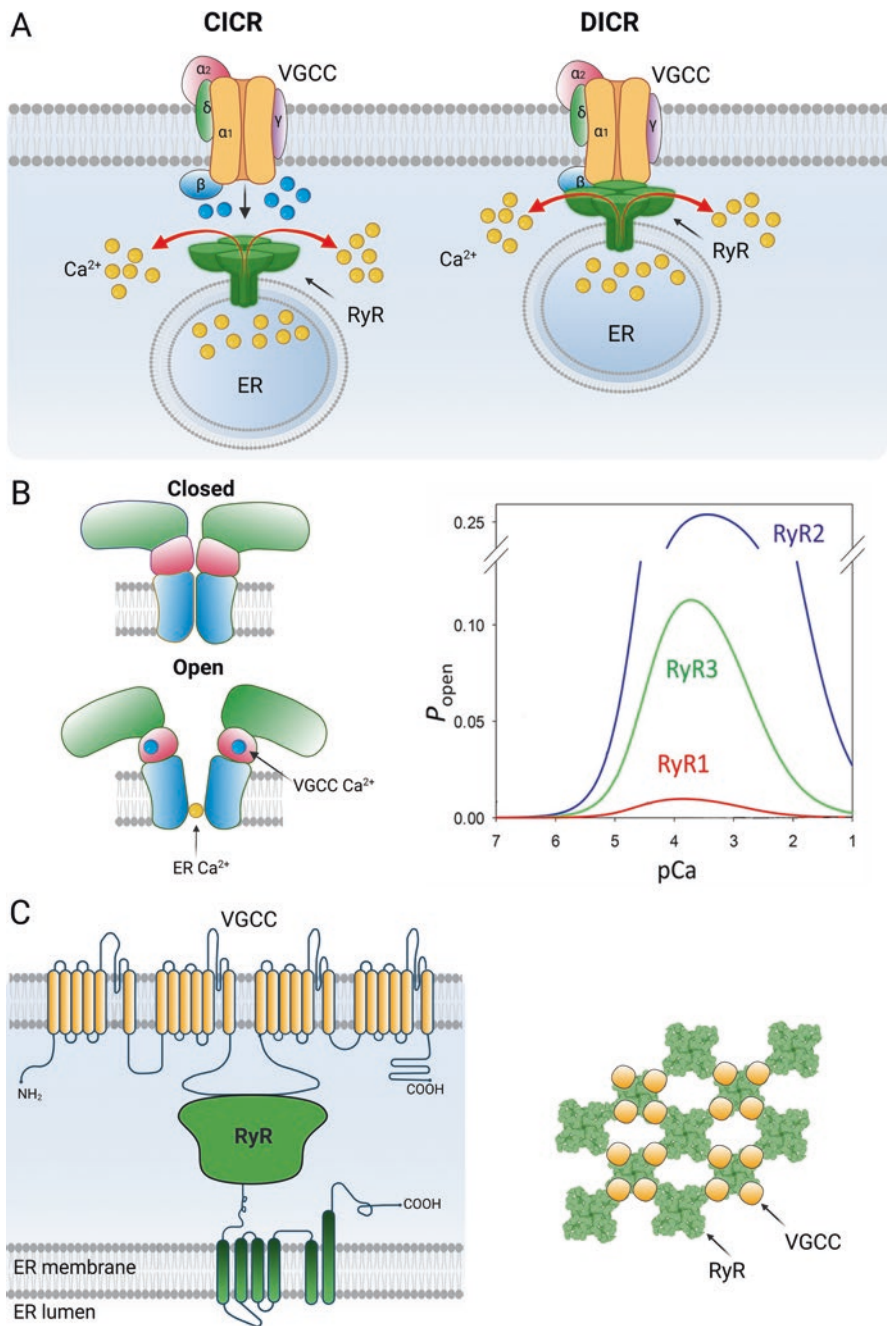
(100 Hz) stimulation is more severe in the knockout mice than in the wild-type [98]. Therefore, the developmental switch from  $\text{Ca}_v2.2$  to  $\text{Ca}_v2.1$  could enhance synaptic efficacy.

