Review

Old proteins, developing roles

The regulation of calcium channels by synaptic proteins

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Abbreviations: CASK, calcium/calmodulin-dependent serine protein kinase; CNS, central nervous system; CSP, cysteine string protein; HVA, high voltage activated; LVA, low voltage activated; Mint-1, munc-18-interacting protein; PKA, protein kinase A; RIM, rab3 interacting molecule; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, soluble NSF attachment receptor

Key words: calcium channel, syntaxin, SNARE, neurotransmission, synapse, alternative splicing

Coupling of presynaptic voltage-gated calcium channels to the synaptic release machinery is critical for neurotransmission. It was traditionally believed that anchoring calcium channels close to the calcium microdomain dependent release machinery was the main reason for the physical interactions between channels and synaptic proteins, however in recent years, it is becoming clear that these proteins additionally regulate channel activity, and such processes as channel targeting and alternative splicing, to orchestrate a much broader regulatory role in controlling calcium channel function, calcium influx and hence neurotransmission. Calcium signalling serves a multitude of cellular functions and therefore requires tight regulation. Specific, often calcium-dependent interactions between synaptic proteins and calcium channels appear to play a significant role in fine-tuning of the synaptic response over development. While it is clear that investigation of a few of the multitude of synaptic proteins will not provide a complete understanding of calcium channel regulation, consideration of the emerging mechanisms by which synaptic protein interactions might regulate calcium channel function is important in order to understand their possible contributions to synaptic transmission. Here, we review the current state of knowledge of the molecular mechanisms by which synaptic proteins regulate presynaptic calcium channel activity.

Calcium Channel Structure

Voltage-gated calcium channels allow entry of calcium into excitable cells in response to a membrane depolarization, and thereby mediate a host of cellular responses including gene transcription, activation of calcium dependent enzymes, and triggering fast neurotransmitter release through vesicle exocytosis. A variety of calcium channel types are expressed in cardiac myocytes, smooth and skeletal muscle, and neurons, where they serve a multitude of physiological roles. The principal determinant of calcium channel function is the pore-forming α1 subunit (Fig. 1), whose primary structure comprises four repeated domains (I–IV) each with six transmembrane spanning segments (S1–6). A calcium-selective pore is formed by the apposition of all four domain re-entrant loops between segments S5 and S6, while the S4 segments act as a voltage sensor that open in response to membrane depolarization. To date, 10 distinct α1 isoforms have been characterized, with a range of biophysical and pharmacological profiles (Table 1).

HVA channels are comprised of the L-type CaV1 family (CaV1.1 through CaV1.4) and the CaV2 family which includes CaV2.1 (P/Q-type), CaV2.2 (N-type) and CaV2.3 (R-type) channels. The LVA family includes and T-type (CaV3.1–3) channels. While the α1 subunit alone is sufficient to form a functional calcium channel, a mature channel is formed by association with additional subunits including an α2-δ subunit plus an intracellular β subunit and possibly a membrane spanning γ subunit (Fig. 1) that influence channel function by regulating activation and inactivation kinetics, voltage dependence, pharmacological properties, expression and membrane targeting.

Exocytosis and Dependence on Calcium Influx

Chemical neurotransmission involves exocytosis of neurotransmitter-filled vesicles from the presynaptic nerve terminal, resulting in neurotransmitter mediated activation of postsynaptic receptors. Calcium entry into the presynaptic nerve terminal is a critical step in evoking this release, and has been demonstrated to occur in a paired, though non-linear fashion. This dependence makes voltage-gated calcium channels critical mediators of neurotransmitter release and hence synaptic function. Calcium is a highly bioactive molecule that controls numerous functions involved in cellular homeostasis, and therefore, the free intracellular calcium concentration is maintained at low levels (50–100 nM). Intracellular calcium needs to rise above a threshold of 20–50 μM to trigger fusion, with half-maximal fusion occurring at around...
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Calcium may rise by 50–100 μM for a few hundred microseconds before it diffuses away or is sequestered by calcium binding proteins, so the exocytotic machinery needs to be in close apposition to the source of calcium entry, in a small microdomain called the active zone. Neurotransmitter exocytosis is a highly regulated, multi-step process that may occur through a host of molecular mechanisms. One well studied mechanism is the formation of a soluble NSF attachment receptor (SNARE) complex that tethers a neurotransmitter-laden vesicle to the presynaptic membrane close to the site of calcium influx. This complex has been well studied primarily due to the highly stable binding interactions that occur between SNARE proteins. Besides their role in vesicle release, proteins of the SNARE complex appear to serve another function: to provide feedback inhibition of voltage-gated calcium channels as first described by Bezprozvanny et al. and studied extensively since.

