# Presynaptic calcium channels: specialized control of synaptic neurotransmitter release

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**Abstract|** Chemical synapses are heterogeneous junctions formed between neurons that are specialized for the conversion of electrical impulses into the exocytotic release of neurotransmitters. Voltage-gated $Ca^{2+}$ (Cav) channels play a pivotal role in this process as they are the major conduits for the $Ca^{2+}$ ions that trigger the fusion of neurotransmitter-containing vesicles with the presynaptic membrane. Alterations in the intrinsic function of these channels, and their positioning within the active zone can profoundly alter the timing and strength of synaptic output. Advances in optical and electron microscopic imaging, structural biology and molecular techniques have facilitated recent breakthroughs in our understanding of the properties of Cav channels that support their presynaptic functions. Here we examine the nature of these channels, how they are trafficked to and anchored within presynaptic boutons, and the mechanisms that allow them to function optimally in shaping the flow of information through neural circuits.

## [H1] Introduction

In their pioneering studies of the squid giant synapse more than 50 years ago, Bernard Katz and Ricardo Miledi established the importance of $Ca^{2+}$ ions as the "essential links in the 'electro-secretory' coupling process of the axon terminal"[1]. It is now well-established that voltage-gated $Ca^{2+}$ (Cav) channels transduce electrical activity into the flow of $Ca^{2+}$ ions that initiate the vesicular release of neurotransmitters at synapses. Although these channels are normally comprised of a pore-forming $\alpha_1$ subunit and two auxiliary subunits [G] ($\beta$ and $\alpha_2\delta$), they interact directly or indirectly with a diverse array of proteins that regulate their function, modulation and localization within the presynaptic terminal.

While electrophysiological approaches have traditionally been used to characterize the biophysical properties of Cav channels and their roles in synaptic transmission (usually in rat and mouse tissue), a number of technical advances in the last decade have paved the way for new insights into how Cav channels participate in neurotransmitter release. In particular, novel imaging approaches have aided quantitative analyses of Cav channels within tiny presynaptic boutons at nanoscale resolution (**BOX 1**). In addition, three-dimensional views of Cav channels, unveiled by cryo-electron microscopy[2,3], have provided a framework for understanding the key molecular determinants that underly the complex functioning of Cav channels at the synapse. In this Review, we will highlight recent progress towards understanding the structure and function of Cav channels, with an emphasis on those features that support the presynaptic roles of these channels in neurons. Most of the work discussed has been carried out in rodents; experiments using other species have been indicated.

## [H1] Characterization of Cav channels

In the decades since the initial electrophysiological characterization of high and low voltage-activated $Ca^{2+}$ currents (termed HVA and LVA currents) in invertebrates[4] — and subsequently in mammalian neurons and cardiac atrial cells[5,6] — the Cav channel subtypes underlying those currents have been characterized (**FIG.1a**). It is now clear that the division between HVA and LVA channels was rather artificial as there is a continuum of activation thresholds among the different Cav subtypes (**FIG. 1b**), which can be further accentuated by different auxiliary subunit combinations, as well as by alternative splicing **[G]** [7-9]. Subsequently the use of pharmacological and biochemical tools, as well as the availability of mouse strains lacking expression of specific $Ca_v$ channel subtypes, has allowed the properties of cloned Cav channel isoforms to be matched more closely with those identified in different tissues (**FIG.1a**).

### [H2] *Dihydropyridines aid identification of Cav1 channels.* Originally

characterized in muscle and neurons[10,11], Cav1 channels form a subset of HVA channels that were designated through electrophysiological experiments as 'L-type' channels, based on their 'long' openings in the presence of dihydropyridine (DHP) agonists[12]. Biochemical purification of DHP-binding proteins from skeletal muscle revealed that these channels (now known as Cav1.1 channels) were comprised of multiple subunits[13]: an $\alpha_1$ ion-conducting subunit (which was called $\alpha_1 S$) and auxiliary $\alpha_2$, $\beta$, $\Upsilon$, and $\delta$ subunits[13,14]. The cloning of the genes encoding $\alpha_1 S$ (*CACNA1S*)[14,15] and subsequently the $\alpha_1$ subunits of Cav1.2 channels from the heart (*CACNA1C*)[16], Cav1.3 channels from the brain (*CACNA1D*)[17], and Cav1.4 channels in the retina (*CACNA1F*)[18,19] revealed key functional differences among Cav1 subtypes (**FIG. 1a**).

For example, Cav1.3 and Cav1.4 were shown to activate at more negative voltages than Cav1.2 (**FIG. 1b**)[20-22].

**[H2] Toxins reveal the presence of distinct non-L-type channels.** The functional characterization of neuronal $Ca_V2$ channels, and the genes encoding their $\alpha_1$ subunits, was greatly aided by the discovery of toxins that specifically block these channels: ω-agatoxin IVA for the 'P/Q-type' channels (now known as Cav2.1 channels and containing $\alpha_1A$) [23], ω-conotoxin GVIA for the 'N-type' channels (now known as Cav2.2 channels and containing containing $\alpha_1B$)[24], and SNX-482 for the 'R-type' channel (now known as Cav2.3 channels and containing $\alpha_1E$)[25]. The genes encoding the $\alpha_1$ subunits of 'T-type' channels (now known as Cav3.1, Cav3.2 and Cav3.3 channels) were then cloned (for review see[26]) (**FIG. 1a**). When expressed in heterologous expression systems, Cav3 channels show very hyperpolarized activation voltages **[G]** (**FIG. 1b**) and rapid inactivation as compared to Cav1 and Cav2 channels, and also do not require auxiliary subunits (for review see[26]).

**[H2] Cav channel topology and involvement in neurotransmitter release.** Like the voltage-gated $Na^+$ channels, Cav $\alpha_1$ subunits have 24 transmembrane segments organized into four homologous domains (I-IV), each with 6 transmembrane alpha-helical segments (S1-S6, **FIG. 1c**). The S5 and S6 segments of each domain form the channel pore **[G]** and selectivity filter **[G]** , with S1-S4 contributing to a voltage-sensing domain (VSD), as further elucidated by the recent structural studies of Cav1.1[2] and Cav3.1[3]. The main Cav channels involved in transmitter release in the central and peripheral nervous systems are Cav2.1 and Cav2.2, whereas Cav1.3

and Cav1.4 are key to the functioning of the specialized ribbon synapses **[G]** of the cochlea and retina. The current nomenclature for Cav channels is based on the identity of the ten $\alpha_1$ subunits identified in the mammalian genome[27] (**FIG. 1a**). In this Review, we will adhere to the convention that 'CavX' will refer to the channels containing the corresponding $\alpha_1$ subunit.

**[H1] Auxiliary Cav subunits**

The properties of Cav1 and Cav2 channels are modulated by their auxiliary subunits, in ways that are key to their differing roles in neurotransmitter release.

*[H2] β subunits are cytosolic modulators of Cav function.* Encoded by four genes (*CACNB1-4*), the β subunits of Cav channels ($\beta_1$- $\beta_4$) regulate various channel properties, including their levels at the cell surface and their voltage-dependent activation and inactivation (for review see[28]). Cav β subunits bind to an $\alpha_1$-interaction domain (AID) in the I-II linker of Cav1 and Cav2 channels [29]. The $\beta_2$ variant confers Cav channels with relatively slow voltage-dependent inactivation — an effect that can be modified further by alternative pre-mRNA splicing[30,31]. In the brain, $\beta_4$ is the predominant ß isoform found in association with Cav2 channels[32]. By contrast, $\beta_2$ is the major Cav β subunit that is thought to interact with Cav1 channels at cochlear and retinal ribbon synapses[31,33].

*[H2] $\alpha_2\delta$ subunits are post-translationally processed extracellular subunits.* The two polypeptides ($\alpha_2$ and $\delta$) making up the auxiliary $\alpha_2\delta$ subunit are encoded by the same gene, of which there are four (*CACNA2D1-4*) (for review see [34]). The gene product encodes a pre-protein, which is proteolytically processed into $\alpha_2$ and $\delta$[35,36].

These proteins are associated with the external leaflet of the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor[37]. $\alpha_2\delta$-1-3 are all expressed in the brain, whereas $\alpha_2\delta$-4 is most abundant in the retina[38].

$\alpha_2\delta$ interacts via its von Willebrand factor A (VWA) domain primarily with the first extracellular loop of domain I of Cav1 and Cav2 $\alpha_1$ subunits[2,39,40] and can regulate the activation and inactivation of Cav channels, although the strength of this regulation varies among Cav subtypes (for review see [34]). The $\alpha_2\delta$ subunits also increase the cell surface expression of these channels, in a manner that requires the presence of Cav $\beta$ subunits[41]. While in complex with presynaptic Cav channels (or possibly independently), $\alpha_2\delta$ subunits may engage in trans-synaptic interactions with proteins that regulate axonal wiring or other processes[42-45].

*[H2] γ subunits.* Although $\gamma_1$ is a component of the skeletal Cav1.1 channel complex[2], none of the $\gamma$ subunits co-purify with Cav2 subtypes in the brain[32] and thus are unlikely to be major components of neuronal Cav channel complexes. Although $\gamma_2$ (also known as stargazin) was first interpreted to be a Cav subunit[46], it and the subsequently cloned $\gamma_{3-8}$ are now understood to be auxiliary subunits of AMPA glutamate receptors[47]. Thus, they will not be considered further here.

**[H1] Trafficking of Cav channels**

*[H2] Auxiliary subunits support Cav channel assembly and trafficking.* In neuronal cell bodies, the formation of the Cav channel complex begins with the translation and folding of $\alpha_1$ in association with the endoplasmic reticulum, a process that may be enhanced by $Ca^{2+}$ binding to the selectivity filter within the pore[48] (**FIG.**

**1c**). The binding of a Cav β subunit to the cytosolic face of $\alpha_1$ promotes the maturation of the channel complex (**FIG. 2a**), protecting it from polyubiquitination and endoplasmic reticulum-associated proteasomal degradation, thus increasing its forward trafficking[49-51].

Where in the assembly and trafficking pathway $\alpha_2\delta$ associates with the channel complex is largely unknown. Since it binds to extracellular sites on $\alpha_1$ (**FIG. 1c**), the $\alpha_2\delta$ pre-protein (that is, the uncleaved form of the protein) could co-assemble with $\alpha_1$ in the endoplasmic reticulum, where they are both translated (**FIG. 2a**). Given that the uncleaved $\alpha_2\delta$ subunit does not allow $Ca^{2+}$ influx through the channel, such co-assembly with $\alpha_2\delta$ could prevent nascent Cav1 and Cav2 channels from opening and thus leaking $Ca^{2+}$ out of the endoplasmic reticulum, potentially avoiding toxic elevations in cytosolic $Ca^{2+}$ [52]. While the $\alpha_2\delta$ pre-protein shows only 'immature' (that is, endoplasmic reticulum enzyme-mediated) glycosylation, the proteolytically processed $\alpha_2$ and $\delta$ subunits exhibit 'mature' (that is, Golgi enzyme-mediated) glycosylation, a process that is modulated by interaction with the multi-functional trafficking protein low density lipoprotein receptor-related protein-1 (LRP1)[45]. This indicates that cleavage of the $\alpha_2\delta$ pre-proteins into mature $\alpha_2$ and $\delta$ subunits probably begins to occur in the Golgi apparatus (**FIG. 2a**).