## 6 Ryanodine Receptor-Mediated $\text{Ca}^{2+}$ Release Contributes to Neurotransmitter Release

The ER is a complex network that extends throughout the neuron, including the soma, dendrites, axons, and presynaptic terminals [90, 99–106]. Due to the vast amount of ER present in neurons, it has been suggested that the ER functions as a neuron-within-a-neuron [7]. The ER is a crucial intracellular  $\text{Ca}^{2+}$  store, and  $\text{Ca}^{2+}$  release from the ER is facilitated by two types of ionotropic receptors on the ER membrane: inositol 1,4,5-triphosphate receptor ( $\text{InsP}_3\text{R}$ ) and ryanodine receptor (RyR). The ER  $\text{Ca}^{2+}$  store is refilled by sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) pumps [6]. Although the ER contains a significant amount of  $\text{Ca}^{2+}$ , the concentration of free  $\text{Ca}^{2+}$  is maintained within the range of 100–500  $\mu\text{M}$  due to buffering by  $\text{Ca}^{2+}$ -binding proteins [107].  $\text{Ca}^{2+}$  release from the ER plays an essential role in presynaptic functions, particularly in the process of neurotransmitter release. In this subsection, the focus is on the role of RyRs in neurotransmitter release.

The RyR channel plays a critical role in mediating intracellular  $\text{Ca}^{2+}$  release. Structurally, the channel is composed of four subunits, each of which contains multiple transmembrane domains that interact with the ER membrane. The amino-terminal domain of the RyR protein is particularly large and interacts with various regulators, including  $\text{Ca}^{2+}$ , ATP, caffeine, calstabin1 (FKBP12), calstabin2 (FKBP12.6), calmodulin, and VGCCs. Cryo-EM studies have revealed the binding sites for some of these regulators [108–121], shedding light on the molecular mechanisms of RyR gating. There are three isoforms of the RyR protein (RyR1, RyR2, and RyR3) encoded by separate genes, with each isoform exhibiting distinct expression patterns in different cell types. Dysregulation of RyR activity has been linked to numerous pathologies, including cardiac arrhythmias, malignant hyperthermia, and neurodegenerative diseases.

The RyR channel can be activated by two different mechanisms:  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) and depolarization-induced  $\text{Ca}^{2+}$  release (DICR) (Fig. 6a). In skeletal and cardiac muscles, RyR1 and RyR2 are the predominant isoforms, respectively. Skeletal muscle cells contain RyR1 in the sarcoplasmic reticulum (SR) membrane, which interacts with the plasma membrane's dihydropyridine receptor (DHPR),  $\text{Ca}_v1.1$ , at the location of transverse tubules. Specifically, one RyR1 channel's cytosolic domain interacts with four DHPRs (a tetrad) (Fig. 6b). In contrast, RyR2 in the heart is activated by CICR, which depends on  $\text{Ca}^{2+}$  influx through  $\text{Ca}_v1.2$ . Skeletal muscles also express RyR3 at a lower level, which is also activated by CICR. The physical interactions occur specifically between  $\text{Ca}_v1.1$  and RyR1



**Fig. 6** Ryanodine receptors (RyRs) may be activated by two different mechanisms:  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) and depolarization-induced  $\text{Ca}^{2+}$  release (DICR). (a) Diagram depicting CICR and DICR. (b) In CICR,  $\text{Ca}^{2+}$  entering the cell through voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs)



[121–126]. The interactions are mediated by  $\text{Ca}_v1.1$ 's intracellular loop between the III and IV repeat domains and RyR1 cytosolic “feet” [121–126] (Fig. 6b). The activation of RyR1 is coupled to the depolarization-induced conformational changes of DHPRs rather than DHPR-mediated  $\text{Ca}^{2+}$  influx.

RyR channels, regardless of their isoform, can be activated by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) but their sensitivities to  $\text{Ca}^{2+}$  can vary. The effects of  $\text{Ca}^{2+}$  on RyR activity depend on its concentration, as it can either activate or inhibit the channels. RyRs are typically activated by lower concentrations of  $\text{Ca}^{2+}$ , while higher concentrations tend to inhibit their activity. This is likely due to  $\text{Ca}^{2+}$  binding to different sites with varying affinities within the channels (Fig. 6b). Further detailed explanations of the theories behind CICR and DICR are available in a review article by Eduardo Rios [127].