CaV2 Channels are Critical in Neurotransmitter Release

Calcium entry must be tightly controlled both temporally and spatially to ensure appropriate neurotransmitter release. Synaptic calcium entry is mainly controlled by the activities of CaV2 calcium channels. The biophysical properties of these channels make them well suited to allow for effective delivery of calcium into the nerve terminal due to their rapid activation and relatively large single channel conductance (between 15 and 20 pS). Moreover, they are

Table 1 Overview of biophysical properties, distribution and function of the voltage-gated calcium channel family

| Name | Subunit | Current | Activation | Distribution | Function |
|------|---------|---------|------------|-------------|----------|
| CaV1.1 | α1S | L-type | HVA | Skeletal muscle | Excitation-contraction coupling, calcium homeostasis |
| CaV1.2 | α1C | L-type | HVA | CNS, heart, smooth muscle, endocrine | Excitation-contraction coupling, hormone secretion, gene regulation |
| CaV1.3 | α1D | L-type | HVA | CNS, pancreas, kidney, cochlea, hear | Hormone secretion, synaptic transmission auditory systems |
| CaV1.4 | α1F | L-type | HVA | Retina, immune cells | Tonic neurotransmitter release in the retina |
| CaV2.1 | α1A | P/Q-type | HVA | Nerve terminals, dendrites | Neurotransmitter release, dendritic CaV2+ transients (mature role) |
| CaV2.2 | α1B | N-type | HVA | Nerve terminals, dendrites | Neurotransmitter release, dendritic CaV2+ transients (developmental role) |
| CaV2.3 | α1E | R-type | HVA | Nerve terminals, dendrites, cell bodies | Neuronal excitability, neurotransmitter release (developmental role) |
| CaV3.1 | α1G | T-type | LVA | CNS, hear | These functions apply to all of CaV3.1, CaV3.2, CaV3.3: Pacemaker activity, burst firing, oscillatory behavior, hormone secretion |
| CaV3.2 | α1H | T-type | LVA | CNS, heart, smooth muscle, liver, kidney | |
| CaV3.3 | α1I | T-type | LVA | CNS | |

Figure 1. Subunit structure of calcium channels. The primary structure of the voltage-gated calcium channel α1 subunit consists four homologous regions (I–IV), each with six transmembrane spanning domains (S1–6). Segments S4 are voltage-sensitive and a re-entrant P-loop between S5–6 forms a divalent cation selective pore. Synaptic proteins, G-proteins and calcium-binding proteins interact with the cytoplasmic intracellular linkers and N- and C- termini. Specifically, synaptic protein interactions primarily occur in the II–III linker at the synaptic protein interaction (synprint) motif. Channel properties are modified by interaction with auxiliary subunits α2δ, β and γ.
subject to intrinsic calcium feedback inhibition, that allows for fine tuning of calcium entry (reviewed in refs. 30 and 31). Distribution of these channels is highest in nerve terminals, but they are also expressed in axons and dendrites (Table 1).25,26