### [H2] Sorting and trafficking Cav channels to presynaptic sites of action.

In the trans-Golgi network, Cav channels are packaged into trafficking endosomes (or trafficking vesicles) for sorting to their final destination (**FIG. 2a**). Although $\alpha_2\delta$ can be transported alone to the plasma membrane and into neurites [52]

(**FIG. 2a**), Cav2 channels require $\alpha_2\delta$ to be present for optimal trafficking into presynaptic terminals[52-54]. For Ca$_V$2.2, it has been shown that this process is regulated by the adaptor protein AP-1 binding to identified motifs in region encoded by an alternatively spliced exon in the proximal C-terminus (exon 37a) and that $\alpha_2\delta$ is required for the effectiveness of AP-1 in this trafficking mechanism[55]. The proteolytic cleavage of $\alpha_2\delta$ in the Golgi may expose this motif, mediating exit of the channel complex from the trans-Golgi network into trafficking endosomes[55]. $\alpha_2\delta$-1 has been identified at the electron microscopic level in axonal endosomes[56], and biochemical analysis indicates that these $\alpha_2\delta$-1 subunits are largely in the mature cleaved form[52], in agreement with the hypothesis that $\alpha_2\delta$ proteolytic cleavage may be a prerequisite for entry into this trafficking pathway in neurons.

Within a single neuron, the Cav subtypes present in the soma, dendrites, and presynaptic terminals can vary substantially[57]. Like other neuronal proteins, the Cav subtypes destined for these compartments may be sorted differentially at key checkpoints[58]. Cav channels interact with a variety of synaptic proteins, some of which affect the presynaptic levels of these channels. However, it is not always evident whether such protein interactions are required for the initial targeting and transport of Cav channels to presynaptic terminals or whether they are involved in maintaining their clustering within presynaptic sites of action.

The segregation of trafficking endosomes containing presynaptic cargo is mediated by multiple Rab proteins[59,60] and appears to occur at a pre-axonal exclusion zone within the axon hillock, prior to the axon initial segment[61] (**FIG. 2b**). Following sorting, the anterograde transport of vesicles along microtubules employs kinesin motors, with different kinesins being involved in dendritic and axonal trafficking[62,63] (**FIG. 2b**). Presynaptic components of the active zone may traffic as

pre-formed units[64], and be inserted together into the presynaptic membrane to enlarge active zones during synaptic plasticity[65]. $\alpha_2\delta$ may be important in this regard, since its overexpression in hippocampal neurons increases presynaptic Cav channel abundance as well as the size of the active zone cytomatrix[66]. Whether Cav channels are co-transported with other active zone proteins has not been established for Cav2 channels, but has been studied with respect to Cav1.4 channels in retinal photoreceptors. It has been shown that Cav1.4 is transported together with other presynaptic proteins (such as Munc13, CAST1 and RIM2) to the presynaptic terminal[67].

## [H1] Synapse-specific Cav functions.

In order to encode a wide range of information that can vary with development and experience, synapses are remarkably diverse in terms of their complement of proteins, and this diversity extends to Cav channels. Studies aimed at defining the contributions of specific Cav subtypes to neurotransmitter release have traditionally relied on electrophysiological recordings of postsynaptic responses to presynaptic stimulation. However, the precision of this approach is somewhat limited by the non-linearity of the relationship between $Ca^{2+}$ entry and vesicular release (for recent review see [68]). More recently, many novel techniques and preparations have improved our ability to directly identify presynaptic Cav channels and the $Ca^{2+}$ signals they mediate at the active zone (**BOX 1**), and have significantly advanced our understanding of the contributions of Cav channels to vesicle release properties.

**[H2] Cav2 channels regulate exocytosis at most synapses.** The fusion of synaptic vesicles with the presynaptic membrane can occur in the absence of stimulation (spontaneous release) or within milliseconds (synchronous release) or up to tens of seconds after (asynchronous release) the arrival of an action potential into the terminal (for review see [69]). Most synapses utilize Cav2.1 and/or Cav2.2 channels to coordinate synchronous release, but there are few generalities that can be made regarding which Cav2 subtype(s) predominates at different synapses. Blockers of Cav2.1 and/or Cav2.2 blunt glutamatergic transmission at synapses in many brain regions[70,71]. While most GABA-ergic neurons in the cerebellum utilize primarily presynaptic Cav2.1 channels[72,73], those in the hippocampus rely on a more variable complement of Cav channels. For example, at synapses formed with dentate granule cells, GABA release is triggered by Cav2.2 or Cav2.1 channels in the presynaptic terminals of interneurons expressing cholecystokinin (CCK) or parvalbumin (PV), respectively[74].

Owing to their small size, most presynaptic terminals are not amenable to pharmacological characterization of resident Cav channels by direct patch-clamp recordings. This hurdle is overcome in analyses of large, electrotonically compact glutamatergic terminals, such those of the dentate granule cells that form synapses with CA3 pyramidal cells in the hippocampus. In patch-clamp recordings of these 'mossy fiber' boutons, Cav2.1 is most efficiently activated by action potentials, with contributions of Cav2.2 and Cav2.3 becoming more evident as action potential width is increased[75]. Consistent with its relatively negative activation voltage, Cav2.3 is the main Cav channel that is recruited by subthreshold depolarizations of mossy fiber boutons, which may account for the prominent role of this Cav2 subtype in plasticity of mossy fiber synapses[76,77] and spontaneous release[78]. Within negative voltage

ranges, such as those present in neurons at or below resting potentials, Cav3 channels have also been found to indirectly modulate neurotransmitter release at various synapses[79,80].

**[H2] Matching Cav2 subtypes to meet synapse demands.** Within a single terminal, the presence of multiple Cav2 subtypes with distinct properties may help to diversify synaptic responses to reflect changes in action potential firing patterns, such as changes in frequency or bursting activity. However, some synapses may require less variability in stimulus–response properties. For example, the Calyx of Held is a synapse requiring rapid and temporally precise glutamate release within the sound localization circuit of the mature auditory brainstem[81]. To accommodate this need, the distance between Cav channels and exocytotic release sites (that is, the coupling distance) at the mature Calyx of Held synapse is very small (<30 nm), such that the opening of only a few Cav channels to form a 'nanodomain' of elevated intracellular $Ca^{2+}$ can trigger vesicle fusion[82] (**BOX 2**). By contrast, before the maturation of this synapse the requirements for Cav channel coupling distance appear less stringent. It has been shown that $Ca^{2+}$ chelators such as EGTA (which have a slow $Ca^{2+}$ binding rate) can blunt release at the Calyx of Held before but not after hearing onset, suggesting that exocytosis at immature Calyx of Held synapses relies on the presence of a 'microdomain' of elevated intracellular $Ca^{2+}$, resulting from the opening of multiple $Ca_v$ channels that are only loosely coupled (up to ~100 nm) to readily-releasable vesicles[83].

As occurs at other synapses[84], the maturation of neurotransmission at the Calyx of Held is accompanied by a shift from a mixed population of Cav2 subtypes[82,85] to one that is dominated by Cav2.1[72,83,86]. Indeed, Cav2.1 is

distinguished from other Cav2 subtypes at most mature central synapses in exhibiting nanodomain coupling [87]. Recent evidence suggests that this property of Cav2.1 channels may relate to their unique tethering by active zone proteins. Quantitative SDS-digested freeze-fracture replica immuno-electron microscopy (SDS-FRL EM; **BOX 1**) of mature parallel fiber–Purkinje cell synapses in the cerebellum has revealed that Cav2.1 is selectively clustered within the active zone, where it is surrounded by Ca$_V$2.2 towards the perimeter of the active zone [88] (see part d of the figure in **BOX 2**). Deletion of Munc13-3, one of three members of the Munc13 family, disrupts the differential localization of Cav2.1 and Ca$_V$2.2 channels at this synapse and the developmental transition from microdomain coupling, supported by both subtypes, to nanodomain coupling supported only by Cav2.1[88]. However, the effects of Munc13-3 in regulating coupling and localization of Cav2 subtypes may differ between synapses[82] (also see **BOX 2**). Filament-forming septins have also been implicated in the developmental switch between microdomain and nanodomain coupling[89,90].

The presence of a mixed population of Cav2 subtypes may also enable the diversification of $Ca^{2+}$ sources in order to match distinct presynaptic responses to variations in activity. For example, at mossy fiber-CA3 synapses in the adult mouse hippocampus, Cav2.3 does not contribute to the $Ca^{2+}$ signals that support the fast neurotransmitter release evoked by single action potentials; however, when neurons are subjected to tetanic stimulation causing brief trains of presynaptic action potentials, Cav2.3 can induce long-term and post-tetanic potentiation. This suggests that Cav2.3 is localized at a greater distance from neurotransmitter release sites than other Cav2 subtypes [76]. At this synapse, Cav2.3 channels are brought into play by tetanic stimulation, which boosts presynaptic $Ca^{2+}$ to levels that can support these

forms of potentiation [76]. At Schaffer collateral synapses in the adult mouse hippocampus, the contributions of Cav2.1, Cav2.2, and Cav2.3 also vary with different stimulus frequencies, which can affect the filtering features that are caused by GABA receptor-mediated inhibition of these synapses[91]. These findings suggest that the pathological up- or down-regulation of a particular Cav2 subtype (for example, as a result of a disease-causing mutation) could profoundly alter the information-encoding properties of synapses containing mixed Cav2 subtypes.

### [H2] Slowly inactivating-Cav1 channels encode sensory information at ribbon synapses.

Vision, hearing and balance rely on transmission at ribbon synapses in the retina and inner ear. In contrast to the phasic, action potential-mediated neurotransmitter release that occurs at conventional synapses, ribbon synapses are specialized for analog transmission. They feature continuous glutamate release that is modulated by graded changes in the membrane potential (for review see[92]). One specialized feature of these synapses is the synaptic ribbon, an organelle found in cochlear and vestibular hair cells, as well as in retinal photoreceptors and bipolar cells. Among other functions, the ribbon primes vesicles for exocytosis and helps to maintain glutamate release during prolonged stimulation[93] (**FIG. 3**).

A second feature of ribbon synapses is their reliance on slowly inactivating Cav1 channels: Cav1.3 in inner hair cells (IHCs) and Cav1.4 in photoreceptors[94-96]. In both cell types, slow voltage-dependent inactivation of Cav1 channels is thought to be conferred in part by the $\beta_2$ subunit[97-99]. $Ca^{2+}$-dependent inactivation (CDI), a process that is mediated by calmodulin **[G]** (CaM) binding to a consensus IQ-domain in the C-terminal domain of Cav1 channels[100], is also suppressed in these channels;

however, the mechanisms underlying this suppression for native Cav1.3 and Cav1.4 channels are distinct (**FIG. 3**).