All three RyR isoforms are expressed in mammalian brains. Analysis of RyR expression in brain tissues from several mammalian species indicates that (1) RyRs are expressed in neurons throughout the brain; (2) the three different RyR isoforms are differentially expressed in brain tissues; (3) a specific brain area often expresses more than one RyR isoform; and (4) RyR2 is the predominant isoform in most brain areas [128–135] (see also <https://mouse.brain-map.org/>). For example, mRNAs of all three RyR isoforms are detected in the mouse hippocampus, with RyR2 being the most abundant [129, 135, 136].

Only a limited number of studies have examined the molecular mechanisms underlying RyR activation in neurons. While it is widely accepted that neuronal RyRs are activated via CICR [7, 137], there is also evidence to support the occurrence of DICR in neurons. For example, depolarization of mouse hypothalamic magnocellular neurons has been shown to increase the frequency of RyR-mediated  $\text{Ca}^{2+}$  syntillas (brief focal  $\text{Ca}^{2+}$  transients) at presynaptic terminals where RyR1 expression is detected, even in the absence of extracellular  $\text{Ca}^{2+}$ . This effect can be blocked by nifedipine, a DHPR antagonist, indicating that membrane depolarization may trigger RyR-mediated  $\text{Ca}^{2+}$  release from the ER through coupling between a VGCC and RyR1 [138]. Similarly, in the hypothalamic neurohypophysial system, depolarization has been found to induce ER  $\text{Ca}^{2+}$  release and neuropeptide secretion even in the absence of extracellular  $\text{Ca}^{2+}$ , and these effects can be antagonized by nifedipine, ryanodine, or BAPTA-AM, suggesting that direct coupling between  $\text{Ca}_v1$  and RyRs can trigger ER  $\text{Ca}^{2+}$  release [139]. In the rat hippocampus,  $\text{Ca}_v1.3$  and RyR2 colocalize and physically interact. Acute depolarization of hippocampal neurons by a high  $[\text{K}^+]_o$  (60 mM) led to RyR-dependent  $\text{Ca}^{2+}$  release even in the absence of  $\text{Ca}^{2+}$  in the extracellular solution [140]. Taken together, these findings

←  
**Fig. 6** (continued) activate the RyR by binding to an activation module of the receptor. Adapted from reference [116]. The open probability ( $P_{\text{open}}$ ) of RyRs consisting of RyR1, RyR2, or RyR3 all exhibit bidirectional responses to elevating  $\text{Ca}^{2+}$  concentrations but differ in their  $\text{Ca}^{2+}$  sensitivity. (c) In DICR, depolarization-induced conformational changes of VGCCs activate the RyR through physical interactions with the receptor. The intracellular loop between the II and III repeat domains of a VGCC physically interacts with the cytosolic domain of a RyR, and each RyR channel interacts with 4 VGCCs. (Created with [BioRender.com](https://BioRender.com) except the right panel in (b), which was adapted from reference [127])



suggest that both DICR and CICR may occur in neurons, and that DICR is not exclusively mediated by coupling between  $\text{Ca}_v1.1$  and RyR1, as concluded from studies with skeletal and cardiac muscles.

