Published in final edited form as:
Trends Neurosci. 2013 October ; 36(10): 598–609. doi:10.1016/j.tins.2013.06.008.

Control of Neuronal Voltage-Gated Calcium Ion Channels From RNA to Protein

Diane Lipscombe[#], **Summer E Allen**, and **Cecilia P. Toro**[*]
Department of Neuroscience, Brown University, Providence, RI 02912

Abstract

Voltage-gated calcium (Ca_V) ion channels convert neuronal activity into rapid intracellular calcium signals to trigger a myriad of cellular responses. Their involvement in major neurological and psychiatric diseases, and importance as therapeutic targets, has propelled interest in subcellular-specific mechanisms that align Ca_V channel activity to specific tasks. Here we highlight recent studies that delineate mechanisms controlling the expression of Ca_V channels at the level of RNA and protein. We discuss the roles of RNA editing and alternative pre-mRNA splicing in generating Ca_V channel isoforms with activities specific to the demands of individual cells; the roles of ubiquitination and accessory proteins in regulating Ca_V channel expression; and the specific binding partners which contribute to both pre- and post- synaptic Ca_V channel function.

Keywords

voltage-gated calcium channels; alternative splicing; splicing factors; synaptic transmission; synaptic proteins; ubiquitination

Ten Ca_V Genes, Thousands of Different Ca_V mRNAs, and Many More Functionally Different Proteins

> "...different Ca currents show so many differences in fundamental properties that we find it easier to assume that there are more than one type." – Hagiwara & Byerly (1981)[1]

Voltage-gated calcium ion channels are present at every critical step of information transfer in the nervous system from signal detection to perception, and from neuronal development to programmed apoptosis. Strategically located at points of sensory detection and on both sides of the synapse, Ca_V channels have a defining role in integrating signals and influencing synaptic strength. All but one of the ten mammalian genes that encode the main α_1 subunit of Ca_V channels are expressed in the nervous system (Box 1). Each Ca_V gene has a distinct expression profile reflecting its functional specialization to support specific cellular tasks.

[#] Corresponding author. Diane_Lipscombe@brown.edu.
[*]Current address: Oregon Hearing Research Center, Oregon Health & Science University, Portland, OR 97239

For more than 50 years the functional diversity of Ca_V currents among different cell-types has been attributed to the expression of multiple channel types [1]. Indeed, individual neurons can express multiple Ca_V genes, consistent with the varied subcellular distribution of calcium channel subtypes controlling a range of neuronal functions. The value of highly selective toxins and drugs used to tease out the relative contributions of Ca_V channels within single cells is hard to overstate. Yet this is not always straightforward: for example, until recently, the absence of pharmacological tools to distinguish $Ca_V1.2$ and $Ca_V1.3$ channels [2], which collectively give rise to most dihydropyridine-sensitive Ca currents in neurons, has stalled progress in defining their individual contributions. Even when specific toxins are available, the challenges associated with isolating the functional importance of closely related Ca_V channels are magnified several-fold when considering the thousands of channel isoforms potentially generated from single genes by alternative pre-mRNA splicing and RNA editing.

All ten mammalian *CACNA1* genes contain multiple sites of alternative splicing; each gene has the potential to generate thousands of unique splice-isoforms controlled by the expression and relative activities of cell-specific splicing factors (Figs. 1, 2). Typically these sites are located in coding regions that tolerate sequence variation and that are not essential for structural integrity: N-termini, cytoplasmic loops I-II and II-III, extracellular linkers that connect transmembrane α-helices S3 and S4 in domains III and IV, and the C-termini (Figs. 2A, 2B, 3). Consequently, these hyper-variable-like regions are hotspots for cell-specific posttranslational modification, second messenger action on, and protein binding to Ca_V channels (Figs. 3, 4). The tethering of appropriate Ca_V channels to presynaptic active zones or to postsynaptic specializations depends on specific nanodomain-level protein interactions (see Figs. 5, 6); as discussed below for the most recently discovered of these interactions. A common feature across these studies is the ability to resolve molecular-level detail of specific RNAs and proteins within subpopulations of neurons, and within functionally distinct subcellular compartments. Novel techniques have been developed and applied to identify: splicing factor binding sites on Ca_V channel pre-mRNAs, Ca_V channel RNAs in specific populations of neurons, and Ca_V proteins at active zones. These methodologies marry disciplines to yield a more comprehensive, multi-dimensional view of Ca_V channels and their regulation *from RNA to Protein*. We highlight these and other recent studies elucidating cell-specific control of Ca_V RNA processing, posttranslational Ca_V protein modifications, and Ca_V protein-protein interactions. Collectively this work demonstrates exciting progress made in defining the molecular mechanisms underlying the expression of Ca_V channel isoforms in specific neurons, and in linking their expression to critical cell functions.

Cell-specific alternative pre-mRNA splicing and RNA editing regulate Ca_V channel function

"...the more you look, the more you see." (Robert M. Pirsig, 1974)

Alternative pre-mRNA splicing is particularly prevalent in the mammalian brain, and is essential for normal neuronal development, axon targeting, neuronal excitability, and neural circuit formation. This form of pre-mRNA processing occurs in the cell's nucleus, and it is controlled by the concerted actions of a set of cell-specific, RNA binding proteins called splicing factors. *Trans*-acting splicing factors bind to consensus *cis*-sequence motifs in pre-mRNAs to influence the action of the spliceosome, promoting or repressing inclusion of alternatively spliced exons, and promoting or repressing the use of alternative splice acceptor or donor sites at intron/exon boundaries (Fig. 1). Nova, rbFox and polypyrimidine track binding (PTB) families of splicing factors direct inclusion or skipping of alternatively spliced exons in a number of neural gene pre-mRNAs including *CACNA1* genes (Fig. 2). These splicing factor protein families have the capacity to either repress (silence) or enhance exon inclusion, in different target pre-mRNAs or within the same pre-mRNA, depending on

the position of their consensus binding motifs, either upstream (5') or downstream (3') relative to the target exon [3-7] (Figs. 1, 2C). Genome-wide maps of splicing factor binding sites reveal certain common features in the mechanism of action of certain splicing factors and bring us closer to defining a working splicing code[8]. These splicing factor genomic maps have advanced our understanding of cell-specific regulation of channel structure and function. In addition, several studies have shown that alternative splicing can be controlled by epigenetic factors [9], signaling factors including cellular protein kinases and phosphatases[10], and by cell-specific alternative splicing of splicing factors themselves[5,6].

Splicing factors that target CACNA1 pre-mRNAs—*CACNA1* pre-mRNAs are targets of splicing factors from the Nova, rbFox, PTB, and Muscleblind-like 2 (Mbnl2) RNA-binding protein families [4-7](Fig. 2D). Moreover, multiple rbFox splice isoforms act on the same *CACNA1* pre-mRNA exon, but with different consequences [11]. Importantly, developmental changes in the patterns of Ca_V splice isoform expression can parallel the changes in expression levels of key splicing factors. For example, as PTB expression levels fall in the developing brain, levels of neuronal PTB (nPTB) increase [12,13]. PTB controls the choice between a pair of mutually exclusive exons in *CACNA1C* ($Ca_V1.2$), e8 and e8a, via binding to motifs in *CACNA1C* pre-mRNA upstream of e8a [14] (Fig. 2C). As PTB levels decrease during development, the ratio of e8a- to e8-containing $Ca_V2.2$ mRNAs increases because PTB no longer represses e8a. In this case, nPTB does not substitute for PTB as a repressor of e8a [14]. E8a and e8 encode alternate forms of transmembrane S6 in domain I of $Ca_V1.2$ in a region that is critical for normal channel function (Fig. 2B). E8a and e8 are of special interest because certain mutations in either exon were shown to cause the severe multi-organ hereditary disorder, Timothy Syndrome [15]. The severity of neurological symptoms in individuals with Type I and Type II Timothy Syndrome depends on which exon carries the mutation, presumably reflecting the different expression patterns of e8 and e8a in the brain [16].