Compared to the properties of Cav1.3 channels expressed in heterologous expression systems, these channels undergo very little CDI when expressed in IHCs[101]. The mechanism underlying the loss of CDI in these cells is thought to involve $Ca^{2+}$-binding proteins (CABPs), a family of CaM-like proteins that suppress CDI, in part by displacing CaM from its binding site on Cav1 channels (**FIG. 3a**). Like CaM, CABPs possess 4 EF-hand $Ca^{2+}$ binding domains, one of which is non-functional[102]. Due to its abundant expression in IHCs and ability to suppress CDI of Cav1.3 upon co-transfection in cell lines[103-105], CABP2 initially emerged as a promising candidate for opposing CDI of Cav1.3 in IHCs. However, in IHCs of CABP2 knock-out mice, voltage-dependent inactivation of Cav1.3 was increased, but CDI was unaffected[106]. It is thought that this was perhaps due to the overlapping expression of another CABP family member, CABP1, in IHCs[103,104] (**FIG. 3a**). Additional mechanisms that could suppress CDI in IHCs include alternative splicing of the mRNA encoding $Ca_v1.3$ [107] and interactions of Cav1.3 with other synaptic proteins[108].

Unlike Cav1.3, long splice variants of Cav1.4 show little CDI even in the absence of CABPs, due to the presence of a C-terminal modulatory domain (CTM) that competes with and/or modifies CaM binding to the channel[109,110]. Cav1.4 splice variants lacking some or all of the CTM, such as one lacking exon 47 (Cav1.4Δex47, Fig.3b), show strong CDI when expressed heterologously[111,112]. CABP4 is a CABP family member that is enriched in photoreceptor synaptic terminals[113] which interacts with the CaM binding site and suppresses CDI of Cav1.4Δex47[114] (**FIG. 3b**). Thus, it

is expected that Cav1.4Δex47, and perhaps other CTM-lacking variants, would show limited CDI in the retina.

# [H1] Cav organization at the synapse

Quantitative proteomic analyses suggest that the presynaptic interactome of Cav2 channels includes ~200 proteins, although not all of these are direct interactions[32]. The intimate association of Cav channels with proteins of the active zone could ensure that vesicular release occurs rapidly upon arrival of an action potential[115], and is aligned with the relevant postsynaptic elements at the nanoscale level[116] (**FIG. 4a**).

## [H2] *A web of protein interactions regulate presynaptic Cav channel clustering.*

Rab3-interacting molecules (RIMs) are key organizers of the active zone and bind via their PDZ domains to a conserved motif (DxWC) in the C-terminus of Cav2.1 and Cav2.2 channels[115,117-119] (**FIG. 4a**). RIMs are also indirectly linked to these channels through association with RIM binding proteins (RBPs), that bind to proline-rich motifs (PxxP) in the C-terminal domain of both Cav1 and Cav2 channels[120]. In mice lacking RIM variants and/or RBPs, the density of Cav2.1 channels is reduced in synaptic terminals[115,117,118,121]. The presynaptic abundance of Cav1.3 channels is also reduced in IHCs in the absence of RIMs and RBPs[122,123], indicating a conserved role for these proteins at different types of synapses.

Despite the evidence supporting a requirement for RIMs and RBPs for controlling presynaptic levels of Cav channels, ectopic expression of a Cav2.1 protein lacking the RIM and RBP binding sites at the Calyx of Held of Cav2.1 knock-out mice leads to normal presynaptic Cav current density[124]. A possible explanation

for these discrepant results is that RIM may regulate presynaptic abundance of Cav2.1 channels via tertiary interactions with Cav β subunits (**FIG. 4a**). In support of this idea RBPs are known to interact with Cav β subunits[118] and RIM is known to bind to CAST/ELKS[125], a core active zone protein that binds to $\beta_4$ [126]. Deletion of CAST/ELKS decreases presynaptic $Ca^{2+}$ influx at inhibitory hippocampal synapses[127], and reduces the clustering of Cav2.1 channels at the Calyx of Held[128] and at Drosophila neuromuscular junctions[129]. There is likely to be redundancy in this interconnected network of proteins[130], such that disrupting one point of contact may be compensated by other Cav protein interactions.

The interaction between mature $\alpha_2\delta$ and $\alpha_1$ subunits is also critically important for the clustering of Cav channels in presynaptic terminals[52,54,131]. Genetic inactivation of $\alpha_2\delta$-1 and $\alpha_2\delta$-4 reduces the active zone localization of Cav2.2 in primary afferent terminals in the spinal cord dorsal horn[53] and Cav1.4 in photoreceptor synaptic terminals[42,132]. In the case of photoreceptors, Cav1.4 channels are targeted to synaptic terminals but the presynaptic level of these channels is reduced in the absence of $\alpha_2\delta$-4[42,132].

It has been proposed that $\alpha_2\delta$s may engage in trans-synaptic protein interactions that ensure appropriate alignment of pre- and post-synaptic signaling complexes. In support of this idea, deletion of $\alpha_2\delta$-2 causes misalignment of presynaptic Cav1.3 channels with postsynaptic glutamate receptors at IHC synapses[43]. The formation of rod to rod-bipolar cell synapses in the retina is thought to require the interaction of presynaptic $\alpha_2\delta$-4 with the postsynaptic protein extracellular leucine-rich repeat fibronectin type III domain containing-1 (ELFN1) [42]. $\alpha_2\delta$ was also found to interact with neurexins that interact trans-synaptically with neuroligins[133]. However in a subsequent study, although $\alpha_2\delta$-1 mediated the effects

of α-neurexin on Cav2.1 function, no specific association could be demonstrated [134], and this interaction is not required for postsynaptic recruitment of GABA receptors at inhibitory synapses[44]. $\alpha_2\delta$ binding to thrombospondins, a family of extracellular matrix proteins, was found to promote excitatory synaptogenesis[135], although in other studies this interaction was shown to be of relatively low affinity and was only demonstrated for thrombospondin-4[136,137]. How such protein interactions with Cav complexes would still allow their mobility within the active zone[66,138] also remains unclear.

**[H2] Molecular coupling of Cav channels to release sites.** The resolution limits of confocal microscopy have previously hindered quantitative analyses of Cav coupling via imaging of fluorescently tagged channels. This hurdle has been overcome with the advent of super-resolution microscopy methods (such as stimulated emission depletion microscopy (STED) and stochastic optical reconstruction microscopy (STORM)) and SDS-FRL EM (**BOX 1**). STED has shown that two isoforms of the positional priming protein, unc13, form distinct scaffolding complexes that cluster at different distances from Cav channels and might underlie the developmental tightening of the coupling between $Ca^{2+}$ entry and release at the Drosophila neuromuscular junction[139]. The mammalian ortholog of unc13, Munc13, associates with RIM and ELKS at particular synapses[140], and with syntaxin at vesicular release sites[141]. In agreement with this, Munc13 proteins are hypothesized to form a juxta-membrane ring around each vesicular release site, together with an inner ring of oligomerized synaptotagmins, anchoring the soluble NSF attachment protein (SNAP) receptor (SNARE) proteins **[G]** in order to allow vesicular fusion to occur on $Ca^{2+}$ entry[142] (**FIG. 4a**).

The search for mechanisms that facilitate the coupling of Cav2 channels to vesicle release led to the identification of the 'synprint' site—a sequence in the cytoplasmic II-III linker of $Ca_V2.1$ and $Ca_V2.2$ that binds to SNAREs and other exocytotic proteins (for review see[143]). When injected into some neurons, peptides corresponding to the synprint site inhibit synaptic transmission, interpreted as competitive displacement of SNAREs from Cav2 channels, thus loosening the coupling of these channels to release sites[144,145]. However, mammalian synprint peptides inhibit neurotransmitter release when introduced into invertebrate neurons in which Cav2 channels lack the synprint sequence, and so it is thought that they may interfere with vesicle dynamics independent of disrupting Cav2-synaptic protein interactions[146]. For example, the synprint interacts with AP-2 adaptor proteins involved in clathrin-mediated endocytosis, and membrane capacitance recordings indicate that injected synprint peptides disrupt synaptic vesicle endocytosis rather than exocytosis at the Calyx of Held synapse[147].

Are there proteins that interact with Cav channels that directly regulate their coupling to exocytosis? An intriguing result in this regard is the distinct coupling efficiencies of Cav2.1 channels with (Cav2.1+ex47) and without (Cav2.1Δex47) exon 47[148]. This exon encodes the binding sites for a number of active zone proteins including RIM and RBPs. At hippocampal synapses, Cav2.1+ex47 channels exhibited higher mobility as well as activity-dependent accumulation in synaptic nanodomains that strengthened coupling and supported greater neurotransmitter release and paired-pulse depression[148]. Similar results were not, however, obtained at the Calyx of Held, where a 19 amino acid sequence upstream of exon 47 was found to be necessary for rapid vesicle release[124]. This sequence is poorly conserved in Cav2.2 and Cav2.3 and therefore may represent a molecular

determinant that affords Cav2.1 the positional advantage in controlling fast vesicular release at this synapse. Whether this site interacts with proteins that enhance Cav2.1 coupling, or rather promotes channel conformations that facilitate coupling, remains to be discovered.

## [H1] Presynaptic Cav channel regulation

Modulation of presynaptic $Ca^{2+}$ entry is one of the key mechanisms through which neurotransmitter release can be rapidly up- or down-regulated, and is mediated by multiple different mechanisms. In neurons, the exquisite sensitivity of such forms of neuromodulation relates to the non-linearity of the dependence of vesicular release on $Ca^{2+}$ entry, such that a small change in the level of presynaptic $Ca^{2+}$ can profoundly impact the amount of neurotransmitter released.

### *[H2] Modulation of presynaptic Cav function by active zone proteins.* In addition to regulating the presynaptic density of Cav channels and their coupling to vesiclular release, proteins within the active zone can also directly regulate Cav function. This was initially demonstrated for proteins of the synaptic vesicle release machinery — such as syntaxin, SNAP25 and synaptotagmin — which interact with Cav2 $\alpha_1$ subunits (reviewed in[143]) (**FIG.4a**).

Through the interaction of their C2B domains with Cav β subunits (**FIG. 4a**), RIM proteins can augment neurotransmitter release by markedly prolonging Cav2 and Cav1 $Ca^{2+}$ currents[108,118,149]. However, analysis of short RIM2γ variants (which contain the C2B domain but not the PDZ and PxxP sequences that mediate direct and indirect interactions with the Cav2 C-terminal domain) suggests that the ability of

RIM proteins to support neurotransmission may be due to their effects in regulating Cav2 channel abundance and/or tethering near vesicle release sites. Although RIM2$\gamma$ slows inactivation of Cav2 channels in transfected HEK293T cells, it does not rescue neurotransmitter release defects in RIM1/2 double knock-out neurons[150]. In rod photoreceptors, RIM1 and 2 do not affect the clustering of Cav1.4 channels at the synaptic ribbon, but strongly enhance the opening of these channels that is required for evoked glutamate release[151]. Thus, the impact of RIM proteins on Cav channel function and the consequences for neurotransmitter release may vary with Cav channel subtype and in different types of synapse.