Of the HVA channels, P/Q- and N-type channels have a clearly established role in mediating the majority of presynaptic calcium entry.24 The R-type channel also localizes presynaptically and may participate in neurotransmitter release,32 although its contribution is smaller and varies with brain region.33,34 With a few exceptions, it has been suggested that calcium channel isoforms are developmentally regulated with N- and R-type common in immature terminals, later favoring P/Q-type in maturity.34,35 In the CNS, P/Q-type channels are often associated with excitatory synaptic transmission, whereas N-type channels tend to be involved in inhibitory neurotransmission.36-38 Furthermore, calcium influx through P/Q-type channels is able to trigger neurotransmitter release more efficiently than N- or R-types39 suggesting it may be the primary mediator of mature neurotransmission. Indeed, in certain synapses, P/Q-type channels are located at the center of the active zones, whereas N-type channels are localized to peripheral sites.39 Yet, both channel subtypes share common features such as their abilities to interact with the SNARE complex. The distribution and distinct localization of channel isoforms within the active zone suggests that positioning of the channel relative to the SNARE apparatus may be a more important factor in channel efficacy than the isoform per se.

Presynaptic nerve terminals can produce a range of synaptic output depending on the compliment of calcium channel isoforms they contain, and the specific modulatory influences acting on each isoform. The nerve terminal has been referred to as a 'functional patchwork' with clusters of N-, P/Q-, or R-type, or all three, mediating a range of possible synaptic release patterns.40,41 This distribution may be due to specific targeting influences on each channel in the synapse,42 a result of developmental influences34,35,43 or of plasticity-related changes in the terminal.44 Additionally, nerve terminals may release transmitter in response to a local or global (volume-averaged) rise in intracellular calcium with varying patterns of release (reviewed in ref. 16). To account for the 10^5–10^6 increase in local calcium, the stoichiometry between calcium channels and exocytotic machinery appears to be highly non-linear, with possibly multiple calcium channels clustering close to docked vesicles in an active zone to ensure summating calcium microdomains.16,45,46 Hence, for release processes that rely on local calcium microdomains, it is critical that voltage-gated calcium channels are closely associated with the release machinery and other synaptic proteins, as outlined below.

Calcium Channel Binding and Regulatory Domains

The intracellular linkers between domains I–II, and II–III, and the C-terminus region of the channel are important targets for channel regulation by protein kinases and interacting proteins. The I–II linker is a known target for G-proteins and protein kinase C (reviewed in refs. 47 and 48). The long cytoplasmic C-terminus associates with a number of proteins that influence channel targeting, and is a critical region that is involved in calcium feedback regulation mediated by its association with calmodulin (reviewed in ref. 49). The II–III linker region is critical for association with synaptic proteins that control neurotransmitter release, and as we will outline below, also serves as a subcellular targeting motif. This region contains synaptic protein binding motifs including the Synaptic Protein Interaction (synprint) site that are necessary for colocalization of channels to the exocytotic machinery and hence functional synaptic transmission (Fig. 1).27,50 In N-type channels (rat Ca_v2.2), the synprint site is an 265aa residue stretch spanning residues 718–983,51,52 although smaller subregions within this motif are in fact involved in binding to SNARE proteins such as syntaxin 1A and SNAP-25.52 In the rabbit brain P/Q-type channel (BI) isoform, the synprint motif encompasses residues 722–1036.51 In contrast, the rat brain P/Q-type channel (rBA) isoform lacks part of the synprint region and thus loses syntaxin 1A association, although it is able to interact with other synaptic proteins.51 R-type channels lack the synprint site altogether, but retain the ability to functionally associate with the SNARE complex.23,52 There are a number of P/Q-type and N-type channel splice isoforms with sequence deletions and variations in the synprint region. Such differences in the synprint motif in N-, P/Q- and R-type channels may underlie observed changes in the ability of specific synaptic proteins to bind, accounting for different regulatory effects exerted on these isoforms.
Functional Interactions Between Presynaptic Calcium Channels and SNARE Proteins

Target (t-)SNAREs syntaxin 1A, SNAP-25 and vesicle (v-) SNARE synaptobrevin (VAMP) form the SNARE core complex that brings the vesicle and target membranes into close apposition, leading to fusion and exocytosis (Fig. 2). The syntaxin family started with the discovery of two 35 kDa proteins, syntaxin 1A and 1B that had primarily neuronal distribution and interacted with synaptotagmin I. This family now comprises 15 members (not including splice variants) with ubiquitous expression, and differing abilities to target interacting proteins to specific subcellular loci (reviewed in ref. 56).