CACNA1B ($Ca_V2.2$) pre-mRNAs are targets of Nova2, and several Nova2 binding sites align with previously identified alternatively spliced exons. For example, *hi*gh-*t*hroughput *s*equencing combined with *c*ross-*l*inking *i*mmuno*p*recipitation methods (HiTS-CLIP) showed that Nova2 binding sites are located within the intron immediately upstream, and overlapping, a short six-nucleotide exon e31a in *CACNA1B* pre mRNAs [17] (Fig. 2C). The positioning of RNA binding motifs for Nova2 upstream of e31a predicts Nova2 repression of e31a. The HiTS-CLIP data are consistent with earlier reports that e31a-containing $Ca_V2.2$ mRNAs are found at very low levels in the rodent brain where Nova2 is expressed. By contrast, e31a-containing $Ca_V2.2$ mRNAs dominate in peripheral ganglia, which do not express Nova2 [18]. The same splicing factor can act differently on multiple alternatively spliced exons within the same pre-mRNA. This is the case for Nova2; in contrast to its silencing action on e31a, it binds motifs downstream of e24a to augment e24a expression in brain (Fig. 2C, 2D; [18]). Intriguingly, there are clusters of Nova2 binding sites in other regions of *CACNA1B* that do not map to known alternatively spliced exons, and the functional significance of these is not yet known.

RbFox and Nova splicing factor protein families have overlapping networks of synaptic protein targets, consistent with the collective action of multiple splicing factors on several alternative *CACNA1* exons [17] (Fig. 1, 2C, 2D). Loss of rbFox2 results in reduced expression of Nova1, perhaps reflecting regulation of Nova1 pre-mRNA splicing by rbFox2 [17]. Several important studies have shown profound consequences—including gross neurodevelopmental abnormalities—of disrupting or eliminating splicing factors which regulate alternative splicing of multiple targets [5,7,19]. However, because the splicing of hundreds of neuronal pre-mRNAs—including those that encode other splicing factors—are

disrupted in these studies, the relative contribution of splicing events within a single gene family, as as *CACNA1,* cannot be distinguished.

Individual Alternatively Spliced Exons Have Measurable Cellular and Behavioral Consequences—Many individual splice sites in *CACNA1* genes are evolutionarily conserved, and it is often assumed that each adds functional value and fitness to the cells in which they are expressed. Another less considered possibility is that the functional impact of any individual splicing change may not be discernible at the cellular or behavioral level. Alternative pre-mRNA splicing of genes may have evolved as essential proteins operate over a broader dynamic range than would be possible from the activity of a single protein. This feature of alternative splicing could be critical during development, particularly in neurons that adapt to and continue to function in the face of dramatic changes in morphology and activity [20]. Yet there are several notable examples showing that exon choice in an influential gene can indeed modulate behavior. For example, reproductive dominance in honeybees is controlled by alternative splicing of exon 5 in a gene homologous to the *gemini* transcription factor of *Drosophila* [21], and cell-specific expression of a TRPV1 splice isoform confers unique infrared sensing capabilities to vampire bats [22].

Recently, the cellular and behavioral consequences of an alternatively spliced exon in the mouse *Cacna1b* gene were demonstrated. Enhanced expression of the nociceptor-specific exon 37a splice isoform of $Ca_V2.2$ increases both cellular sensitivity to inhibition by activated -opioid receptors and behavioral sensitivity to spinal morphine-induced analgesia [23] (Fig. 4). Cell-specific expression of e37a in wild-type mice may help explain why $Ca_V2.2$ channels in nociceptors are particularly sensitive to inhibition by drugs, transmitters, and hormones that act through G protein-coupled receptors. Additional alternatively spliced exons in *Cacna1b* and in the closely related *Cacna1a* ($Ca_V2.1$) and *Cacna1e* ($Ca_V2.3$) genes modify amino acid sequences in the cytoplasmic domains of Ca_V channels. The actions of G proteins and second messengers on other ion channels may also be influenced by cell-specific alternative splicing of ion channel pre-mRNAs in other parts of the nervous system.

Cell-specific inclusion of exons in *Cacna1* genes can also impact disease states. Already mentioned are the different symptoms in Type I and Type II Timothy Syndrome determined by which exon, e8a or e8 in *CACNA1C* carries the mutation [16]. Other examples include the different consequences of *Cacna1a* ($Ca_V2.1$) mutations associated with familial hemiplegic migraine on the short (Δ47) and long (+47) splice isoforms of $Ca_V2.1$ [24], and an epilepsy causing mutation in mouse *Cacna1h* ($Ca_V3.2$) that results in different channel phenotypes depending on whether alternative exon 25 is expressed [25].

RNA editing of CACNA1D mRNA—Additional cell-specific alterations in amino acid sequence can arise from RNA editing, introducing potentially even finer specialization of Ca_V activities. In RNA editing, posttranscriptional deamination of adenosine to inosine (read as guanosine during translation) is catalyzed in RNA by the ADAR (adenosine deaminase acting on RNA) family of enzymes. Evidence for different RNA edited versions of Ca_V channel mRNAs was first reported for the *Drosophila* CaV2 homologue *cacophony (Dmca1A*) (Table 1) [26]. Recently, central nervous system (CNS)-specific editing of mammalian $Ca_V1.3$ RNAs by the ADAR protein ADR2 has been carefully documented and shown to generate four distinct sequences within the C-terminus IQ domain: IQDY, MQDY, IRDY, and MRDY [27]. By contrast, only one IQ sequence, IQDY, is found in $Ca_V1.3$ channels expressed outside of the CNS [27]. The IQ domain forms part of a critical calmodulin-binding site in Ca_V channels that mediates calcium-dependent inhibition. Calcium-dependent inhibition functions as an important feedback control on subsequent calcium entry; cytoplasmic calcium inhibits the further gating of the ion channel through

which it flowed. Editing of the IQ site (e.g. to MQ) reduces calcium-dependent inactivation and as a consequence is thought to participate in shaping rhythmicity of action potential firing in neurons of the suprachiasmatic nucleus [27].

Cell-specific RNA editing could exert similar control over Ca_V channels in other populations of neurons, and could alter amino acid sequences of other critical domains that regulate specific functions. Because of the potential importance of $Ca_V1.3$ channels in promoting calcium-dependent cell death in dopaminergic neurons in the substantia nigra pars compacta and the proposed connection between $Ca_V1.3$ activity and Parkinson Disease [28], it will be interesting to know which edited versions of $Ca_V1.3$ RNAs dominate in these neurons. Mapping the cell-specific expression patterns of ADAR2 protein and mapping ADAR2 binding sites to specific RNAs would provide valuable information in this regard. However, as is the case for splicing factors, ADAR2 activity depends on several factors including phosphorylation-dependent prolyl-isomerase Pin1 that controls its nuclear localization [29], the ubiquitin E3 ligase WWP2 that promotes its proteasomal degradation [29], as well as other second messengers.