**[H2] *Regulation by G-protein coupled receptors.*** A prominent route by which some G-protein coupled receptors (GPCRs) suppress neurotransmitter release is through inhibition of Cav2 channels, with Ca$_\mathrm{V}$2.2 being more sensitive to this form of modulation than Ca$_\mathrm{V}$2.1. Generally mediated by GPCRs that couple to G$_i$/G$_o$ proteins, this inhibition requires the precise positioning of GPCRs within the vicinity of the channels[152] (**FIG. 4b**). The inhibition involves a slowing of channel activation which was first characterized in dorsal root ganglion neurons[153,154], and subsequently shown to be mediated by the G-protein $\beta\gamma$ subunits[155-157]. G$\beta\gamma$-mediated inhibition of Cav2 channels is voltage-dependent in that it can be relieved by strong or repeated depolarizations which cause the unbinding of G$\beta$Y from the channel[158]. It was subsequently found that Ca$_\mathrm{V}$ $\beta$ subunits are required for voltage-dependent inhibition of Cav2 channels by G$\beta\gamma$[159,160]. Although G$\beta\gamma$ was found to interact with a QxxER motif within the AID, this site was previously identified to be partially occupied by Ca$_\mathrm{V}\beta$[161]. Thus other sites, including a sequence of conserved residues in the N-

terminus of all the $Ca_V2$ channels, represent additional essential determinants of $G\beta\gamma$ modulation[162] (**FIG. 4b**).


**[H2]** *The modulatory role of presynaptic lipids*. The importance of the active zone lipid composition has been little explored with respect to presynaptic Cav function. Cholesterol is an important component of presynaptic membranes[163], and GPI-anchored proteins, including $\alpha_2\delta$, are concentrated in cholesterol-rich regions of the membrane, raising the possibility that cholesterol may contribute to a mechanism for clustering presynaptic channels[164]. Furthermore, phosphatidylinositol 4,5-bisphosphate (PIP2), a minor component of the intracellular leaflet of the plasma membrane, is important for the binding of RIM and other synaptic proteins, including synaptotagmin, to the cell membrane[165,166]. Indeed, rapid elevation of PIP2 in the membrane of chromaffin cells, by photo-uncaging, potentiated exocytosis[167] and, on a slower time scale, lowering presynaptic PIP2 in calyx of Held terminals slowed endocytosis[168].

It is also well-documented that Cav channels can be directly regulated by PIP2 (for review see[169]): the activation of $G_q$-coupled GPCRs depletes PIP2 via activation of phospholipase C, and thus inhibits $Ca_V$ currents[170] (**FIG. 4b**). This represents yet another potential mechanism for presynaptic Cav channel modulation, although it has not yet been demonstrated in synapses.


**[H2]** *Presynaptic modulation by protein kinases.* The contribution of Cav channels to neurotransmitter release can be modulated by various protein kinases, including protein kinase C (PKC). In sympathetic neurons, the activation of PKC by

phorbol-12-myristate-13-acetate (PMA) increases currents mediated by Cav2.2 channels by opposing G-protein inhibition[171]. The mechanism for this regulation involves PKC phosphorylation of the $G\beta\gamma$ binding site in the I-II linker of Cav2.2[172] (**FIG. 4b**) and could provide a means whereby $G_q$-linked receptors coupled to PKC could facilitate neurotransmitter release[173]. As shown in Aplysia neurons, PMA can also increase neurotransmitter release by promoting the insertion of Cav2 channels in the presynaptic membrane[174].

Cyclin-dependent protein kinase (CDK5) is another protein kinase implicated in the modulation of Cav channels and presynaptic function. An inhibitor of CDK5, roscovitine, was used to show that CDK5 phosphorylates the II-III loop of $Ca_V2.1$, which reduces $Ca_V2.1$ currents and inhibits the interaction of these channels with SNAP-25 and synaptotagmin[175]. While this mechanism could explain the effects of roscovitine in inhibiting neurotransmitter release, it is important to note that roscovitine has mixed agonist/antagonist effects on Cav channels and may regulate neurotransmission independent of CDK5[176,177]. In an alternative approach, a dominant-negative CDK5 construct was used to show that CDK5 phosphorylates a conserved serine residue in the C-terminal domain of Cav2.2. This was found to cause an increase in somatic Cav2.2-mediated currents (**FIG. 4b**), and also facilitates synaptic vesicle docking and release, possibly through enhanced Cav2.2 interactions with RIM1 and other SNAREs[178] (**FIG. 4a**). However, a later study that used optical imaging of presynaptic boutons showed that CDK5 inhibits action potential evoked Cav2.2-mediated $Ca^{2+}$ signals and vesicular release — effects that were reversed by the phosphatase calcineurin[179]. These disparate results could be due to distinct effects of CDK5 with respect to Cav2.2 channels in the soma

compared to presynaptic terminals and indicate the importance of the balance in kinase and phosphatase activities for the synaptic functions of Cav channels.

Ca$_V$ β subunits also represent a hub for phosphorylation by multiple kinases and for other post-translational modifications (for reviews see[28,180]). While the impact of Ca$_V$ β phosphorylation on the functions of presynaptic Cav channels has yet to be explored, the interaction of Cav β with the Rem-Gem-Kir (RGK) protein, Rad, has been shown to be critical for PKA-mediated potentiation of Cav1.2 channels. However, the mechanism involves PKA phosphorylation of Rad itself, which prevents Rad-mediated inhibition of Cav1.2 [181]. The Cav β-Rad interaction also confers PKA-dependent regulation to Cav2.2 [181], and therefore could contribute to facilitation of presynaptic Ca$^{2+}$ currents by neuromodulators.

**[H2] CaM and other Ca$^{2+}$ binding proteins.** As has been discussed for Cav1 channels, CaM also regulates Cav2 channels; however, the mechanisms involved are distinct. In Cav2.1 channels, the association of CaM with a Ca$^{2+}$ sensor (CaS) binding site in the C-terminal domain causes CDI (**FIG. 4b**), but also a potentiation of the Cav2.1 current (Ca$^{2+}$-dependent facilitation (CDF)) which is apparent during repetitive stimuli[182,183]. CDI and CDF of Cav2.1 have both been reported to contribute to short-term synaptic plasticity at a variety of synapses, which can be modified by CABPs and related CaS proteins (reviewed in[184]). However, many of these results were obtained in electrophysiological recordings at room temperature with relatively high concentrations of extracellular Ca$^{2+}$. Under more physiological conditions, CDF is relatively nominal and does not contribute significantly to short-term facilitation at synapses in the hippocampus, cerebellum, and brainstem[185]. CABP1, binding to the CaS site, prevents CDF and enhances CDI of Cav2.1[186], and

causes short-term depression of PV-expressing interneuron-CA1 pyramidal neuron synapses in the hippocampus[187]. Thus, CaS proteins could oppose CDF of Cav2.1 under physiological conditions in nerve terminals, helping to limit the potential for vesicle depletion at some synapses.

The modest level of Cav2.1 CDF is expected to offset the effect of CDI in promoting synaptic depression[187]. This could be prominent at CA3–CA1 hippocampal synapses, which rely on both Cav2.1 and Cav2.2, since Cav2.2 channels undergo CDI but not CDF[188] (**FIG. 4b**). The lack of CDF in Cav2.2 arises from differences in regions corresponding to the IQ-like domain and upstream sequences of Cav2.2 and Cav2.1[189]. The inability of Cav2.2 to undergo CDF may be beneficial in nociceptive dorsal root ganglion neurons and sympathetic neurons where, if present, CDF would oppose the inhibition of Cav2.2 by neurotransmitters acting on presynaptic GPCRs in the control of pain transmission and sympathetic outflow.

# [H1] Presynaptic Cav channelopathies

Considering how relatively modest changes in Cav channel function and/or positioning within the active zone can dramatically influence synaptic output, it is perhaps not surprising that genetic variations causing dysregulation of Cav channels are linked to a variety of nervous system disorders in humans.

***[H2] Synapse-specific impact and effects on neuromodulation.*** Missense mutations in the *CACNA1A* gene lead to numerous neurological disorders[190]. The

best studied in terms of its impact on synaptic transmission is familial hemiplegic

migraine type 1 (FHM-1), a rare form of migraine with aura. Two FHM-1 mutations in

*CACNA1A*, R192Q and S218L, cause an increase in the open probability of the

$Ca_V2.1$ channel and a large negative shift in the voltage-dependence of its

activation[190]. Studies of knock-in mice bearing these mutations indicate that both

mutations lead to increased action potential-evoked $Ca^{2+}$ influx and a greater

probability of glutamate release at cortical pyramidal cell synapses[191,192]. In contrast,

at the calyx of Held, peak Cav2.1 current density is actually reduced, and action

potential-evoked $Ca^{2+}$ currents are smaller in S218L knock-in mice than in wild type

mice. However, the large (~10 mV) negative shift in the half-maximal activation

voltage enables the opening of some S218L mutant channels at this synapse at the

resting potential, increasing basal levels of intracellular $Ca^{2+}$. As a consequence,

calyces from the knock-in mice still exhibit a gain-of-function, with greater

spontaneous release, faster recovery from synaptic depression, and stronger

synaptic strength than those of wild type mice[193].

Although GABA release at synapses between fast-spiking interneurons and

cortical pyramidal cells relies primarily on Cav2.1, transmission at these synapses is

surprisingly unaltered in FHM-1 mutant mice[191]. Moreover, the enhanced activation

of Cav2.1 in pyramidal cells is not found for these channels in GABA-ergic

interneurons of FHM-1 mutant mice[194]. GABA-ergic neurons may express long

Cav2.1 splice variants containing exon 47, in which the effects of FHM-1 mutations

are nominal[195]. Alternatively, the complement of Cav β subunits, which can affect the

impact of some FHM1 mutations[196], may differ in excitatory and inhibitory

interneurons. Regardless of the underlying mechanism, heightened glutamate

release in the absence of corresponding alterations in inhibitory synaptic inputs to

cortical pyramidal cells is thought to fuel cortical spreading depression, a neurophysiological hallmark of migraine aura in humans, that is also observed in the FHM-1 mutant mice[190].

**[H2] *Presynaptic Cav channels and neuropsychiatric disease.*** Genes encoding Cav channel subunits have emerged as prominent risk alleles for a variety of neuropsychiatric disorders. In the largest genome-wide analysis of schizophrenia to date, genes encoding Cav channel subunits (*CACNA1C, CACNB2,* and *CACNA1I*) and Cav-interacting proteins (RIMs and neuroligins) were identified among the 108 disease-associated loci[197]. Moreover, autism-associated mutations in the RIM3 gene were found to disrupt the effects of RIM3 in suppressing Cav2.1 inactivation, resulting in impaired neurotransmitter release[198]. Mice lacking the Cav $\beta$-anchoring and regulatory protein (BARP), which suppresses Cav2 and Cav1.2 channel function[199], show an enhancement in phenotypes that are characteristic of schizophrenia and autism such as working memory, flexibility, and sociability [200]. Thus, our understanding of how dysregulation of Cav channels gives rise to neuropsychiatric disease could benefit from studies of mouse strains lacking expression of such Cav-modulatory proteins.

Fragile X syndrome is a genetic form of intellectual disability, often associated with autism, which is characterized by a loss of expression of Fragile X mental retardation protein (FMRP). One of the pleiotropic effects of FMRP knockout is disruption of synaptic function [201]. Cav2 channels have been shown to interact with FMRP [202] and FMRP overexpression was found to decrease the forward trafficking of Ca$_V$2.2 [203]. Furthermore loss of FMRP causes an increase in presynaptic Ca$^{2+}$ entry and vesicular release in both primary afferent and hippocampal presynaptic

terminals [202,203]. Thus, interference with normal Ca$_V$2 trafficking and function may be one of the many ways in which of loss of FMRP has a disruptive effect on synaptic function in Fragile X syndrome.