Presynaptic RyRs have been shown to play a role in regulating the frequency and amplitude of miniature postsynaptic currents (minis). For example, in the rat cerebellum, the frequency of inhibitory minis recorded from Purkinje cells was increased by 10  $\mu\text{M}$  ryanodine but decreased by 100  $\mu\text{M}$  ryanodine. The higher concentration of ryanodine also reduced the proportion of large-amplitude minis whereas the lower concentration of ryanodine showed no effect on the amplitude [141]. These opposite effects were attributed to activation and blockade of RyRs, respectively [141], because ryanodine can lock RyR in a sub-conductance state at submicromolar or low micromolar concentrations but block it at high micromolar concentrations [142]. In the rat hippocampus, nicotine increased the frequency of glutamatergic minis and the fraction of large-amplitude events. These effects were mimicked by the RyR activator caffeine but blocked by 100  $\mu\text{M}$  ryanodine [143]. At the *C. elegans* NMJ, presynaptic RyRs were found to be important for the occurrence of minis. Null mutants of *unc-68*, which encodes the only RyR of *C. elegans*, showed greatly reduced mini frequency and essentially no large-amplitude events, while postsynaptic receptor sensitivities to acetylcholine and GABA remained normal [144]. A more recent study suggested that minis at the *C. elegans* NMJ depend on two different VGCCs, UNC-2 (a  $\text{Ca}_v2$ ) and EGL-19 (a  $\text{Ca}_v1$ ), and that EGL-19 functions through UNC-68 [90]. Taken together, these findings indicate that presynaptic RyRs can increase the frequency of minis and promote the occurrence of large-amplitude events.

Minis are generally thought to result from sporadic exocytosis of individual synaptic vesicles. The amplitude of minis could potentially be affected by a variety of factors. How might presynaptic RyRs increase the occurrence of large-amplitude minis? One hypothesis is that presynaptic RyRs may promote synchronized multivesicular exocytosis. This hypothesis was mainly based on the results of two studies. In one study [141], large-amplitude minis were thought to be due to multivesicular release because their proportion could be reduced by prolonged exposure to a  $\text{Ca}^{2+}$ -free extracellular solution. In the other study [143], a similar conclusion was reached because a positive correlation was observed between the rise time and mean amplitude of minis. It was reasoned that when multiple synaptic vesicles exocytose at the same time, a lack of absolute synchrony would result in an increased rise time. An alternative hypothesis is that presynaptic RyRs may increase the quantal size (the amount of neurotransmitter released from a vesicle in a single exocytotic event). This hypothesis was mainly based on analyses of synaptic transmission at the *C. elegans* NMJ, where large-amplitude minis are essentially eliminated by null mutations of the RyR gene *unc-68* [144]. Several lines of evidence suggest that RyR-dependent large-amplitude minis at the *C. elegans* NMJ were not due to multivesicular release [144]. First, the proportion of large-amplitude events did not decrease in syntaxin or SNAP25 mutants, which are severely defective in synchronizing synaptic vesicle exocytosis. Second, the rise time of minis was constant regardless of the amplitude. Third, the proportion of large-amplitude events did not

decrease when  $[Ca^{2+}]_o$  was changed from 5 mM to zero [144]. Given the existence of these two competing hypotheses, further studies are needed to determine whether RyR-mediated large-amplitude minis are mono- or multiquantal, and whether this property varies from synapse to synapse.

Presynaptic RyRs are important for evoked neurotransmitter release and synaptic plasticity. At the *C. elegans* NMJ, the amplitude of evoked postsynaptic currents decreased by 40–50% in *unc-68* null mutants compared to wild-type, and this defect was rescued by expressing a wild-type *unc-68* transgene in neurons but not muscle cells [144, 145]. At inhibitory synapses between cerebellar basket and Purkinje neurons, the mean amplitude of evoked inhibitory postsynaptic currents decreased by ~30% when RyRs were blocked with ryanodine, and this effect appeared to be presynaptic because ryanodine also increased the paired-pulse ratio of the evoked responses [146]. In the presynaptic terminal of hippocampal pyramidal neurons, blocking RyRs with ryanodine inhibited paired-pulse facilitation of evoked EPSCs, suggesting a role for presynaptic RyRs in short-term synaptic plasticity [147]. At mossy fiber terminals in the hippocampus, blocking RyRs with the ryanodine receptor blocker TMB-8 reduced presynaptic  $Ca^{2+}$  accumulation and short-term synaptic depression caused by repetitive nerve stimuli. Immunohistochemistry showed that RyR2 is preferentially localized to the axons of hippocampal mossy fibers. These findings suggest that axonal RyR2 enables use-dependent  $Ca^{2+}$  release to facilitate presynaptic forms of synaptic plasticity at the mossy fiber-CA3 synapse [148]. At excitatory synapses between hippocampal CA3 neurons, blocking RyRs with ryanodine during the induction period but not afterward abolished NMDA receptor-dependent long-term depression (LTD), suggesting that presynaptic RyR-sensitive stores are required for LTD induction but not expression [149]. At frog motor terminals, conditioning nerve stimulation (10–20 Hz for 2–10 min) enhanced the amplitude and quantal content of end-plate potentials, and this effect of stimulation can be prevented by blocking RyRs with ryanodine [150].