Structurally, these proteins consist of four helical (H) domains, Ha, Hb, Hc and H3a, b and c and a C-terminal TM domain. Within the syntaxin 1 family, synaptic proteins bind to the H3 domains while the H domains form an alpha helix bundle that directly binds to the N-type II–III linker and to one isoform of the P/Q-type II–III linker (Fig. 2) and ensures that the synaptic core complex is within 50 nm of calcium entry. It may also serve as an auto-inhibitory domain. In addition to anchoring to the synprint site, another region of the H3 domain binds elsewhere on the channel to regulate its biophysical properties.

Neuronal SNAP-25 and its ubiquitously expressed homolog SNAP-23 are members of a family of synaptosomal-associated proteins that interact with syntaxin 1A to form the t-SNARE complex. Both SNAP-25 and SNAP-23 have highly conserved N- and C-termini that form helical domains consistent with the zippering interactions in the SNARE complex. Syntaxin interactions occur at the C-terminal half of the protein while synaptobrevin interactions occur at the N-terminal two-thirds (Fig. 2). The C-terminal also directly interacts with the II–III linker regions of N-type and P/Q-type channels, while early evidence points against its association with R-type channels. Three SNAP-25 splice variants exist, the mature, and two developmentally regulated isoforms: SNAP-25a, found in immature terminals, and SNAP-25b, predominant during synaptogenesis. The latter variant may influence synaptic plasticity.

Finally, the v-SNARE protein synaptobrevin (VAMP) has two neuronal isoforms and is anchored to the vesicle by a C-terminal TM domain, interacting with t-SNAREs by a short motif. It interacts with syntaxin 1A and SNAP-25 but does not bind the II–III linker in any of N-, P/Q- or R-type (Fig. 2).

In both N- and P/Q-type, SNARE protein interactions occur at the synprint site. R-type channels lack the synprint site, however, it has been reported that SNARE proteins may still interact with a homologous region of the II–III linker. In native cells, cleavage of syntaxin by botulinum toxin C affects P/Q-, N- and R-type currents, showing that this protein functionally regulates all types of Ca,2 calcium channels. Interestingly, the cleavage of SNAP-25 and synaptobrevin had no effect on calcium currents suggesting they may not have the same modulatory effect that syntaxin does. Coexpression of syntaxin 1A (or 1B) with N-type or P/Q-type channels results in inhibition of channel activity, by virtue of a strong hyperpolarizing shift in the voltage dependence of steady state inactivation of the channel. A similar effect is observed upon co expression of N-type channels with SNAP-25. For N-type channels, the inhibitory effects of syntaxin and SNAP-25 are reversed when both proteins are co expressed with the channel. In the case of the rabbit brain P/Q-type channel, the inhibitory effects of syntaxin on channel activity are relieved in the presence of both SNAP-25 and synaptotagmin. These findings are consistent with a mechanism by which calcium entry through N- and P/Q type channels is reduced in the absence of a docked vesicle (i.e., in the presence of syntaxin 1A alone), whereas calcium entry is facilitated when the channel is associated with the entire SNARE complex (and a docked vesicle), thus allowing for an optimization of calcium entry through those channels that are most likely to partake in synaptic release events.

Syntaxin 1A (but not 1B) also mediates a second inhibitory action on N-type calcium channels by inducing a tonic G protein inhibition of N-type channels which may involve a syntaxin 1A dependent colocalization of the channel with Gβγ subunits. Unlike the effect of syntaxin 1A on channel availability, this modulation persists in the presence of SNAP-25. Evidence for the functional significance of this effect in neurons is still scant, although it is known that G protein regulation of N-type channels in chick calyces is altered upon treatment with botulinum toxin C and Gβγ subunits have been shown to regulate neurotransmission with their interactions with SNARE complexes.