Mechanisms that Control Numbers of Ca_V Channels at the Cell Surface

Ca_V channel activity depends not only on the pattern of expression of functionally different splice and RNA edited isoforms but also on the overall expression level of Ca_V channel proteins in specific subcellular compartments. Counting Ca_V2 channels at active zones of different synapses by quantitative molecular and ultrastructural analyses recently demonstrated a tight correlation between presynaptic $Ca_V2.1$ and $Ca_V2.2$ channel number and vesicle release probability [30,31]. Whereas the third member of the Ca_V2 gene family, $Ca_V2.3$, was shown (with the exception of the interpeduncular nucleus) to be mostly restricted to postsynaptic structures, particularly dendritic shaft plasma membrane [32]. Thus, the overall activities of presynaptic $Ca_V2.1$ and $Ca_V2.2$ channels have a major influence on the efficacy of transmission at mammalian synapses. We know a great deal about G protein-coupled receptor mediated control of Ca_V2 channels, particularly $Ca_V2.2$. These well-studied signaling pathways terminate on $Ca_V2.2$ channels and account for short-term presynaptic effects of many neurotransmitters, hormones and drugs that modulate synaptic transmission. By contrast, the cellular mechanisms that control the overall number of Ca_V channels at active zones—which likely contribute to long-term changes in synaptic plasticity—were only recently elucidated [31].

Not surprisingly, cellular mechanisms analogous to those critical in setting the overall expression levels of postsynaptic receptors, including the ubiquitin proteasome system (UPS) [33], control surface expression of presynaptic $Ca_V2.1$ and $Ca_V2.2$ channels [34-36]. In addition, protein-Ca_V2 channel interactions described recently appear critical in establishing the number of Ca_V2 channels specifically at presynaptic terminals [30,37,38] (Fig. 5). Such molecular interactions are dynamic and subject to modulation, thereby regulating the density and number of presynaptic Ca_V2 channels during synaptic plasticity [30,31].

Ubiquitin Regulates Ca_V1 and Ca_V2 channels—Ubiquitination of several neuronal proteins is a critical posttranslational mechanism to modify the trafficking, endocytosis and activity of synaptic receptors and ion channels to adjust synaptic strength [34,39]. Ubiquitin covalently attaches to intracellular lysines of target proteins and, depending on the type of conjugate (i.e., monoubiquitination or polyubiquitination), it can promote internalization, modify protein function, or target protein for degradation via the UPS [40]. Activity-dependent ubiquitination of postsynaptic AMPA receptors is described and known to regulate synaptic plasticity. Until recently, Ca_V channels were rarely considered important

targets of ubiquitin despite functional evidence that ubiquitin-dependent changes in synaptic efficacy involve presynaptic components [41]. Four reports now show that $Ca_V1.3$, $Ca_V1.2$, and $Ca_V2.2$ channels are all targets of posttranslational modification by ubiquitin [34-36,42]. These reports implicate the UPS in controlling activity-dependent pre- and postsynaptic calcium levels. Ubiquitination of $Ca_V\alpha_1$ subunits is influenced by their association with $Ca_V\beta$ subunits—known to regulate plasma membrane targeting of Ca_V—and by cell-specific alternative splicing. $Ca_V\beta$ subunit binding to $Ca_V1.2$ and $Ca_V2.2$ reduces $Ca_V\alpha$ ubiquitination and protects channels from proteasomal degradation [34,36]. Similarly, $Ca_V2.2$ channel splice isoforms with restricted expression (e37a-containing) have reduced ubiquitination compared to other broadly expressed isoforms (e37b-containing) and are associated with increased channel current densities [35]. Thus, the influence of the UPS on $Ca_V2.2$ channels is cell-specific, reflecting the particular expression profile of certain alternative splice forms. $Ca_v2.2$ was recently identified as a target of ubiquitin based on a large-scale proteomics analysis of diGly-modified lysine residues of proteins expressed in a human colorectal carcinoma cell line [43]. Modified lysines in the region of $Ca_v2.2$ encoded by e37a and e37b were not identified in this screen, suggesting that ubiquitination at this site is cell-specific. Future large-scale proteomics analyses that enrich for neuronal ion channels, and other membrane proteins, hold promise for mapping major sites of posttranslational modifications of critical synaptic proteins.

$Ca_V\alpha_2\delta$ and RIM regulate Ca_V2 expression—Two protein families have recently grabbed the limelight as essential for trafficking or tethering Ca_V2 channels to nerve terminals: $Ca_V\alpha_2\delta$ and RIM (Fig. 5, Fig. 6). $Ca_V\alpha_2\delta$-1, first shown to interact with Ca_V channels ~25 years ago, is a glycosylphosphatidylinositol (GPI)-anchored extracellular protein [44]. This auxiliary subunit of Ca_V2 channels promotes membrane trafficking, speeds channel activation and inactivation, and binds the analgesics gabapentin and pregabalin. $Ca_V\alpha_2\delta$-1 has now been shown to act as a rate-limiting factor controlling the number of $Ca_V2.1$ channels at presynaptic terminals [45]. Evidence for a limiting factor in Ca_V2 channel trafficking to presynaptic nerve terminals emerged from studies in cultured hippocampal neurons. Whereas somatic Ca currents were reliably enhanced in cultured neurons transiently expressing exogenous $Ca_V2.1$ or $Ca_V2.2$ channels, synaptic transmission was not [45,46]. However, co-expressing $Ca_V\alpha_2\delta$-1 along with $Ca_V2.1$ or $Ca_V2.2$ channels in these neurons augmented channel function, facilitated Ca_V2 coupling to exocytosis, and enhanced synaptic transmission [45] (Fig. 5).

RIM proteins are also critical Ca_V2 channel partners at nerve terminals. The reported actions of RIM proteins on Ca_V2 channels are diverse: they augment $Ca_V2.1$ currents in presynaptic calyx of Held terminals [37], decrease voltage-dependent inactivation (VDI) of $Ca_V2.2$ channels, interfere with the inhibitory actions of μ-opioid receptors on $Ca_V2.2$ channels expressed in HEK-293 cells [47], and slow $Ca_V1.3$ channel inactivation in inner hair cells [48]. But their importance in tethering Ca_V2 channels to active zones is central to explaining their *in vivo* function [38,49]. Meticulous high-resolution analyses of the molecular and morphological architecture of glutaminergic synapses show that RIM protein numbers scale proportionately with presynaptic $Ca_V2.1$ channel numbers at active zone areas [30]. Moreover, the numbers of these complexes scale with and predict vesicle release probability, consistent with critical function [30] (Fig. 6).

Controlling Subcellular Targeting of Ca_V channels

Synapse-specific location of Ca_V2 channels—$Ca_V2.1$ and $Ca_V2.2$ channels couple differentially to neurotransmitter vesicle release machinery according to synapse type. In hippocampal interneurons, $Ca_V2.1$ channels mediate release of neurotransmitter from parvalbumin (PV)-expressing fast spiking basket cells while $Ca_V2.2$ channels mediate

release of neurotransmitter from cholesystokinin (CCK)-expressing basket cells [50,51]. The physical distance between presynaptic channels and calcium sensors of exocytosis are predicted to be different between $Ca_V2.1$ and $Ca_V2.2$. These predictions as based on the differential ability of BAPTA and EGTA – that are fast and slow calcium chelators, respectively – to inhibit synaptic events mediated by $Ca_V2.1$ and $Ca_V2.2$ channels [50,51]. $Ca_V2.1$ channels at PV nerve terminals are predicted to be within nanometers of the calcium sensor that leads to synchronous GABA release, whereas $Ca_V2.2$ channels at CCK terminals are predicted to be within micrometers of the Ca sensor and are thought to underlie the highly asynchronous GABA release of CCK neurons [50-52]. However, the precise molecular identity of $Ca_V2.2$ and $Ca_V2.1$ splice isoforms at these synapses is not yet determined. It is therefore possible that different splice isoforms of Ca_V2, that are known to influence binding to release machinery, contribute to synapse-specific differences in calcium-dependent exocytosis.