***[H2] Altered interactions with synaptic proteins at ribbon synapses.*** The impact of disease-causing mutations in Cav channel-interacting partners is well-illustrated at photoreceptor synapses, where a mutation causing premature truncation of CABP4 (R216X) results in congenital stationary night blindness type 2 (CSNB2)-related visual impairment[204]. The truncation removes the C-terminal EF-hand domains of CABP4 and prevents the capacity of CABP4 to enhance the voltage-dependent activation of long Cav1.4 variants[113,205] (**FIG. 3b**). Loss of such regulation by CABP4 is expected to decrease the availability of Cav1.4 channels at the membrane potential of photoreceptors in darkness , thus reducing the sensitivity of neurotransmitter release modulation to light stimuli. A remarkably similar mutation in CABP2 (F164X) causes an autosomal recessive hearing impairment. Like R216X in CABP4, F164X deletes the C-terminal EF-hands of CABP2, which greatly impairs its ability to bind Ca$^{2+}$ and to regulate Cav1.3[105] (**FIG. 3a**). Such defects in Cav1.3 function could underlie the abnormalities in glutamate release at IHC synapses and of hearing that have been observed in CABP2 knock-out mice[106]. However, CABP2 and CABP4 are likely to interact with a variety of other effectors presynaptically, and so disease-causing mutations in the encoding genes could have complex effects on synaptic function.

**[H1] Conclusions and future directions**

Advances in multiple techniques, including electron microscopy and super-resolution imaging, electrophysiology, time-resolved optical techniques and simulations have greatly enhanced our current knowledge of the principles governing the organization and function of Cav channels in various types of synaptic boutons[82,84,116,206]. The picture that emerges is that the exquisite molecular diversity of mature synapses and the changes in synaptic function that occur during development in many cases depend on alterations in presynaptic Cav channel properties. The up- or down-regulation of Cav channels within the active zone can be achieved via a myriad of mechanisms, allowing for input-specific forms of neuromodulation onto the dendritic arbour of individual neurons (**FIG. 5a, b**). In cell types with multiple active zones (such as IHCs) the distinct recruitment of different Cav channel-regulatory mechanisms at different release sites offers a convenient mechanism for generating the heterogeneous forms of afferent activity that may be needed to cover a wide dynamic range of sensory stimuli[207] (**FIG. 5c**).

Yet, there are still many gaps in our understanding. We still do not know the routes by which Cav channels are trafficked to the active zone, and whether they are conserved for the different Cav channels found at conventional and ribbon synapses. With improved methods to label Cav channels for ultrastructural analyses, we will be able to identify the organelles that carry Cav channels to and from the plasma membrane and determine whether the insertion and retention of Cav channels in the active zone are dynamically regulated by the pattern of electrical activity. These approaches will be particularly informative for studies of how differential sorting and trafficking of Cav2.1 and Cav2.2 might underlie the distinct coupling of these Cav2 subtypes to neurotransmitter release[57,82,85].

As members of the presynaptic interactome of Cav channels continue to be identified, advances in imaging technologies[208] should help resolve the temporal and spatial dynamics of key interactions of Cav channels with synaptic proteins in relation to vesicle release, not only for synapses in culture but also in intact neural circuits. *In vivo* proximity labeling techniques that can identify components of Cav microenvironment [181], combined with synapse-specific proteomic strategies[209] are now in place to quantify how activity-dependent modifications in Cav complexes contribute to synaptic plasticity in both healthy and diseased states of the nervous system.

The differences in the intrinsic properties and modulation of Cav subtypes in heterologous expression systems has led to the assumption that such differences contribute to the distinct roles of Cav channels at various synapses. An important challenge for future studies will be to validate this assumption in intact neural circuits. Viral methodologies that enable the expression of the entire Cav $\alpha_1$ subunit[124] in neurons should facilitate future analyses both ex vivo and in vivo of structure–function relationships of Cav channels and the ways in which they contribute to presynaptic function in mature and developing circuits. Furthermore, the importance of G-protein modulation of presynaptic Cav channels in a variety of neurological contexts can now be resolved at the level of individual synapses using cell type-specific targeting of GPCR ligands[210].

As the combination of techniques that are available to researchers continues to evolve, it is clear that new results will continue to surprise in this field for many years to come.

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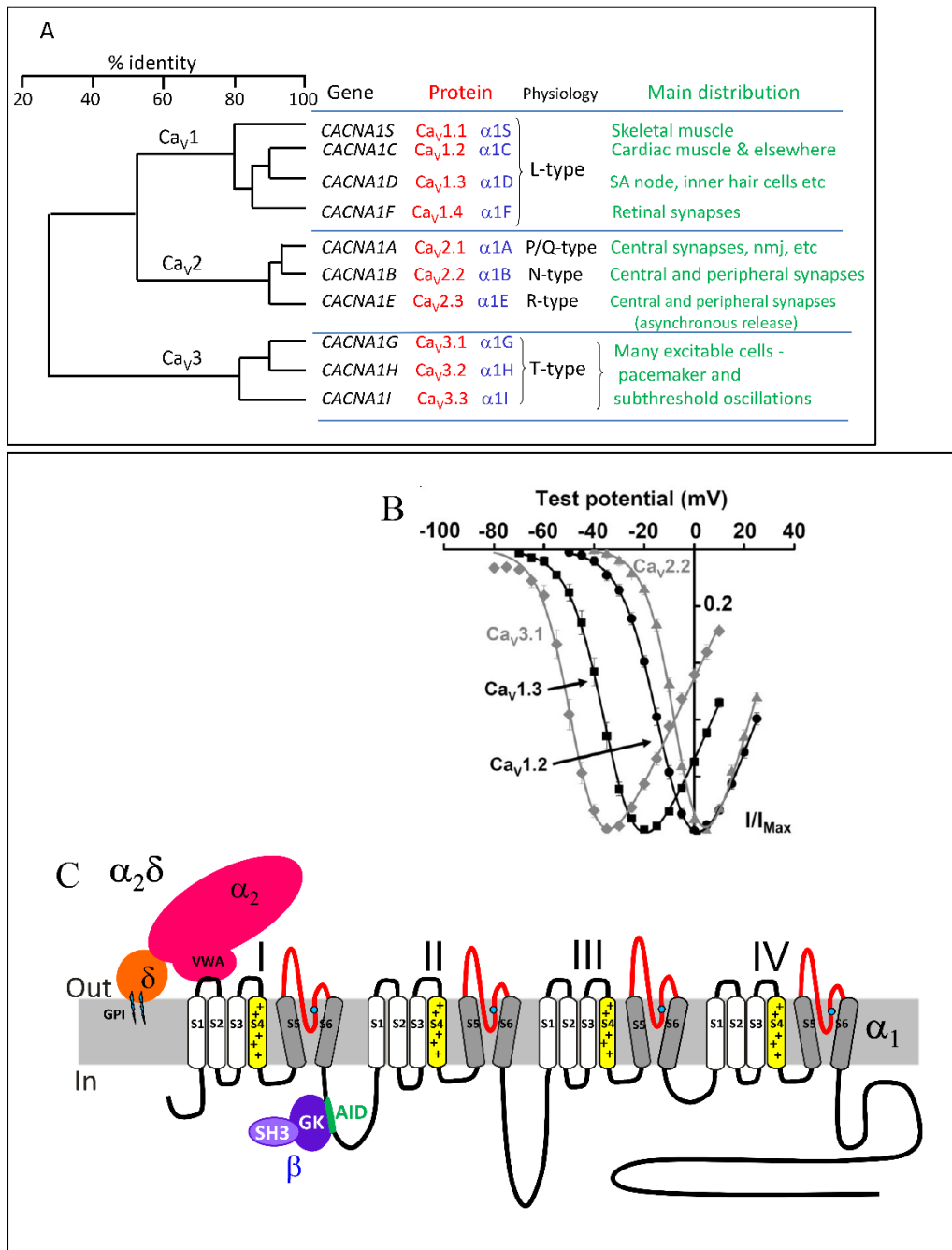
**Author contributions**

The authors contributed equally to all aspects of the article.

**Competing interests**

The authors declare no competing interests.

# Figures

## Fig. 1| Cav channel nomenclature and properties



**a|** Schematic showing Cav channel homology (the % identity between protein sequence of the difference Cav channel isoforms), human genetic nomenclature and protein classification. The channels are divided into 3 main groups, Ca$_V$1, Ca$_V$2 and Ca$_V$3, based on homology, and then subdivided according to the individual gene products. The original name for each of the cloned α$_1$ subunits, as well as the names derived from electrophysiological experiments are also shown. The tissue distribution and main functions of each isoform are listed. **b|** Normalized current-voltage relationships for Cav currents recorded from tsA201 cells. A comparison of the activation voltage ranges of Cav3.1, Cav1.3, Cav1.2 and Cav2.2 channels shows

that there is a continuum of activation voltages for the different channels, rather than a clear division into low voltage activated (LVA) and high voltage activated (HVA) channels[7-9,20-22] **c|** Schematic illustrating Cav channel subunit interactions[2,29,34]. The $\alpha_1$ subunit, contains four homologous domains (I-IV) each with 6 transmembrane segments (S1-S6), an S4 voltage sensor (yellow), containing a motif of positively charged amino acid residues, and a P-loop between S5 and S6 (red segments), which comprise the pore domain, containing key acidic residues (generally glutamate, cyan circles) involved in the selectivity filter. The $\alpha_2\delta$ subunit is shown with its GPI anchor linking it into the membrane and von Willebrand factor A (VWA) domain binding to the first extracellular loop of the $\alpha_1$ subunit. The two domains of the β subunit (Src homology domain (SH3) and guanylate kinase-like domain (GK)) are also shown, with the GK interacting with the α1-interaction domain (AID) motif on the I-II linker intracellular loop. Part b is reproduced, with permission from ref[7]. NMJ, neuromuscular junction.