A third regulatory modality linked to the synprint motif is subcellular targeting of the channel. Work from Mochida et al. shows that the presence of the synaptic protein interaction site is an important determinant of subcellular targeting of P/Q-type channels. Similarly, splice variants of human N-type channels lacking the synprint region are still targeted to axonal compartments, but are excluded from the active zone. Conversely, R-type channels which do not contain a synprint region can still target to the presynaptic nerve terminal and participate in neurotransmitter release. Collectively, these findings indicate that while the synprint region may be an important determinant of subcellular distribution of Ca,2 channels in neurons, it is unlikely to be the only determinant, and its significance may vary with channel subtype.

Calcium-Dependence of Synaptic Protein Regulation

In order to trigger exocytosis, intracellular calcium in highly localized presynaptic microdomains rises from 100 nM to 100 μM in a matter of a few hundred microseconds. Several reports have examined the calcium dependent binding affinities of syntaxin 1A and SNAP-25. Sheng et al. showed syntaxin 1A and SNAP-25 exhibit a biphasic calcium dependence in their binding to the synprint region which occurs independently of the putative calcium sensor synaptotagmin I. A 10–20 μM local increase in calcium produced maximal binding affinity between the SNARE complex and N-type channel synprint region, whereas lower affinities were observed in the absence of calcium, or at concentrations above 100 μM, the latter additionally increasing the affinity of the SNARE complex for synaptotagmin I. Together, this would allow the dissociation of the SNARE complex from the N-type calcium channel, permitting vesicle fusion and leaving the channel available for subsequent docking interactions. In P/Q-type channels, the structure of the II–III linker in rB1 and B1 channel isoforms appears to be a critical determinant in the calcium-dependence of SNARE protein binding. The B1 isoform interacts with syntaxin, SNAP-25 and synaptotagmin I independent of calcium concentration whereas
the rBa isoform interacts with synaptotagmin I and SNAP-25 (but not syntaxin 1A) with similar affinity at similar calcium concentrations as in N-type channels. Although our knowledge of these interactions is incomplete, it is evident that while the fundamental mechanism of neurotransmission is essentially the same, a number of scenarios exist where different calcium sensitivity would result in distinct regulation of exocytosis and just as likely, calcium channel activity per se.

**Functional Interactions of Presynaptic Calcium Channels with Other Synaptic Proteins**

**Synaptotagmins.** The synaptotagmin family contains 12 isoforms (I–XII). Synaptotagmins I–V are primarily found in neurons while the other isoforms express ubiquitously. Synaptotagmin I is the best-characterized member of the family. This 65 kDa vesicle associated protein with calcium sensing properties contains a small N-terminal intravesicular domain, a TM-spanning domain and a C-terminal portion with two calcium binding C2 domains: C2A and C2B. It is the putative calcium sensor that permits the final step of exocytosis. Synaptotagmin I acts as a clamp, forming a complex with syntaxin 1A and SNAP-25 (Fig. 2), preventing spontaneous fusion and accelerating fusion in response to rising intracellular calcium. Calcium binding causes a conformational change that dissociates synaptotagmin I from the SNARE complex. Interestingly, this formation can also occur independent of calcium binding. In addition to binding the SNARE complex, the synaptotagmin I C2B and C2A domains bind the synprint motifs in N-type, and in an analogous II–III linker motif in R-type channels, which places the calcium binding domains intricately close to the source of calcium influx, helping to account for the speed of exocytosis.