Alternative splicing of auxiliary Ca_V subunits, as well as other interacting proteins, is also likely to contribute to the functional diversity among different synapses. For example, the four *CACNA2D1-4* genes that encode mammalian $Ca_V\alpha_2\delta1$-4 subunits are each subject to alternative pre-mRNA processing. Similarly, the *Rims1* and *Rims2* genes generate five principal RIM proteins by the use of independent promoters (RIM1α, RIM1β, RIM2α, RIM2β, and RIM2γ) and both *Rims* genes contain sites of alternative splicing [49]. This provides substantial capacity for synapse-specific differences in Ca-dependent transmitter release. Finally, asynchronous transmission can occur as a consequence of prolonged Ca_V2 channel openings, a phenomenon that is significantly augmented in $Ca_V2.1$ channels associated with $Ca_V\beta_{2a}$ subunits [53]. $Ca_V\beta$ subunits strongly influence the rate of Ca_V2 channel inactivation, and $Ca_V\beta_{2a}$ subunits in particular slow channel inactivation significantly compared to $Ca_V\beta_1$, $Ca_V\beta_3$, and $Ca_V\beta_4$ subunits (e.g. see [53]).

Proteins in Nanodomains with Synaptic Ca_V2 Channels—Although lacking resolution at the level of specific synapses, a proteomics screen combined with affinity purification and high-resolution quantitative mass spectroscopy has identified over 200 proteins that interact closely with Ca_V2 proteins in rodent brain [54]. This valuable data set includes a subset of proteins isolated from brain synaptosomes that were previously identified to interact with $Ca_V2.2$ channels [55] as well as cytoskeletal and chaperone proteins and a complex of novel proteins involved in both exocytosis and endocytosis (Fig. 6). Functional analyses, validating the significance of several of these protein-protein interactions, suggest the interactions are critical to the sub-cellular specialization of Ca_V2 channels. For example, the collapsin response mediator protein 2 (CRMP2)-$Ca_V2.2$ interaction controls $Ca_V2.2$ channel current densities in sensory neurons [56,57]. Disrupting this interaction using a cell permeable blocking peptide reduced $Ca_V2.2$ trafficking to presynaptic terminals, spontaneous excitatory postsynaptic currents in the spinal dorsal horn, and inflammatory and neuropathic pain [56]. The absence of gross behavioral or motor effects in these mice supports a unique role for CRMP2-$Ca_V2.2$ in maladaptive responses in sensory neurons to noxious stimuli. Other protein interactions that, like CRMP2, are mediated via C-termini of Ca_V2 channels may be important in stabilizing the presynaptic molecular architecture (Fig. 6).

Perhaps the best-studied region of Ca_V2 channels is the synprint site located in the II-III intracellular linkers of $Ca_V2.1$ and $Ca_V2.2$ (Fig. 3). The synprint region binds synaptic protein SNAREs (Syntaxin 1A and SNAP-25) and synaptotagmin (Fig. 6). This tripartite interaction, Ca_V2/synprint/synaptotagmin, so critical to the release process, is reviewed in detail elsewhere [58]. Less studied is the interaction between endocytotic proteins and presynaptic Ca_V2 channels, most notably AP-2 (adaptor protein for clathrin-mediated endocytosis) [54]. AP-2 was shown to associate with $Ca_V2.2$ and $Ca_V2.1$, but not $Ca_V1.2$,

via the synprint region to control endocytosis, but not exocytosis, at calyx of Held nerve terminals [59]. The II-III linker sequence of Ca_V channels is highly variable among *CACNA1* genes (Fig. 3) and it contains several sites of alternative pre-mRNA splicing (Fig. 3). Alternative splicing of $Ca_V2.2$ and $Ca_V2.1$ pre-mRNAs therefore generates II-III splice isoforms that have different capacities to interact with SNAREs [58] and potentially with AP-2.

Proteins that bind and modulate pre and post synaptic $Ca_V1.3$—$Ca_V1.3$ channels are found both pre- and post- synaptically in cochlear inner hair cells (IHCs). Presynaptic $Ca_V1.3$ channels regulate transmitter release, interact with ribeye (the ribbon synapse protein that promotes channel clustering), and negatively regulate the size of the ribbon body [60] [61] (Fig. 6). Harmonin also associates with presynaptic $Ca_V1.3$ channels at the onset of hearing in 2 week-old mice. This interaction promotes $Ca_V1.3$ ubiquitination and leads to a decrease in channel surface expression. In the deaf circler mouse, *dfcr*, a mutant allele of the harmonin gene disrupts the harmonin interaction with $Ca_V1.3$, leading to abnormally high $Ca_V1.3$ currents in IHCs [42]. Postsynaptic $Ca_V1.3$ channels also play a critical role in the auditory brainstem where they are required for the normal development of the inhibitory medial nucleus of the trapezoid body (MNTB) to the lateral superior olive (LSO) projection [62,63]. There is substantial reduction in the number of MNTB-LSO axons over the first two weeks of life concomitant with strengthening of the remaining synapses. Thus, postsynaptic $Ca_V1.3$ channels in the LSO neurons are thought to be vital for the development and maturation of inhibitory MNTB–LSO projections [62]. Postsynaptic $Ca_V1.3$ channels isolated from brain are also found in a complex with densin and CaMKII where they localize to dendritic spines [64]. Densin promotes calcium-dependent facilitation of $Ca_V1.3$ channels [64]. Alternative pre-mRNA splicing of *CACNA1D* pre-mRNA must generate functionally distinct presynaptic and postsynaptic $Ca_V1.3$ channels. Defining the major splicing patterns of $Ca_V1.3$ isoforms, their differential expression patterns, and functional properties should provide valuable insight into structural domains essential for presynaptic and postsynaptic function.

Challenges

The number of molecularly distinct Ca_V proteins that can be generated from *CACNA1* genes is stunning. Cell-specific gene expression, alternative pre-mRNA splicing, RNA editing, posttranslational modifications including ubiquitination, miRNA targeting, and subcellular specific protein-protein interactions are all employed according to the demands of the cell.

At the level of RNA, we still lack cell-specific information about which mRNA is expressed and when. Several cell-type specific transcriptome data sets have been generated from the combined application of methods including high throughput mRNA sequencing, pooling of genetically homogenous cells, and enriching for polysomal RNAs [13,65]. These methods are cataloguing *CACNA1* transcripts according to neuronal sub-type and developmental stage, although substantial variability in the composition of certain transcripts has been observed across different studies and stochastic variations in transcriptomes are evident even in genetically similar cell types [13]. Genetically targeted and affinity-based miRNA profiling (miRAP) has also generated the first comparative analysis of cell-specific miRNAs in glutamatergic and GABAergic neurons of neocortex and cerebellum [66], giving insights into miRNA-dependent control of mRNA translation and stability in different classes of neurons. In addition, recent genome-wide mapping of splicing factor binding-sites by CLIP-seq, as already discussed, locates sites of splicing factor binding relative to target exons in pre-mRNAs which, combined with other information, could eventually lead to a splicing code [8]. These, and other sequence-based data sets not mentioned here, are publicly

accessible (http://genome.ucsc.edu/) and recently integrated with the encyclopedia of DNA elements project (ENCODE, http://www.genome.gov/10005107).

At the protein level, major challenges remain but exciting technological advances are being made that combine high resolution imaging with proteomic analyses of single synapses [67]. It should soon be possible to visualize and distinguish among Ca_V isoforms with the spatial resolution needed to place them at individual active zones and synapses. Similarly, it may be possible to define unique patterns of posttranslational modifications, and characterize unique protein-Ca_V interactions that occur in highly localized, specialized subcellular regions of neurons. Such information should reveal the molecular mechanisms that underlie functional specialization at the level of individual synapses, and may suggest new therapeutic strategies to target specific regions or neural circuits within the brain.