**Fig. 2| Synthesis and trafficking of Cav channels**
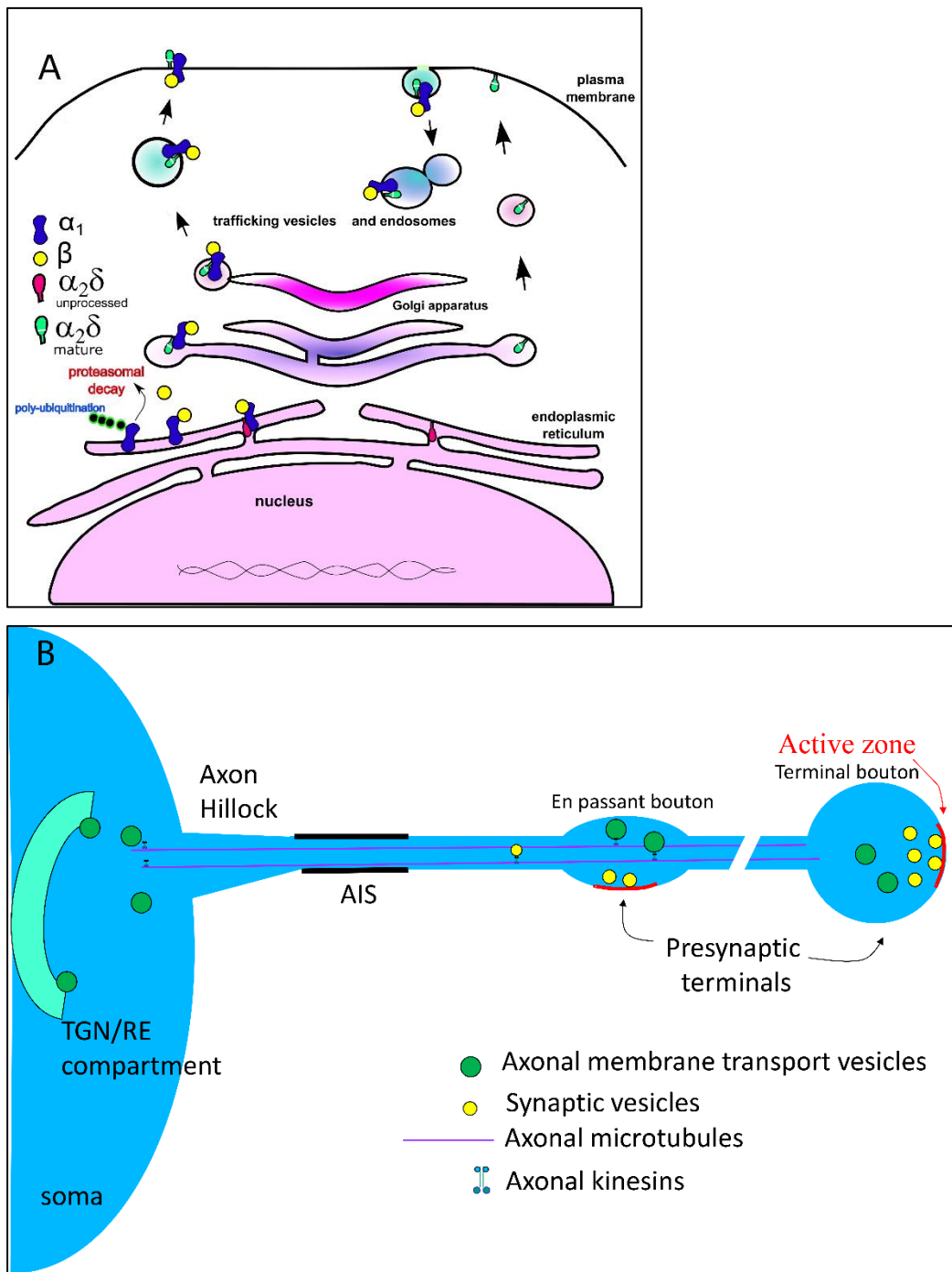


a| The synthesis of the $\alpha_1$ and $\alpha_2\delta$ subunits of Cav2 channels occurs on endoplasmic reticulum-associated ribosomes. The unprocessed form of $\alpha_2\delta$ is therefore synthesised entirely within the endoplasmic reticulum and attached to the endoplasmic reticulum membrane by a GPI anchor. In the endoplasmic reticulum, the $\alpha_1$ subunit associates with the ß subunit, which is a cytoplasmic protein. This interaction protects the $\alpha_1$ subunit from polyubiquitination and ER-associated degradation via the proteasome. The $\alpha_2\delta$ subunits are heavily glycosylated in the endoplasmic reticulum (there are up to 18 N-glycosylation sites to which glycans are attached) and the glycosyl moieties are further processed in the Golgi apparatus[45], which is also probably the main site of its proteolytic cleavage into $\alpha_2$ and $\delta$[45] to form

the mature protein. $\alpha_2\delta$ proteins may associate with the $\alpha1$ and ß subunit complex in the endoplasmic reticulum or in the Golgi apparatus. The complex is subsequently transported in trafficking endosomes and incorporated into the plasma membrane by fusion. The calcium channel complexes are also subject to endocytosis and are recycled to the plasma membrane via recycling endosomes[55,211]. The $\alpha_2\delta$ proteins are also able to reach the cell surface alone. **b|** The transport of axonal membrane transport vesicles containing Cav channels destined for active zone membranes involves binding of axonal cargo-containing transport vesicles (axonal endosomes) from the trans-Golgi network / recycling endosome compartment to microtubules in the pre-axonal exclusion zone at the axon hillock[61,212]. The axon initial segment (AIS) represents a specialized region in which particular $Na^+$ and $K^+$ channels are concentrated, and action potentials are initiated, which may also restrict axonal trafficking. The cargo destined for en passant or terminal boutons is attached by axonal kinesins to axonal microtubules, and is released from the microtubules at presynaptic sites, as are synaptic vesicles [213].

**Fig. 3| Regulation of Cav1 channel inactivation at ribbon synapses.**
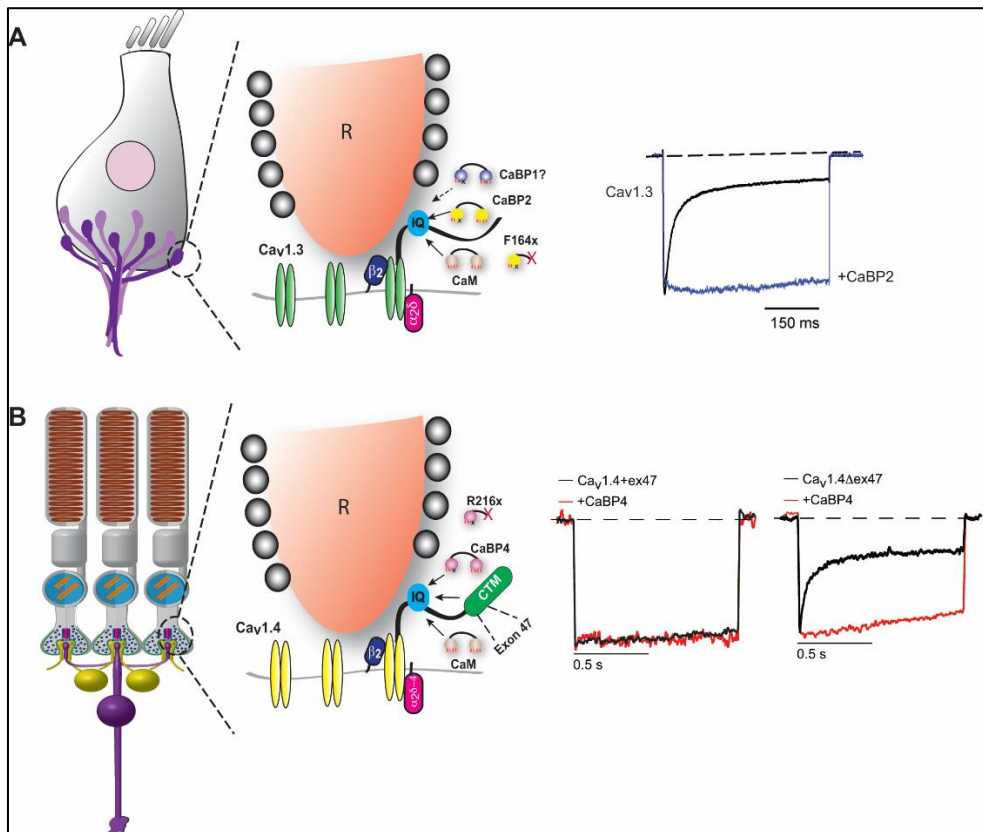


The schematics show the location of Cav1 channels at the active zones of inner hair cells (IHCs) and photoreceptors near the synaptic ribbon. The traces on the right show the normalized $Ca^{2+}$ current recorded from HEK293T cells expressing Cav1 channels alone or Cav1 channels together with a calcium-binding protein (CABP). **a|** In many cell-types, Cav1 channels are thought to be constitutively associated with calmodulin (CaM) which is bound to an IQ-domain (IQ) in the C-terminal domain of the channel. The binding of 4 $Ca^{2+}$ ions to the N- and C-terminal lobes of CaM triggers a conformational change in the channel that favors $Ca^{2+}$-dependent inactivation (CDI) (reviewed in[100]). In IHCs, Cav1.3 channels exhibit limited inactivation in comparison to that present in other cell types[21,101]. This is due in part to the co-assembly of Cav1.3 with $\beta_2$ subunits, which cause slow voltage-dependent inactivation[33]. In addition, CABP1 and/or CABP2 are $Ca^{2+}$ binding proteins with a dysfunctional $Ca^{2+}$ binding site in the N-terminal lobe (red x). These CABPs are thought to compete with CaM for binding to the channel thus suppressing the effects of CaM on inactivation. Right panel shows that $Ca^{2+}$ currents inactivate more slowly in cells cotransfected with Cav1.3 and CABP2 than in those transfected with Cav1.3 alone[105,106]; CABP1 has a similar effect (not shown) [101,104]. A mutation (F164X) that causes premature truncation of CABP2 inhibits its ability to suppress inactivation of Cav1.3 and causes autosomal recessive hearing loss[105]. **b|**In photoreceptor terminals, Cav1.4 channels, which are associated with $\beta_2$ and extracellular $\alpha_2\delta-4$ subunits, show little CDI. Channels containing exon 47 (Cav1.4+ex47) possess a C-terminal modulatory domain (CTM) that is thought to compete with CaM for binding

to the channel (left current trace)[109]. Splice variants lacking exon 47 (Cav1.4Δex47) show stronger CDI, which may be due to a reduced ability of the CTM to compete with CaM for binding to the channel (right purple current trace). CABP4 slows inactivation of Cav1.4Δex47, possibly because the loss of exon 47 enables CaBP4 to compete effectively with CaM for binding to the channel[114]. A mutation (R216X) that causes premature truncation of CABP4 inhibits its ability to suppress CDI of Cav1.4 and causes vision impairment in humans[205]. Trace in part a is adapted, with permission from[105]. Traces in part b are adapted, with permission from ref 114.