In N-type, P/Q-type and R-type channels the binding of the synaptotagmin I C2A domain to the II–III linker modulates the activation kinetics of the channel, an effect which is lost in channel mutants with compromised C2A binding. As syntaxin 1A and synaptotagmin I compete for synprint binding, the functional effect of this mutation may be due to impairment of synaptotagmin’s ability to reverse syntaxin 1A inhibition of the channel. The physical association of synaptotagmin I to the II–III linker is a requirement for N-type channel mediated exocytosis although it is yet unknown if this interaction alone has any regulatory effect on the channel. Synaptotagmin I may thus be able to exert a modulatory effect on calcium channel function both directly and through modulating interactions with syntaxin 1A and SNAP-25. While the synaptotagmin family is involved in trafficking synaptic vesicles, it remains to be determined if it regulates calcium channels trafficking.

**Sec family.** nSec1 (munc-18) is the most studied neuronal isoform from the Sec1 family of proteins. There are six mammalian family members including nSec1A and B, munc-18b (muSec1) and munc-18c and munc13-1, 13-2 and 13-3. nSec1 binds with high affinity to syntaxin 1A and to the SNARE complex, stabilizing its closed conformation which antagonizes priming by preventing binding to SNARE partners and as mentioned earlier, syntaxin 1A-mediated inhibition of N-type channels. nSec1 is a critical regulator of the exocytotic machinery with null mutants exhibiting no neurotransmission. Recent studies suggest that nSec1 may be the key regulator of the machinery, controlling every step of the exocytotic pathway and dictating how closely SNAREs interact. It also seems important for determining which SNARE protein isoforms interact, specifically ensuring interactions with syntaxin 1A/synaptobrevin 2. In other systems, nSec1 promotes SNARE complex formation showing that it may have additional regulatory functions not yet explored. Additionally, it has been shown to transport syntaxin 1A from the golgi to the cell surface in epithelial cells and CHO cells which indicates it may have a similar role in neurons. Munc-13 appears to be necessary for dissociation of nSec1 from the SNARE complex. Coexpression of nSec1 eliminates the enhanced N-type channel inactivation promoted by syntaxin 1A, however when expressed alone, nSec1 is unable to influence the voltage-dependence of N-type channels suggesting it has no direct regulatory effect over the channel. Little is known about the possible calcium channel regulation by other Sec family members.

**Cysteine string protein.** Cysteine String Proteins (CSP) are a family of two 32–34 kDa proteins, practically identical except for their C-termini, with cysteine rich domains that localize to the vesicle membrane in the drosophila synapse. Mammalian CSP was found to regulate presynaptic calcium channels although it was not initially clear whether this action was the direct modulation of calcium influx or regulation of exocytotic machinery. CSP interacts with the N-type and P/Q-type channel synprint motif (Fig. 2), and in case of N-type channels, appears to trigger a tonic G protein inhibition that involves on one hand a colocalization of the channel with Gβγ, and on the other, a GEF-like activity that leads to activation of Gα subunits. In addition, CSP may enhance channel function via a recruitment of channels to the plasma membrane. CSP also indirectly regulates channel activity by virtue of its competition with syntaxin 1A for the channel, and perhaps by its direct interactions with syntaxin 1A.

A decrease in calcium sensitivity has been seen in CSP null Drosophila mutants while calcium currents remain unchanged and CSP overexpression produces enhanced calcium-dependent exocytosis. PKA-mediated phosphorylation of CSP dramatically reduces binding affinity for syntaxin 1A suggesting a possible mechanism by which PKA might indirectly affect calcium channel activity. Similar interactions and phosphorylation effects are seen with synaptotagmin I binding. Both syntaxin 1A and synaptotagmin I have defined roles in modulating calcium channels directly and through other synaptic proteins, in a calcium concentration dependent manner. Thus, the differing affinities of CSP for these proteins may provide a mechanism by which syntaxin 1A and synaptotagmin 1 mediated control can be regulated, however, much of this remains speculative and needs to be supported experimentally, especially in light of findings showing that P/Q-type channel activity in CSP null mice is relatively normal.