At the gene level, several hereditary diseases and disorders originate from point mutations or tri-nucleotide expansions in *CACNA1* genes but a major, as yet unexplained finding implicates *CACNA1C* ($Ca_V1.2$) in determining an individual's susceptibility to a range of psychiatric illnesses. In the most recent and largest genome wide association study of its kind, four risk loci were identified with significant and overlapping links to autism spectrum disorder, attention-deficit/hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia [68]. Two of these risk SNPs are located in calcium channel genes, *CACNA1C* ($Ca_V1.2$) and *CACNB2* ($Ca_V\beta_2$) [69] [70,71] [72]. The widespread expression of $Ca_V1.2$ channels in the body including muscle cells of the cardiovascular system, endocrine cells, and smooth muscle adds to the intrigue. The cellular mechanisms described here that individualize Ca_V channel function according to cell-type, and in particular alternative pre-mRNA splicing, could be selectively disrupted in individuals exhibiting psychiatric illness without involvement of non-CNS systems.

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Box 1. *By Any Other Name*

Voltage-gated calcium (Ca_V) ion channels are highly specialized plasma membrane proteins that convert small changes in the membrane potential into rapid, localized increases in intracellular calcium. Only a limited number of ion channels, including several ligand-gated ion channels, allow the passage of calcium. However, Ca_V channels are exquisitely selective for calcium over other cations as long as extracellular calcium concentrations exceed ~ 10 M. In the absence of extracellular calcium, Ca_V channels lose their high selectively to calcium and readily conduct a range of cations.

Most Ca_V channels have a dual function: they support ionic current that changes the membrane potential, and they permit the flow of calcium across the plasma membrane that serves as an intracellular second messenger. In mammals, ten genes encode the ten major $_1$ subunits of Ca_V channels (*CACNA1A* through *CACNA1I*, and *CACNA1S*). They have unique expression profiles, cellular function, pharmacology, and are associated with various diseases. The numerous aliases used to identify and distinguish the different Ca_V channel genes, proteins, and currents are shown for seven different species (Table I).

The major $_1$ subunits of Ca_V channels fall into three classes based on sequence homology Ca_V1 (1.1, 1.2, 1.3, and 1.4), Ca_V2 (2.1, 2.2, and 2.3) and Ca_V3 (3.1, 3.2, and 3.3). The classes are functionally distinguished by a combination of pharmacological tools and biophysical properties, although pharmacological differences among Ca_V1 (dihydropyridine-sensitive), Ca_V2 (ω-agatoxin-IVA inhibits $Ca_V2.1$; ω-conotoxin GIVA inhibits $Ca_V2.2$), and Ca_V3 channels more reliably distinguish among Ca_V sub-types. Pharmacological, genetic and functional studies have helped delineate the different cellular roles controlled by Ca_V channels. Presynaptic calcium entry, and subsequent transmitter release is mediated by Ca_V2 (mainly $Ca_V2.1$ and $Ca_V2.2$) channels throughout the nervous system, by $Ca_V1.3$ in inner hair cells [73] and by $Ca_V1.4$ in retina [74,75]. Dendritic postsynaptic Ca_V1 (mainly $Ca_V1.2$) channels regulate calcium entry that controls gene expression [76,77]. Dendritic $Ca_V2.3$ channels [32] are implicated in acquisition of dendritic phenotype [78] and oscillatory burst discharge in the reticular thalamus[79]. Postsynaptic $Ca_V1.3$ channels underlie pacemaking in certain cells [80], are important for brain stem neuron development [63], and are implicated in calcium-dependent death of dopaminergic neurons [2]. The roles of presynaptic Ca_V2 channels in synaptic transmission and short-term plasticity [58] and the role of postsynaptic Ca_V1 channels in activity-dependent gene expression in neurons have been reviewed elsewhere [81]. A functional, correctly targeted Ca_V channel depends on its association with other calcium channel subunits including $Ca_V\beta$, $Ca_V\alpha_2\beta$, and other proteins – some of these aspects of Ca_V channel regulation are detailed in other recent reviews [82].

Highlights

Highlights for “Control of Neuronal Voltage-Gated Calcium Ion Channels From RNA to Protein”

- How many different voltage-gated calcium channels are there and should you care?
- All mammalian *Cacna1* genes have the potential to generate hundreds of Ca_V channels
- Cell-specific mechanisms control Ca_V channel function at RNA and Protein levels according to cell type
- Cell-specific protein-protein interactions control sub-cellular Ca_V channel trafficking and function
- Cell-specific and subcellular expression patterns of Ca_V isoforms is important for disease and treatment developement

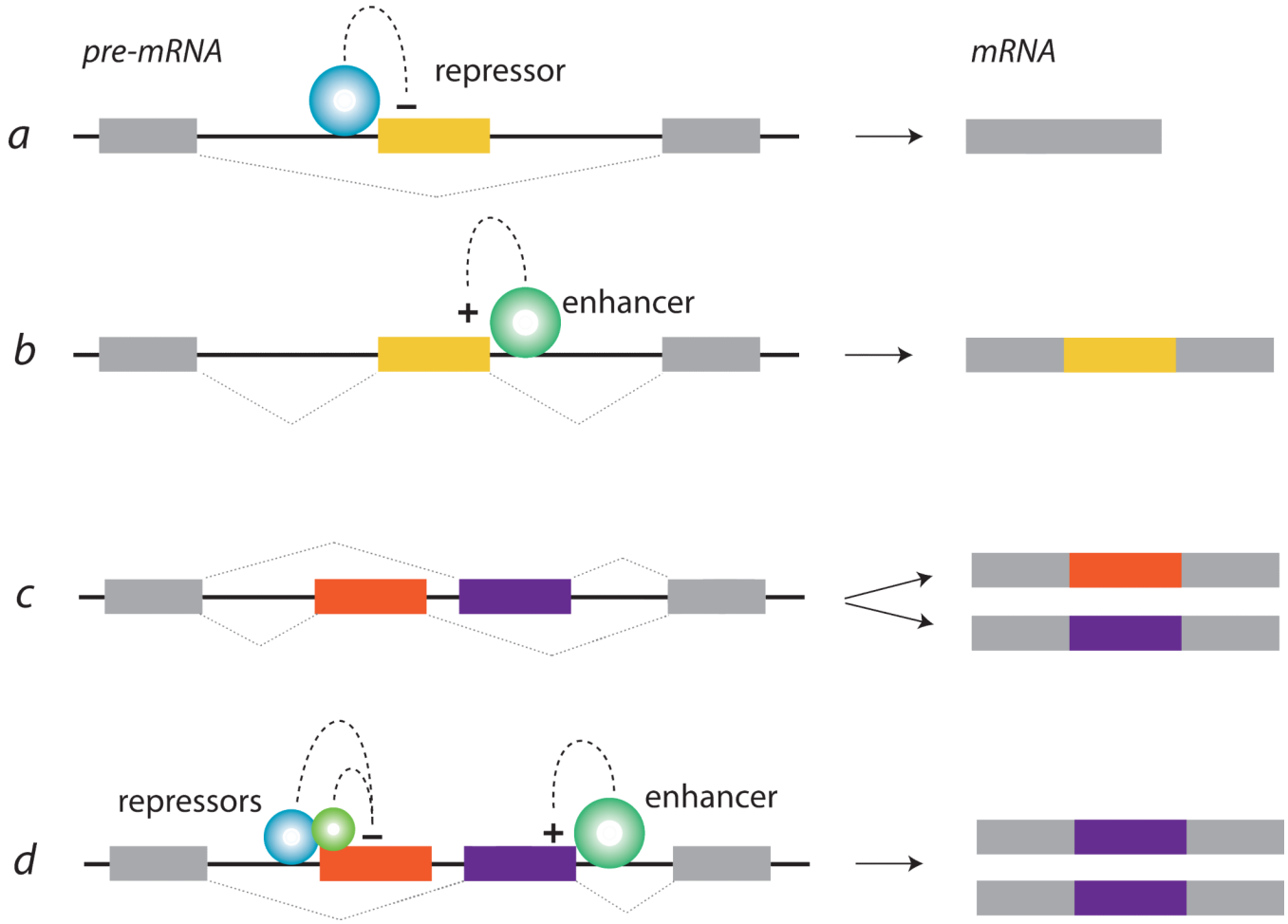
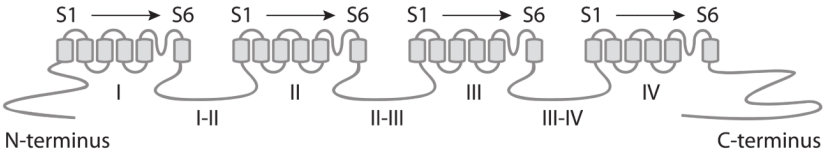