**Fig. 4| Organization and modulation of Cav2 channels in synapses**
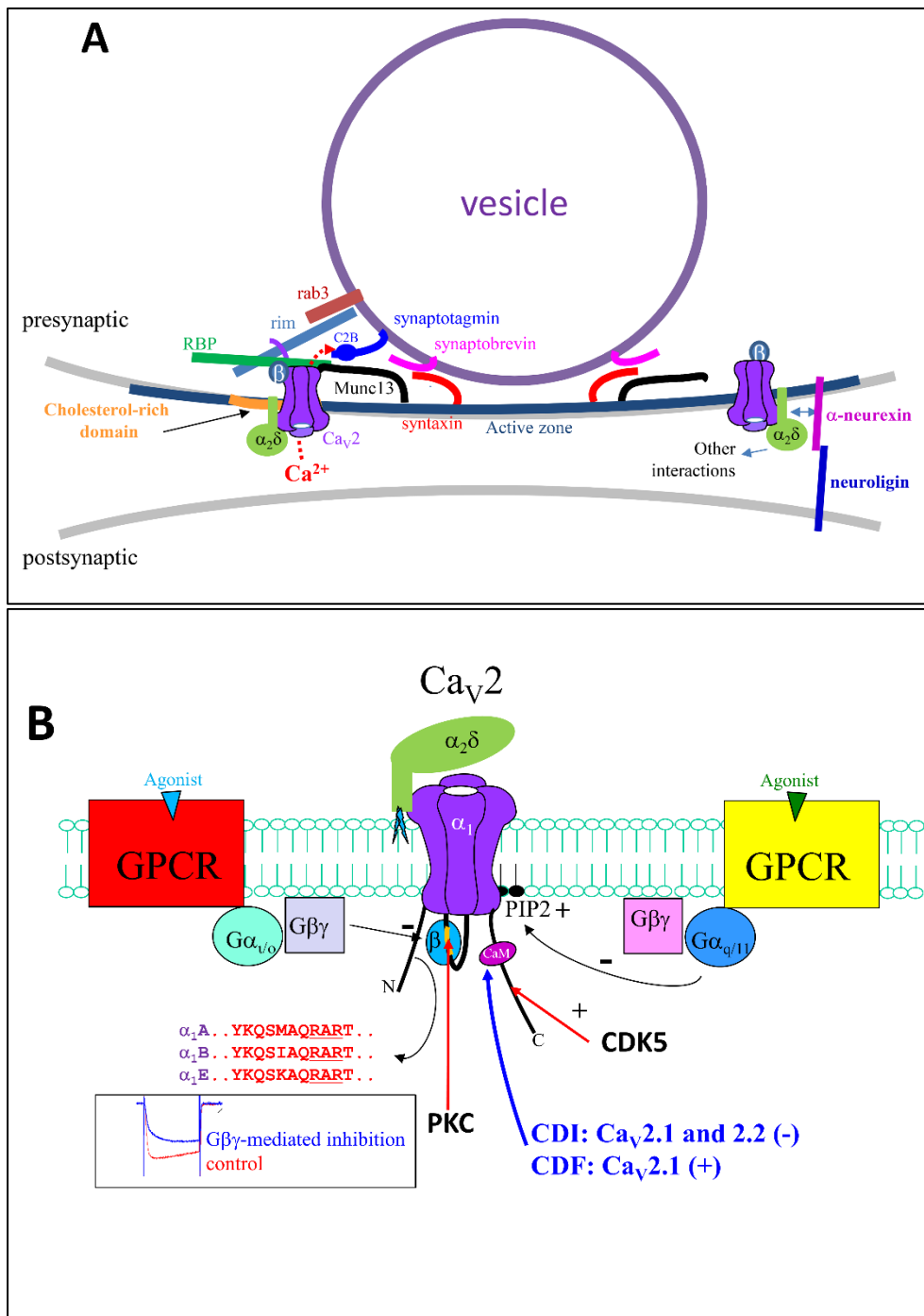


a| Schematic depicts some of the proteins known to be involved in anchoring the Cav2 channels sufficiently near to synaptic vesicles to form a nanodomain within the presynaptic active zone (for reviews see [69,87]). These include Rab3, synaptotagmin (the major $Ca^{2+}$ sensor, with one of its C2 domains shown) and synaptobrevin, all of which are associated with the vesicular membrane. Rab3-interacting molecules (RIM) and RIM binding proteins (RBP) are cytosolic, whereas Munc13 and syntaxin are associated with the plasma membrane. The association of the Cav ß subunit with the channel is shown. Ca$_V$ß interacts with RBP and also with another scaffolding protein, CAST/ELKS (not shown). The $\alpha_2\delta$ subunit is extracellular, and may (via its GPI anchor) preferentially associate with cholesterol rich lipid raft membrane

domains. It also mediates effects on Cav channels of α-neurexins, which also interact with neuroligin (postsynaptic except in *Caenorhabditis elegans*). Other interactions of α$_2$δ subunits are also likely to occur at the synapse but have not been depicted for clarity. **b**| Schematic showing some of the pathways modulating Ca$_V$2 calcium channel function. Some G protein-coupled receptors (GPCRs) inhibit Ca$_V$2 channel activity via their Gßγ subunits. This requires the presence of a conserved RAR motif in the N-terminal sequence of the channel, and also involves Cav ß binding to the I-II linker[159,162]. GPCRs coupled to G$_{q/11}$ inhibit Cav2 channels by reducing levels of PIP$_2$ (and activating PKC, which phosphorylates the AID[214] and elsewhere). Modulation involving the C-terminal domain of Cav2 channels includes CDK5-mediated phosphorylation of a conserved serine of Ca$_V$2.2, which increases channel open probability[178]. Ca$^{2+}$-dependent inactivation (CDI) (in Cav2.2 and Cav2.1) and Ca$^{2+}$-dependent facilitation (CDF (in Ca$_V$2.1) are mediated by calmodulin (CaM) binding to sites in the proximal C-terminal domain[182,183].

**Fig. 5| Differential synaptic recruitment of Cav channel regulatory mechanisms**
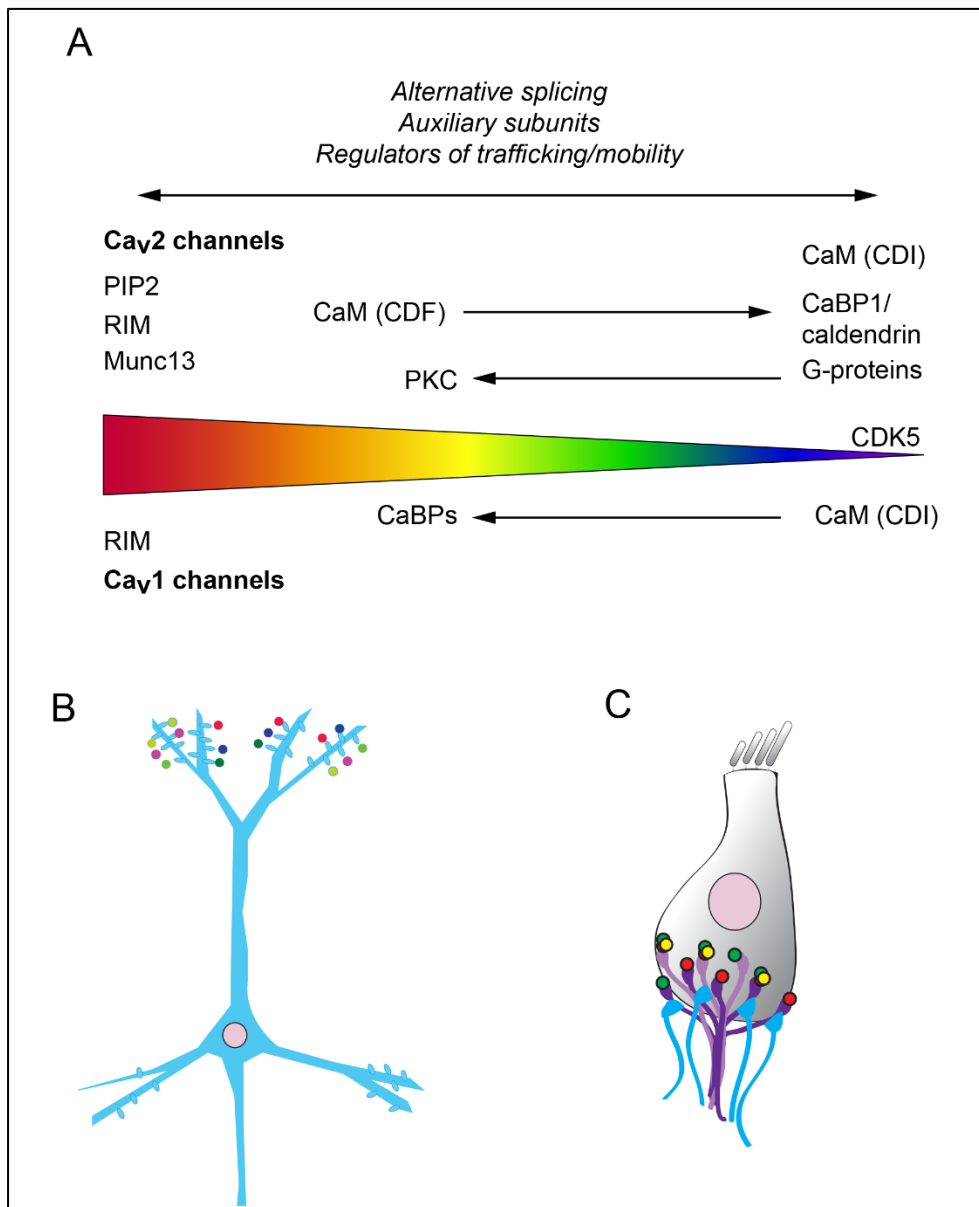


**a|** Cav2 channel–protein interactions that promote or inhibit presynaptic Cav channel function (depicted along the color-coded gradient to indicate strength of stimulatory (red) and inhibitory (blue) modulation) are major determinants of the amount of neurotransmitter release at the synapse[88,100,105,106,108,113,115,117,118,121-123,153,154,170-172,175,179,182,183,186]. Arrows between proteins indicate that they exhibit opposing forms of regulation. Distinct forms of alternative splicing, different auxiliary subunits, and bidirectional regulators of trafficking/mobility are also expected to have either stimulatory or inhibitory effects on presynaptic Cav channel function.**b|** These diverse modulatory mechanisms may add to observed variations in the nanoscale topographies of synapses, resulting in heterogeneity in synapse strength and plasticity that is needed for the computational robustness of neural circuits[215]. **c|** In cell-types that utilize multiple active zones such as inner hair cells (IHCs), inter-synaptic differences in Cav channel modulation and therefore neurotransmission could increase the complexity of information that can be encoded and integrated postsynaptically. The individual active zones of a single IHC provide input to

functionally distinct spiral ganglion afferent neurons. Such presynaptic diversity of Cav channel properties likely contributes to the ability of spiral ganglion neurons to represent the richness of sound information through their variable firing rates[207]. Colored arcs represent presynaptic inputs to postsynaptic sites of a neuron (B) and IHC (C) and are coded to represent intensity of presynaptic Cav Ca$^{2+}$ signals based on modulatory mechanisms described in  part **a**.

**Box 1| Methods to study Cav channel organization and function**



**Properties of presynaptic calcium currents in calyx of Held**
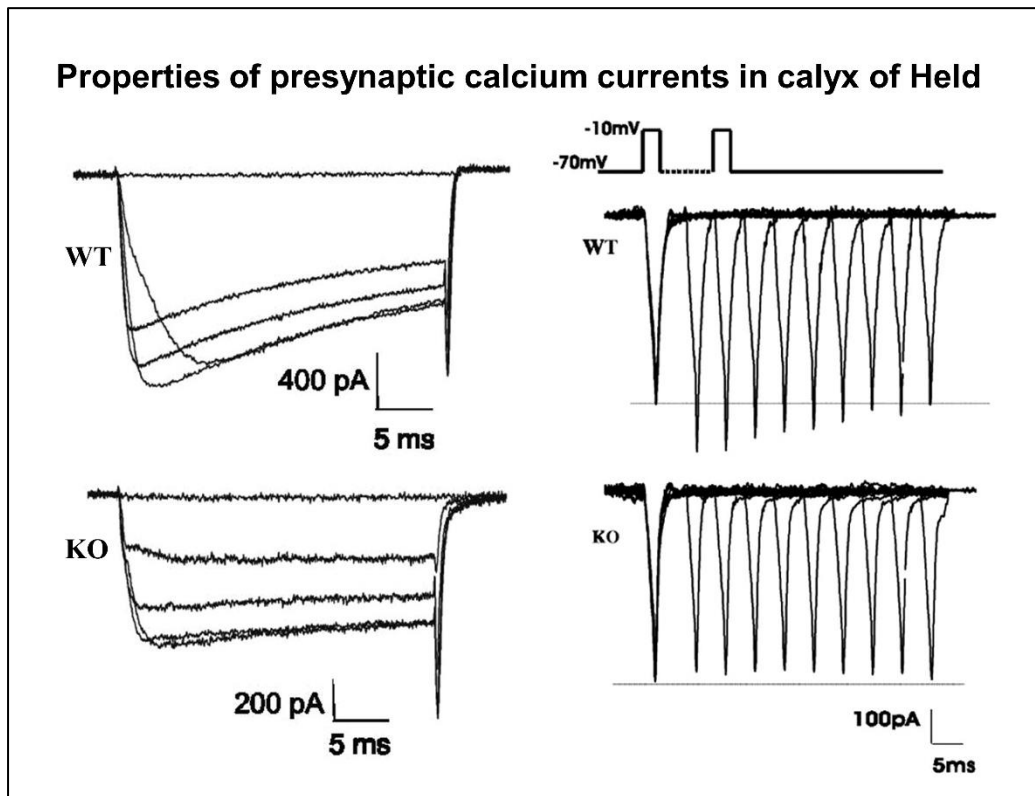
*[b1] Confocal and super-resolution imaging.* The extension of confocal imaging to

form the basis of multiple super-resolution imaging techniques(for review see[216]) has

facilitated the direct visualization of Cav channels in presynaptic terminals and

allowed their physical relationship to other presynaptic proteins to be determined[97].