The function of RyRs in regulating presynaptic  $Ca^{2+}$  signaling has been investigated through  $Ca^{2+}$  imaging in several studies. Using two-photon laser scanning fluorescence microscopy with Oregon Green-1, it was found that ryanodine (100  $\mu$ M) inhibited AP-evoked  $Ca^{2+}$  transients by approximately 50% at the presynaptic terminal of rat cerebellar basket cells [141]. Confocal laser scanning microscopy with Oregon Green 488 BAPTA-1 demonstrated that ryanodine (20  $\mu$ M) inhibited paired-pulse facilitation of  $Ca^{2+}$  transients at presynaptic boutons of hippocampal CA3 neurons [147].  $Ca^{2+}$  imaging using fluo-3 and a signal mass approach showed that 10  $\mu$ M ryanodine increased the frequency of syntillas, while 100  $\mu$ M ryanodine decreased it at isolated mouse hypothalamic magnocellular nerve terminals [151]. Additionally,  $Ca^{2+}$  imaging by expressing GCaMP6 in *C. elegans* motor neurons showed that knockdown of the RyR gene *unc-68* reduced the frequency of  $Ca^{2+}$  transients [145]. These findings support the notion that presynaptic RyRs play a critical role in mobilizing  $Ca^{2+}$ .

The importance of RyRs in regulating neurotransmitter release is also demonstrated by the effects of mutations of proteins that regulate RyR function or expression. In mice, the conditional double knockout (cDKO) of presenilin 1 and presenilin

2, which are  $\gamma$ -secretases implicated in the generation of amyloid  $\beta$  peptides from an amyloid precursor protein, in presynaptic (CA3) but not postsynaptic (CA1) neurons in the hippocampal Schaeffer collateral pathway inhibits short-term synaptic facilitation and glutamate release. Blockade of RyRs with ryanodine (100  $\mu$ M) mimics the defect of presynaptic cDKO in synaptic facilitation [152]. The presynaptic effects of cDKO are mainly due to reduced RyR protein expression [135]. Additionally, conditional knockout of ATG5, a protein essential to autophagy, augments evoked field excitatory postsynaptic potentials, caffeine-induced elevation of  $[Ca^{2+}]_i$  in axons and presynapses, an action potential train-induced exocytosis, and RyR protein level in hippocampal neurons. The effects of ATG5 knockout on exocytosis can be eliminated by either dantrolene (an RyR antagonist) or RyR knock-down. These results suggest that knockout of ATG5 augments neurotransmitter release by increasing RyR expression [153]. In *C. elegans*, a hypomorphic mutation of *aipr-1*, which encodes an ortholog of human aryl hydrocarbon receptor-interacting protein (AIP), causes great increases in the frequency and amplitude of minis and in the amplitude of evoked postsynaptic currents at the NMJ. The effects of the *aipr-1* mutation may be eliminated by targeted expression of wild-type AIPR-1 in neurons but not muscle cells and occluded by a null mutation of the RyR gene *unc-68*. These findings suggest that a physiological function of AIPR-1 is to restrict RyR-mediated  $Ca^{2+}$  release from the ER [145].