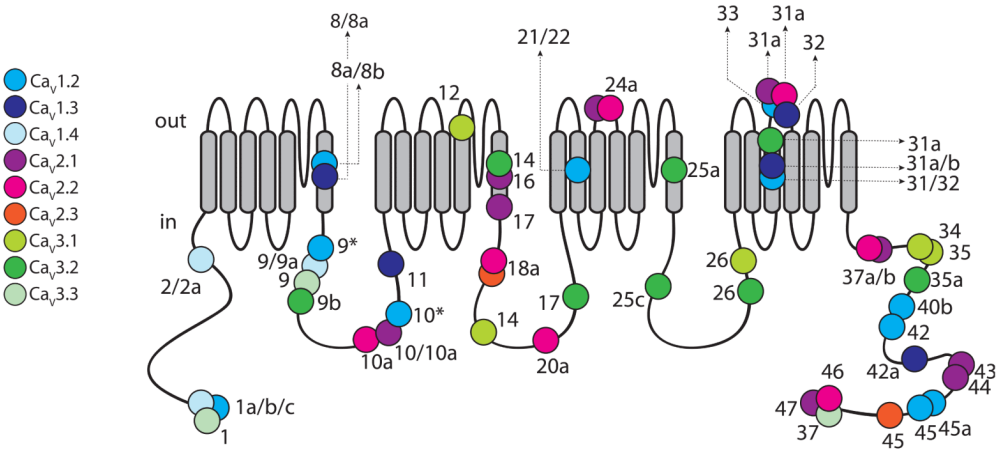
Figure 1. Splicing factors repress and enhance alternative exon inclusion during pre-mRNA processing

Splicing factors bind to consensus nucleotide sequences in introns or target exons of pre-mRNAs to enhance or repress (silence) recognition of the exon by the spliceosome during pre-mRNA processing. Alternatively spliced exons (colored), constitutive exons (gray), and introns (black connecting lines) are illustrated. The position of splicing factor binding, relative to the target exon, is often predictive of splicing factor action. For example, members of the Nova, rbFox, and PTB splicing factor protein families tend to repress or silence exon inclusion when they bind their respective nucleotide sequence binding motifs upstream (a) or within (b) the target exon, and enhance exon inclusion when they bind their respective binding motifs downstream of the target exon (b). Alternatively spliced cassette exons are excluded or included during pre-mRNA splicing (a, b). Mutually exclusive alternative splicing involves two or more exons, only one of which is selected during pre-mRNA processing (c, d). Mutually exclusive exons often start or end with an incomplete codon. In this case, there is a shift in the reading frame in mRNAs that either lack both mutually exclusive exons or contain both, and early protein termination (not shown). Often exon selection is influenced by the concerted action of several splicing factors (d). The expression levels and activities of individual splicing factors depend on many cellular factors including those involved in development, neuronal activity, and defining neuronal subtype.

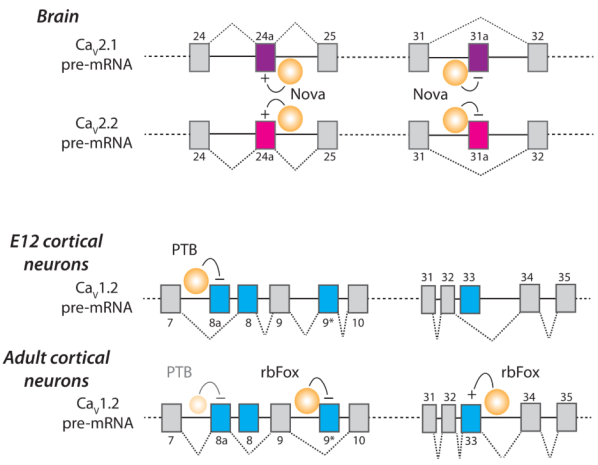
A. Transmembrane spanning (S1-S6), domains (I-IV), and intracellular regions of Ca_V channels



B. Location of alternative exons in Ca_V channels



C. Splicing factors target exons in Ca_V pre-mRNAs



D. Known exons regulated by splicing factors

Channel *Gene*	Exon	Location	Splicing Factor	Ref.
Ca$_V$2.1 *Cacna1a*	↑24a	IIIS3-IIIS4	Nova	[18]
	↓31a	IVS3-IVS4	Nova	[18]
Ca$_V$2.2 *Cacna1b*	↑24a	IIIS3-IIIS4	Nova	[4]
	↓31a	IVS3-IVS4	Nova	[18]
	↓18a	II-III	rbFox	[91]
Ca$_V$1.2 *Cacna1c*	↓8a	IS6	PTB	[14]
	↓9*	I-II	rbFox	[90]
	↑33	IVS3-IVS4	rbFox	[90]
Ca$_V$1.3 *Cacna1d*	↓8b	IS6	rbFox	[5]
	↑11	I-II	rbFox	[5]
Ca$_V$1.1 *Cacna1s*	↑29	IVS3-IVS4	rbFox	[5]

Figure 2. Alternative splicing is extensive in Ca_V channels and is regulated by the action of cell-specific splicing factors

A. Major domains and regions located on a schematic of the α_1 subunit of a Ca_V channel. There are four structurally homologous domains I, II, III and IV. Each domain is comprised of 6 transmembrane spanning α-helices (S1-S6). The intracellular regions that link the domains are labeled I-II, II-III, and III-IV. The N-terminus and C-terminus are intracellular. This naming system is used to identify the major regions of Ca_V channels. For example, the third transmembrane spanning α-helix in domain IV is referred to as IVS3.

B. Location of alternative exons mapped on to a schematic of the α_1 subunit of a Ca_V channel. The 24 transmembrane spanning domains are shown (gray) as well as extracellular

and intracellular regions. The approximate locations of alternative exons are shown (circles) and color-coded to indicate specific Ca_V channel subtypes. Exon numbers are indicated and the numbering system used follows mouse *Cacna1* gene numbering. Alternate first exons are shown at the start of the N-termini, and mutually exclusive alternative exons are designated as X/Y. The following references were used to compile information for $Ca_V1.2$ [14]; $Ca_V1.3$ [83]; $Ca_V1.4$ [84]; $Ca_V2.1$ [85]; $Ca_V2.2$ [83], accession # FJ609386; $Ca_V2.3$[83,86]; $Ca_V3.1$ [87]; $Ca_V3.2$ [88]; $Ca_V3.3$ [89].

C. Splicing factor target exons in Ca_V pre-mRNAs. Two splicing patterns are shown for three splicing factor RNA-binding protein families that have similar mechanisms of action: Nova, Fox and PTB. The first example (top) illustrates the action of Nova on alternatively spliced exon cassettes, e24a and e31a, in $Ca_V2.1$ and $Ca_V2.2$ pre-mRNAs. Nova enhances inclusion of e24a during pre-mRNA splicing of $Ca_V2.1$ and $Ca_V2.2$ by binding to elements in the respective introns downstream of the target exons (Intronic Splicing Enhancer; ISE), whereas Nova represses (or silences) inclusion of e31a during pre-mRNA splicing by binding elements upstream and within or overlapping the target exon (Intronic Splicing Silencer: ISS; Exonic Splicing Silencer: ESS). Nova is expressed in brain where $Ca_V2.1$ and $Ca_V2.2$ mRNAs containing e24a and lacking e31a dominate [18]. In the second example (lower), PTB represses inclusion of mutually exclusive e8a during splicing of $Ca_V1.2$ pre-mRNA. PTB is expressed in embryonic brain and therefore $Ca_V1.2$ mRNAs lacking e8a and containing e8 dominate early during development. In adult cortical neurons, PTB levels are reduced and it no longer represses e8a. By contrast, rbFox is upregulated during development and it represses inclusion of e9* and enhances inclusion of e33 [14,90]. In adult cortical neurons, $Ca_V1.2$ mRNA containing e8a, lacking e9*, and containing e33 dominate [14].