This approach relies either on the existence of antibodies (or potentially nanobodies)

that bind specifically to the channel itself[116,129] or to inserted epitopes or fusion

proteins[53]. It is therefore vital to verify that such epitopes do not affect the function of

the channels or their interactions.

*[b1] Electron microscopy:* Electron microscopic images allowed the first glimpse of

vesicular release[217] and revealed the exquisite detail of presynaptic structures[218].

Quantitative SDS-digested freeze-fracture replica labelling immuno-electron

microscopy (SDS-FRL EM)[206] is currently the method of choice to localize

presynaptic $Ca^{2+}$ channels, and involves freeze-fracture of brain tissue, followed by

immunogold labelling and electron microscopy. Taking this approach, Ca$_V$2.1

antibodies have been used to label the intracellular face of presynaptic active zone

membranes, allowing clusters of channels to be mapped and their relationship to

other presynaptic markers determined[82,88]. Nevertheless, since the efficiency of

antibody labeling can vary between preparations, care must be taken in interpreting

quantitative estimates of Cav channel numbers[82]. More reliable antibodies for Ca$_V$2.2

and Ca$_V$2.3[219] that are amenable to this technique are sorely needed.

***[b1] Direct and indirect electrophysiology*:** Presynaptic Ca$^{2+}$ currents have been

recorded by whole cell or cell-attached patch clamp in a number of accessible

presynaptic terminals, including calyceal terminals[220-222] and mossy fiber en passant

terminals[223]. Such recordings give direct information on the composition of

presynaptic calcium currents, but not their subcellular localization. The use of

specific Cav blockers and knock-out animals can be used to reveal the effect of loss

of particular channels[224]. When combined with the postsynaptic measurement of

synaptic currents, the channels that are involved in release of the neurotransmitter

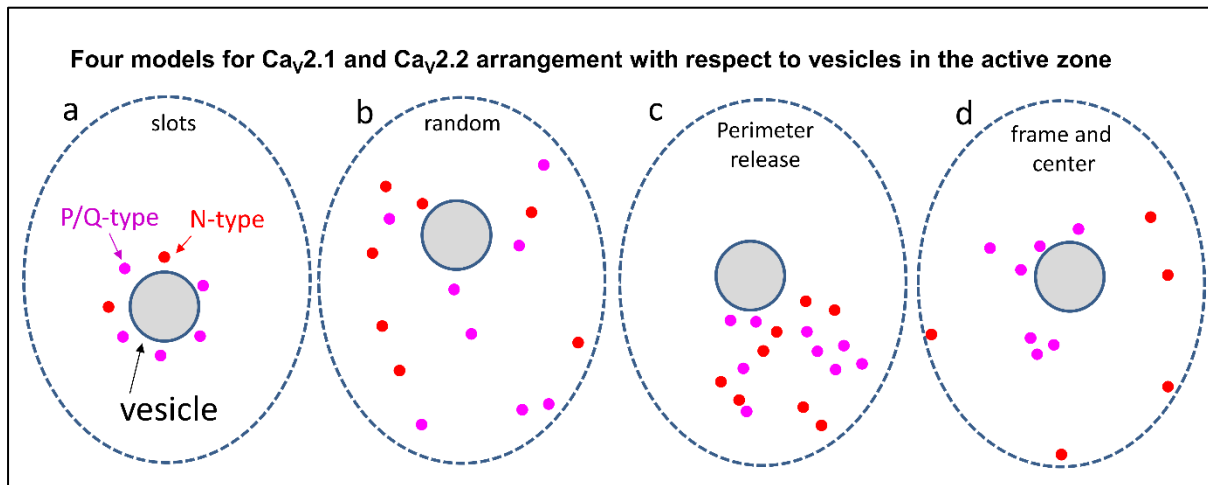can also be identified indirectly (for example see REF[82]).

***[b1] Ca$^{2+}$ imaging.*** Intracellular Ca$^{2+}$ measurements in individual presynaptic

boutons can be obtained using either chemical dyes (such as Magnesium-green or

Fluo-3) or genetically-encoded Ca$^{2+}$ reporters[225]. These measurements can be

performed in cultured neurons, acute or cultured slices or *in vivo*[68,226]. They allow

determination of the specific Cav channels responsible for presynaptic Ca$^{2+}$ entry

resulting from a single action potential stimulation or from trains of action potentials.

***[b1] Imaging vesicular release.*** Genetically-encoded vesicle release sensors

generally rely on the use of a pH-sensitive green fluorescent protein (GFP) that is

targeted to the lumen of the synaptic vesicle. The change in pH when a vesicle fuses

with the membrane results in an increase in fluorescence[227]. Styryl dyes such as FM1-43 which are loaded into synaptic vesicle membranes have also been widely used, although they have lower sensitivity and time resolution[228]. These measurements allow determination of the relationship between $Ca^{2+}$ entry and vesicular release, as well as the specific $Ca_V$ channels responsible for neurotransmitter release resulting from single and multiple action potential stimulation patterns.

***[b1] Glutamate sensors.*** 'Sniffer' outside-out patches containing glutamate receptors have been used to identify the sites of glutamate release[229] and, more recently, novel glutamate sensors have been developed[141,230,231] that can precisely localize release sites and also allow optical quantal analysis to be performed[141]. The effects of $Ca_V$ blockers on release from specific sites can then be determined.

## Box 2| Organization of Cav channels relative to synaptic release sites



Four models for $Ca_V2.1$ and $Ca_V2.2$ arrangement with respect to vesicles in the active zone

a  slots
b  random
c  Perimeter release
d  frame and center

P/Q-type  N-type

vesicle

**[b1] How many Cav channels are required to open to trigger vesicular release?**

This question remains a source of controversy and the answer may vary with synapse geometry, as described below. A combinination of results from ultrastructural imaging studies and electrophysiology (in the presence of fast and slow $Ca^{2+}$ chelators) is required to produce models of the arrangement between channels and release sites. At different synapses, estimates of the number of Cav channels required to open to trigger vesicle release have varied from a single channel in both chick ciliary ganglion terminals [232] and mouse hippocampal Schaffer collateral synapses[233] , to 3 channels or fewer at the rat basket cell-granule cell synapse of rat hippocampus[234], to more than 60 in the immature rat calyx of Held synapse[235].

**[b1] Coupling distance between Cav channels and release sites.** $Ca^{2+}$ channels have been reported to be organized in 'nanodomains' or 'microdomains' around a vesicle release site (for review see [87]). These terms refer to the positioning of Cav channels involved, respectively implying tight (nanodomain) or loose (microdomain) coupling of these channels to vesicular release. In a situation in which nanodomain coupling is present, the opening of one or a few Cav channels could trigger release,

whereas a larger number of Cav channels are required to contribute to the $Ca^{2+}$ elevation that triggers release in a situation in which microdomain coupling is present. The quantitative definition of a nanodomain or microdomain has varied slightly across the many studies that have investigated this point; however, in general the distance between individual channels and the vesicle release site is less than ~20 nm for nanodomains and greater than ~20 nm for microdomains[84] (although it seems clear that there is a continuum of distances [90] and geometries). Indeed, the coupling distance has been defined differently depending on the study. For example it has been estimated from the centre of the synaptic vesicle release site to the nearest perimeter edge of a Cav cluster[82] but it has also been defined by the relative effectiveness of $Ca^{2+}$ buffers with differing $Ca^{2+}$ on rates to supress vesicular release [87] .

*[b1] The arrangement of Cav channels in relation to release sites:* Using the techniques described in Box 1 (and others), many different arrangements have been proposed for the localization of channels in relation to vesicles within active zones (see the figure). Proposals include the tethering of a single channel to each vesicle[232] (not shown) or a circular arrangement of channel-preferring 'slots' surrounding each vesicle[236] (akin to the circular arrangement of SNAREs[142] (see the figure, part **a**), although this is not supported by electron microscopy and other findings, for example [48,82,237]. A random arrangement of $Ca_V2$ channels within the active zone has also been proposed (see the figure, part **b**)[233]. Electrophysiological, electron microscopical and $Ca^{2+}$-imaging data has pointed to a perimeter release model (see the figure, part **c**) for calyx of Held active zones, consisting of a loose cluster of ~20 - 30 $Ca_V2$ channels, with one or more vesicles located 15 - 30 nm from the perimeter of the cluster [82]. Numerical simulations from this model showed a

remarkable correspondence to the average number of channels in a cluster observed using electron microscopy[82]. According to this model, a single channel opening could result in vesicular fusion, albeit with very low probability, although action potential-mediated release normally relies on the opening of multiple channels in the cluster[82]. Unlike calyx of Held synapses, the high release probability of neocortical synapses does not change with maturity, despite the presence of tighter coupling and reorganization of channel topology such that $Ca_V2.1$ channels become the predominant source of $Ca^{2+}$ for neurotransmitter release relative to $Ca_V2.2$[84], as the $Ca_V2.1$ channels cluster nearer to the vesicle (see also the figure, part d). Also seen in parallel fiber–Purkinje neuron synapses of the cerebellum, this type of organization is termed the 'frame and centre' model[88]. More ordered arrays of channels are observed in a variety of neuromuscular junctions[238,239].

**Glossary**

Auxiliary subunits: Subunits that modify the activity and/or trafficking of the pore-forming subunit.

Alternative splicing: The process in which certain exons are included or excluded to produce multiple different mRNAs from a single gene.

Activation voltages: The membrane potentials at which a channel open probability increases, which can be quantified in terms of the voltage for half-maximal activation

Channel pore: The ion conduction pathway of a channel.

Selectivity filter: The part of the channel pore that restricts passage to particular ions, for example for $Ca^{2+}$ ions within a Cav channel.

Ribbon synapses: Synapses characterized by a synaptic ribbon that is specialized for the transmission of sensory information.

Calmodulin: A multi-functional $Ca^{2+}$ sensing protein found in all eukaryotic cells.

Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein (SNAP) receptor (SNARE) proteins: Members of a protein complex that mediates the fusion of vesicles with another membrane, for example the plasma membrane.