Despite the evidence presented above, the role of presynaptic RyRs in controlling neurotransmitter release remains unclear for several reasons. Firstly, certain studies have demonstrated that the blockage of RyRs has little to no impact on synaptic transmission [154–157]. Secondly, the effect of ryanodine on neurotransmitter release often exhibits high variability even in studies claiming that presynaptic RyRs play a role in spontaneous or evoked release. For instance, in a study involving the application of ryanodine (10  $\mu$ M), the frequency of inhibitory minis increased in only 4 out of 10 rat cerebellar Purkinje neurons [141] and 4 out of 12 mouse cerebellar Purkinje neurons [154]. Additionally, ryanodine (100  $\mu$ M) showed a highly variable and often weak effect on evoked neurotransmitter release from rat cerebellar basket cell terminals [146]. Thirdly, while the use of 100  $\mu$ M ryanodine to inhibit RyRs reduced the proportion of large-amplitude minis at cerebellar inhibitory synapses, the use of 10  $\mu$ M ryanodine to activate RyRs did not demonstrate an opposite effect [141]. Finally, ryanodine has been used as a key pharmacological tool in the majority of previous studies. However, ryanodine is a bidirectional modulator of RyRs with poorly defined concentration boundaries for activation and inhibition. For instance, 10–20  $\mu$ M ryanodine has been used either to activate [141, 143, 154] or to block [147, 150, 155, 158] RyRs. Thus, additional analyses involving more specific pharmacological agents or RyR mutants are necessary to better understand the function of presynaptic RyRs in neurotransmitter release.

The function of presynaptic inositol 1,4,5-trisphosphate receptors (InsP<sub>3</sub>Rs) in neurotransmitter release is much less understood. The InsP<sub>3</sub>R is activated by InsP<sub>3</sub>, and its sensitivity to InsP<sub>3</sub> is enhanced by  $Ca^{2+}$  [7]. In the rat barrel cortex, blockage of InsP<sub>3</sub>Rs using 2-aminoethoxydiphenylborane resulted in a reduced frequency of

minis recorded from layer II pyramidal neurons [158]. However, at the *C. elegans* NMJ, synaptic transmission appeared normal in a hypomorphic mutant of *itr-1*, which encodes the only InsP<sub>3</sub>R of *C. elegans* [144].

## 7 Presynaptic Mitochondria May Play a Role in Sustained Neurotransmitter Release

Mitochondria are highly concentrated at the presynaptic nerve terminal and serve at least two critical functions. First, they provide the energy needed by the presynaptic nerve terminal, which may account for up to 10% of the total energy required for neuronal signaling [159]. Second, they may regulate neurotransmitter release by modulating  $[Ca^{2+}]_i$  at the presynaptic nerve terminal.

The interior of the mitochondrion is approximately 200 mV more negative than the exterior, creating a significant driving force for  $Ca^{2+}$  influx.  $Ca^{2+}$  may enter the mitochondrion through undefined uniporters in the inner membrane and exit through  $Na^+/Ca^{2+}$  and  $H^+/Ca^{2+}$  antiporters [160]. During sustained high-frequency nerve stimulation, the concentration of  $Ca^{2+}$  inside the mitochondrion ( $[Ca^{2+}]_m$ ) increases due to enhanced uptake activity. There is ongoing debate about the levels that  $[Ca^{2+}]_m$  can rise to, with estimates ranging from as low as a few micromolar to several hundred micromolar [160].  $Ca^{2+}$  uptake into the mitochondria may occur even when the cytoplasmic  $[Ca^{2+}]_i$  is as low as a few hundred nanomolar [161, 162].

The role of presynaptic mitochondria in neurotransmitter release has been examined in various synapses using pharmacological agents that depolarize the mitochondrial membrane or inhibit the uniporter. These analyses suggest that presynaptic mitochondria serve several functions in synaptic transmission. Firstly, they may accelerate recovery from short-term presynaptic depression. At the rat calyx of Held synapse, a train of stimuli at 200 Hz leads to synaptic depression, indicated by diminishing amplitudes of EPSCs. Following a resting period of 500 ms, the amplitude of EPSCs in response to a single stimulus recovered to ~80% of the first EPSC in the train. However, pharmacological agents that depolarize the mitochondria or inhibit the uniporter resulted in reduced EPSC amplitude recovery due to deficient mitochondrial  $Ca^{2+}$  sequestration [163]. Secondly, presynaptic mitochondria may alleviate synaptic depression in response to sustained nerve stimulation. At the lizard NMJ, the amplitude of end-plate potentials gradually decreases in response to a train of 500 stimuli at 50 Hz. Treatments that depolarize the mitochondria aggravated synaptic depression [164]. Thirdly, mitochondria may contribute to post-tetanic potentiation. At the crayfish NMJ, tetanus stimulation of the motor axon for 7–10 min at 20–33 Hz potentiated subsequent responses to nerve stimulation, which were blocked by pharmacological perturbation of mitochondrial  $Ca^{2+}$  handling [165]. These observations suggest that the regulation of  $Ca^{2+}$  by mitochondria may be essential in controlling neurotransmitter release during sustained nerve stimulation.