D. Alternatively spliced exons of Ca_V channel genes are shown together with splicing factors known to regulate their expression. Cassette exons are either included or excluded. E8a/e8 of $Ca_V1.2$, and e8b/e8a of $Ca_V1.3$ are mutually exclusive (see A and B). Therefore, repression of e8a during pre-mRNA splicing of $Ca_V1.2$ will promote inclusion of e8, and repression of e8b during pre-mRNA splicing of $Ca_V1.3$ will promote inclusion of e8a. $Ca_V1.2$ e8a is strongly repressed by PTB but more weakly repressed by the neuronal homologue, nPTB, despite their similar RNA binding motifs [14]. Based upon references [4,5,14,18,90,91].

Figure 3. The II-III intracellular loop regions of $Ca_V2.1$ and $Ca_V2.2$ have divergent sequences

Amino acid alignments for $Ca_V2.1$ (rat sequence: NM_012918) and $Ca_V2.2$ (rat sequence: AF055477) are shown for about 700 amino acids encoding the II-III intracellular loops as well as sequence in IIS6 and IIIS1 (Clustal Omega software [92]). The approximate locations of boundaries between transmembrane and intracellular domains are indicated. The chemical nature of each amino acid is coded according to the following color scheme: acidic (green) D, E; basic (purple) K, R, H; hydrophobic (orange) A, F, I, L, M, P, V, W; polar (blue) C, G, N, Q, S, T, Y. The black solid bars below the alignments indicate regions of differences in amino acid type between $Ca_V2.1$ and $Ca_V2.2$. White circles indicate amino acids of interest, the amino acid numbers are shown and they are coded according to the following categories: (S) alternative exon, deletion (del.), or synprint area start; (E) alternative exon del., or synprint area end; (I) alternative exon insertion; (P) phosphorylation. References: Alternative e16, e17, and e17a of $Ca_V2.1$ [85]; $Ca_V2.2$ e18a [93] [94]; $Ca_V2.2$ synprint [95]; $Ca_V2.1$ synprint [96]; PKC and CaMKII phosphorylation [97]; $Ca_V2.1$ Δ1 and Δ2 [98]; Ca_V2.

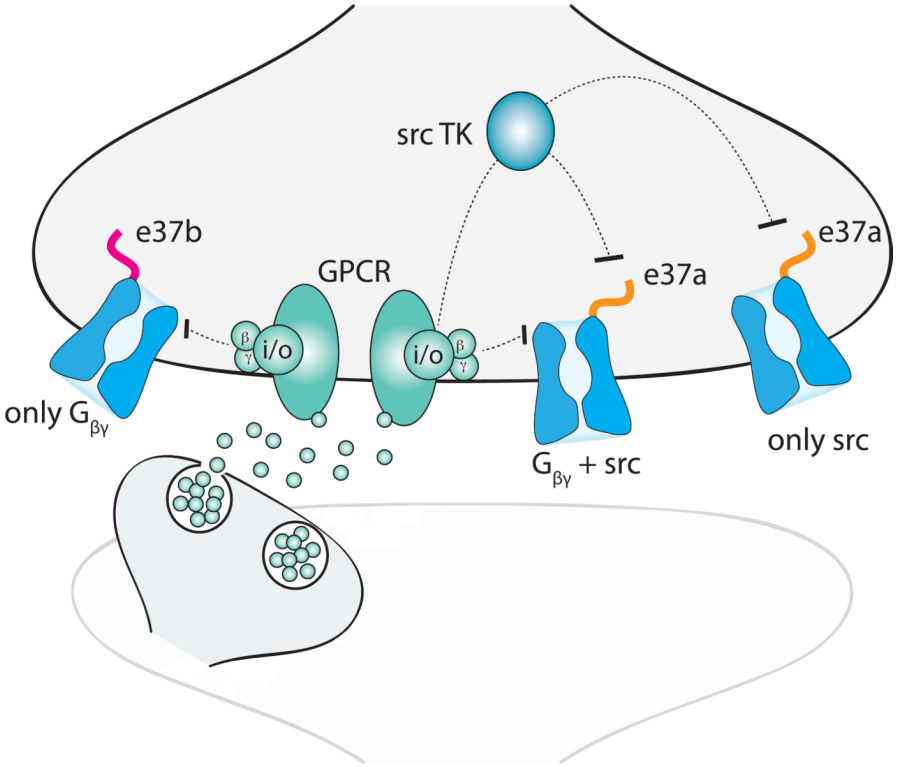


Figure 4. Alternative splicing influences G protein coupled receptor inhibition of $Ca_V2.2$ channels

Illustration showing a $G_{i/o}$ protein coupled receptor (GPCR; green) inhibiting presynaptic $Ca_V2.2$ channels (blue) by different signaling mechanisms. The GPCR is activated by neurotransmitter released from a neighboring neuron. Following activation, the G dimer dissociates from $G_{i/o}$, binds to and directly inhibits both $Ca_V2.2$ channel splice isoforms (e37a (orange) and e37b (pink)). A second G protein-dependent pathway, that requires src tyrosine kinase (TK) activation but that is independent of G, inhibits e37a-$Ca_V2.2$. This schematic is based on data published in [99] [23]. A prediction that follows from these studies is that e37a-$Ca_V2.2$ channels, that are more distant from GPCRs, will not be inhibited by membrane delimited G but may still be inhibited by src TK. This is because the latter mechanism involves a diffusible second messenger.

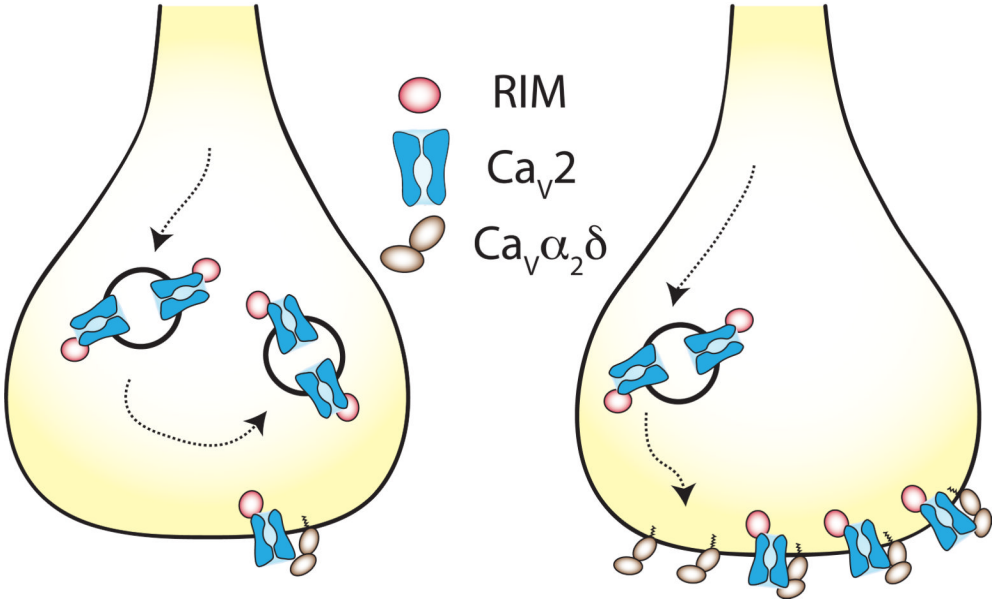


Figure 5. The density of Ca_V2 channels at presynaptic nerve terminals is controlled by RIM and the $Ca_V\alpha_2\delta$-1 subunit