## 8 Are Minis $\text{Ca}^{2+}$ -Dependent?

Traditionally, minis were considered as elementary events of action potential-evoked neurotransmitter release with no physiological significance, resulting from the full-collapse fusion of individual synaptic vesicles. However, over the past two decades, our understanding of minis has significantly progressed with several notable advances. First, minis may also occur through kiss-and-run exocytosis, where the release of neurotransmitters in the synaptic vesicle may be partial (Chapter “[Multiple modes of fusion and retrieval at the calyx of Held synapse](#)”). Second, minis may be essential for several important physiological functions, such as post-synaptic receptor clustering [166], modulation of NMDA receptor subunit composition during development [167], regulation of dendritic protein synthesis [168, 169], maintenance of dendritic spines [170], and action potential firing [143, 171]. Third, minis appear to be different from evoked responses in various ways: minis and evoked responses could result from the release of distinct populations of synaptic vesicles [172]; they could depend on the function of different synaptotagmins, with synaptotagmins 1 and 2 being important for evoked neurotransmitter release [12, 173, 174], and synaptotagmin 12, which does not bind  $\text{Ca}^{2+}$ , being important for minis [175]; minis and evoked responses might be mediated by different  $\text{Ca}^{2+}$  channels in the plasma membrane [176–178]; and they are differentially affected by mutations of synaptobrevin [179], SNAP25 [144, 180], and synaptotagmins [173, 174, 181]. At the *C. elegans* NMJ, with respect of VGCCs, evoked neurotransmitter release is mediated by  $\text{Ca}^{2+}$  entry through UNC-2, a Cav2 channel, while minis depend on  $\text{Ca}^{2+}$  entry through both UNC-2 and EGL-19, a Cav1 channel [91]. Time-resolved “flash-and-freeze” electron microscopy, fluorescence microscopy, and electrophysiology analyses indicate that UNC-2 and EGL-19 act on two spatially distinct pools of synaptic vesicles, including a central pool dependent on UNC-2 and a lateral pool dependent on EGL-19 and RyRs [90].

Despite the physiological significance of minis and their utility in analyzing synaptic transmission, it remains unclear whether the occurrence of minis is dependent on  $\text{Ca}^{2+}$ . Scientific literature often refers to minis as “ $\text{Ca}^{2+}$ -independent” events because they can occur in the presence of  $\text{Ca}^{2+}$ -free or nominally  $\text{Ca}^{2+}$ -free extracellular solutions. However, it is important to note that a significant amount of  $\text{Ca}^{2+}$  may be present in the water used to create nominally  $\text{Ca}^{2+}$ -free solutions, and RyR-mediated  $\text{Ca}^{2+}$  release from the ER may contribute to the generation of minis. Therefore, the term “ $\text{Ca}^{2+}$ -independence” may be not an accurate description of minis. In fact, there is no compelling evidence to suggest that minis can occur in the complete absence of  $\text{Ca}^{2+}$ , with the possible exceptions of minis induced by hypertonic solutions [182] and  $\alpha$ -latrotoxin [183, 184].

The roles of extracellular  $\text{Ca}^{2+}$  and RyRs in minis have been examined in several previous studies. Extracellular  $\text{Ca}^{2+}$  appears to play a varying role in minis across different synapses. For instance, reducing  $[\text{Ca}^{2+}]_o$  from 5 mM to zero in the presence of 5 mM EGTA resulted in an approximate 80% decrease in the frequency of minis at the *C. elegans* NMJ [144, 185]. Conversely, the application of ionomycin, a  $\text{Ca}^{2+}$

ionophore, increased the frequencies of both excitatory and inhibitory minis in hippocampal brain slices [184]. These observations suggest that  $\text{Ca}^{2+}$  influx may induce minis. However, in several synapses investigated, the application of the  $\text{Ca}^{2+}$  channel blocker cadmium or a  $\text{Ca}^{2+}$ -free solution did not affect the frequency of minis [186–190], indicating that  $\text{Ca}^{2+}$  influx may not trigger minis. At the frog NMJ, changing  $[\text{Ca}^{2+}]_o$  showed varying effects on the frequency of minis [191].