Illustration shows that Ca_V2 channel trafficking to, or retention at presynaptic nerve terminals depends on association of Ca_V2 proteins with both RIM and $Ca_V\alpha_2\delta$-1[45] [46] [38,49] [30]. The details of where RIM and $Ca_V\alpha_2\delta$-1 interact with Ca_V2, in which intracellular compartment, are not known but the figure is intended to capture the finding that overexpression of Ca_V2 channels does not lead to an increase in functional presynaptic Ca_V2 channels (left) unless Ca_{V21} is also over-expressed (right) [45] [46]. These data suggest that $Ca_V\alpha_2\delta$-1 limits the insertion into or the stabilization of Ca_V2 channels at the active zones of the presynaptic nerve terminal [45].

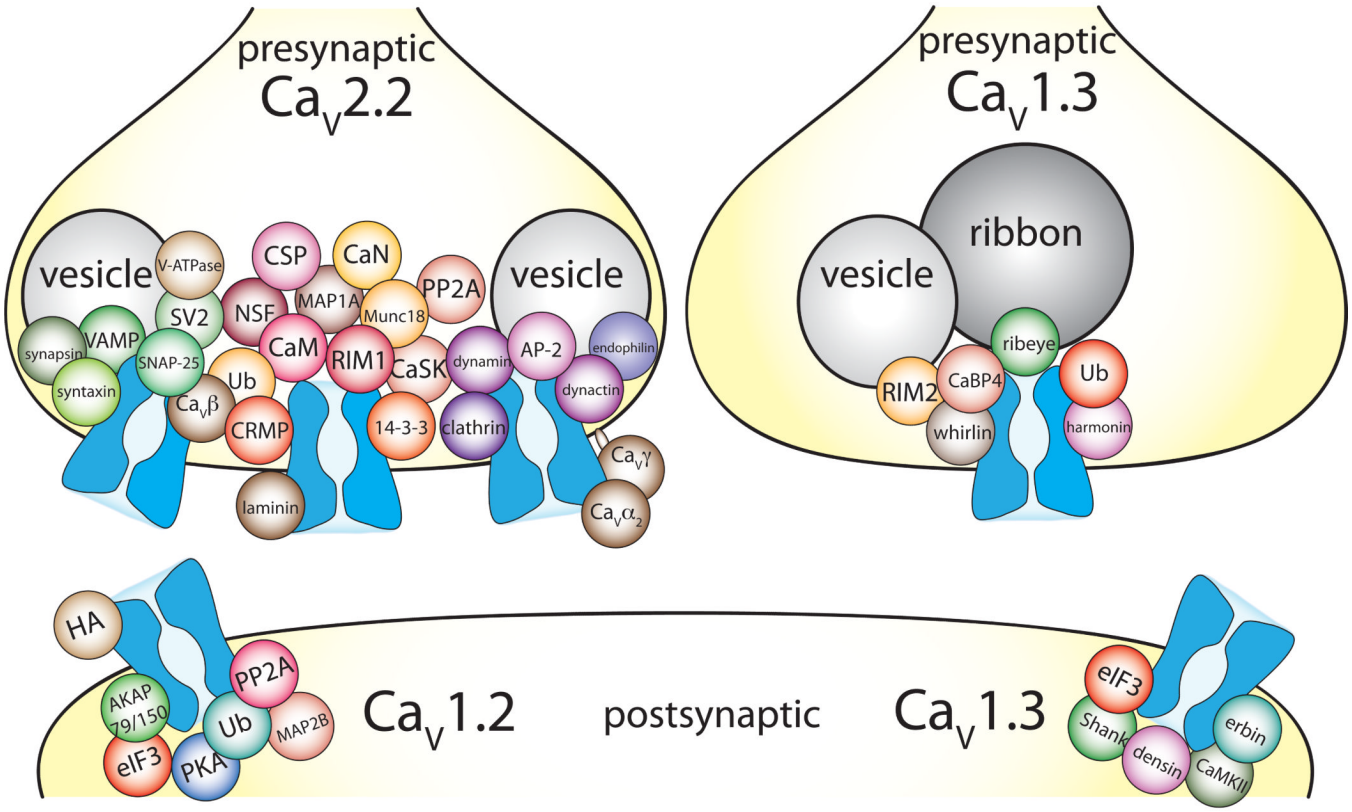


Figure 6. Presynaptic and postsynaptic Ca_V channels are associated with a number membrane anchoring and signaling proteins that define their function

Illustrated are a subset of proteins shown to bind presynaptic $Ca_V2.2$, postsynaptic $Ca_V1.2$, and pre- and postsynaptic $Ca_V1.3$ channels. Not all proteins reported to bind to these Ca_V channels are shown, but those illustrated were confirmed by more than one proteomics screen and/or by a functional assay. In addition, some G proteins, chaperone, and cytoskeletal proteins are not included. The proteins shown are approximately grouped by function, but beyond that there is no significance to location in the synapse or relative to each other. Data were collected using the following references: $Ca_V2.2$: CSP [100], CRMP [101], laminin [102], MAP1A [103], Ub [36] [35], other proteins were identified by [54,55,104]. $Ca_V1.2$: PP2A, [105], PKA [106], AKAP79/150 [107] [108] [109], HA [110], Ub [34,111], eIF3 [112], MAP2B [108] [113], dynamin [112]. $Ca_V1.3$ presynapse: whirlin [114], RIM2 [48], harmonin [42], Ub [42], ribeye [115] [60], CaBP4 [116] [117]. $Ca_V1.3$ postsynapse: Shank [118], erbin [81], densin [64], CaMKII [64], eIF3 [112].

Table I

Voltage-gated Calcium Channel Nomenclature								
Protein name		**Current type**	**Gene name**					
	old		**Human**	**Mouse, Rat**	**Zebrafish**	**Pufferfish**	**Drosophila**	**C.elegans**
$Ca_V1.1$	α_{1S}	L	*CACNA1S*	*Cacna1s*	*cacna1sa,b*	*1S-a,b,c*	*Ca-α1D*	*eql-19*
$Ca_V1.2$	α_{1C}		*CACNA1C*	*Cacna1c*	*cacna1c*	*1C*		
$Ca_V1.3$	α_{1D}		*CACNA1D*	*Cacna1d*	*cacna1da,b*	*1D-a,b,c,d*		
$Ca_V1.4$	α_{1F}		*CACNA1F*	*Cacna1f*	*cacna1f*	*1F-a,b,c*		
$Ca_V2.1$	α_{1A}	P/Q	*CACNA1A*	*Cacna1a*	*cacna1aa,b*	*1A-a,b*	*cac*	*unc-2*
$Ca_V2.2$	α_{1B}	N	*CACNA1B*	*Cacna1b*	*cacna1ba,b*	*1B*		
$Ca_V2.3$	α_{1E}	R	*CACNA1E*	*Cacna1e*	--	*1E-a,b*		
$Ca_V3.1$	α_{1G}	T	*CACNA1G*	*Cacna1g*	*cacna1g*	*1G-a,b*	*Ca-α1T*	*cca-1*
$Ca_V3.2$	α_{1H}		*CACNA1H*	*Cacna1h*	--	*1H-a,b*		
$Ca_V3.3$	α_{1I}		*CACNA1I*	*Cacna1i*	*cacna1i*	*1I*		