Similarly, manipulating the function of RyRs produced varying effects on minis. Blocking RyRs with ryanodine (20–100  $\mu\text{M}$ ) decreased the frequency of minis recorded from rat barrel cortex layer II pyramidal neurons [158], cerebellar Purkinje neurons [141], and hippocampal CA3 neurons [143]. On the other hand, activating RyRs with caffeine or ryanodine (10  $\mu\text{M}$ ) increased the frequency of minis recorded from pyramidal [158] and Purkinje [141] neurons. These observations suggest that RyR-mediated  $\text{Ca}^{2+}$  release from the ER is significant to minis. However, blocking RyRs with ryanodine (30 or 100  $\mu\text{M}$ ) did not exhibit a significant effect on the frequencies of glycinergic minis in rat auditory brainstem nuclei [156] and GABAergic minis in rat hippocampus [157], indicating that RyRs do not mediate minis at these synapses.

It is not clear why manipulations of  $[\text{Ca}^{2+}]_o$  and RyR function have different effects on minis at different synapses. The results presented above do not provide enough evidence to tell whether  $\text{Ca}^{2+}$  is necessary for minis because some minis persisted when either  $[\text{Ca}^{2+}]_o$  or RyR function was removed or blocked. One study has investigated the combined effects of a  $\text{Ca}^{2+}$ -free extracellular solution and RyR dysfunction on synaptic transmission in *C. elegans*. The study found that a null mutation of the RyR gene *unc-68* decreased the frequency of minis by more than 75% at the NMJ in the presence of 5 mM  $[\text{Ca}^{2+}]_o$ , and applying a  $\text{Ca}^{2+}$ -free extracellular solution almost completely eliminated the remaining minis [144]. Another study showed that minis at the *C. elegans* NMJ are essentially abolished by combined deficiencies of UNC-2 ( $\text{Ca}_v2$ ) and EGL-19 ( $\text{Ca}_v1$ ) even in the presence of extracellular  $\text{Ca}^{2+}$  (0.5 mM) [90]. These results suggest that  $\text{Ca}^{2+}$  may be necessary for minis, and that  $\text{Ca}^{2+}$  influx and RyR-mediated  $\text{Ca}^{2+}$  release are the only sources of  $\text{Ca}^{2+}$  that trigger synaptic exocytosis at the *C. elegans* NMJ.

If it is true that  $\text{Ca}^{2+}$  is required for the occurrence of minis, then the application of fast  $\text{Ca}^{2+}$  chelators such as BAPTA-AM might be able to eliminate all minis. However, in previous studies, BAPTA-AM had no effect on the frequency of minis at inhibitory synapses in rat auditory brainstem nuclei [156], excitatory synapses in cultured rat hippocampal neurons [192], and dorsolateral periaqueductal gray neurons of rats [193]. At other synapses, such as the mouse calyx of Held synapse and NMJ and rat barrel cortex layer II pyramidal neurons, a  $\text{Ca}^{2+}$ -free extracellular solution containing BAPTA-AM was able to significantly reduce the frequency of minis but not eliminate them [158, 174]. These observations suggest that either  $\text{Ca}^{2+}$  is not essential to minis at these synapses or that a very tight functional coupling exists between  $\text{Ca}^{2+}$  channels or RyRs and the  $\text{Ca}^{2+}$  sensor of synaptic exocytosis. Therefore, it may be useful to investigate the combined effects of a  $\text{Ca}^{2+}$ -free extracellular solution and RyR mutation on the frequency of minis in other systems to determine whether the findings from *C. elegans* are applicable to other synapses.



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