**RIM.** Rab3 interacting molecules (RIMs) are a family of six large scaffolding proteins found primarily in the presynapse. The protein comprises an N-terminal zinc-finger motif and C-terminal PDZ and C2 domains. RIM interacts with N-type channels at the synprint motif and also weakly with the P/Q-type (Fig. 2). It does not seem to target N-type to the presynaptic nerve terminal. RIM possesses an N-terminal domain that binds to Rab3 and two C2 domains C2A and C2B similar to those found in synaptotagmin I, indicating it may be a calcium sensor. This C2 domain can selectively associate with SNAP-25.
or synaptotagmin I in a calcium-dependent manner (favoring synaptotagmin I binding at concentrations higher than 75 μM). A recent study showed that RIM1 anchors SNAREs close to calcium channels by C-terminal binding to the channel β subunit, thereby prolonging calcium influx by inhibiting channel inactivation. This effect was seen in P/Q-type channels as well as N- and R-type channels, and suggests that RIM may be an important regulator of synaptic calcium channel function.

**Table 2** Effects of selected alternate splicing events in the CaV2 family

| Splice variant | Lacking region | Effect |
|---------------|----------------|--------|
| CaV2.1 rBA II–III | L754-P948 | Reduced current density, depolarizing shift in the voltage dependence of inactivation<sup>138</sup> |
| | R793-P948 | No change<sup>138</sup> |
| CaV2.2 II–III | R756-L1139 | Less sensitive to ω-conotoxin MVIIA, GVIA, loss of syntaxin 1A binding, depolarizing shift in the voltage dependence of inactivation, faster recovery from inactivation<sup>139</sup> |
| Δ1 | K737-A1001 | Loss of syntaxin 1A binding, depolarizing shift in the voltage dependence of inactivation, faster recovery from inactivation<sup>139</sup> |
| | +e18a | Protects from cumulative inactivation<sup>137</sup> |
| | -e18a | Increased expression over development<sup>137</sup> |
| C-terminal | | Susceptible to cumulative inactivation<sup>137</sup> |
| Short (a) | e37a | Retained CASK, Mint-1 binding<sup>125</sup> |
| Long (b) | | Loss of CASK, Mint-1 binding<sup>125</sup> |
| CaV2.3 II–III | +e18a | Decreased expression over development<sup>137</sup> |
| α1E-d | -e19 | Increased sensitivity to current-enhancement, no syntaxin 1A mediated changes<sup>141</sup> |

**CASK and Mint-1.** The modular adaptor proteins CASK (calcium/calmodulin-dependent serine protein kinase) and Mint-1 (Munc-18-interacting protein) are involved in synaptic targeting of channels. This occurs via interactions with C-terminal motifs that bind the CASK SH3 domain and Mint-1 PDZ domain in the N-type and P/Q-type channels, but not R-type channels.<sup>42,50,129</sup> The functional effect of loss of these targeting motifs has been demonstrated in N-type channels where the absence of these interaction motifs prevents synaptic targeting.<sup>42</sup> Disrupting Mint-1/CASK interactions with synaptic calcium channels in invertebrate neurons inhibits synaptic transmission, possibly because of channel mis-targeting.<sup>130</sup> However, as outlined earlier, it is unlikely that Mint-1 and CASK are the only determinants of channel targeting to synaptic sites. An additional proposed role of modular adaptor proteins may be the physical anchoring of calcium channels to release sites<sup>42,131</sup> though recent evidence suggests otherwise.<sup>125,126</sup>

**Regulation by Alternate Splicing**

Alternative splicing is an essential mechanism in synaptic development that allows for a variety of functional variations in the proteins involved in exocytosis. Temporally and spatially controlled channel splicing can dictate (1) intrinsic calcium channel biophysical properties, and (2) the selective expression and association of distinct synaptic protein isoforms that exert regulatory effects on calcium channel function.<sup>132</sup> Additionally, there is evidence that channel-vesicle stoichiometry changes over development, with fewer channels coupled to a single vesicle SNARE complex as the synapse matures,<sup>133</sup> a phenomenon that could possibly be regulated by splicing of either calcium channels or synaptic proteins.

In addition to a number of CaV2.1 and CaV2.2 splice variants that encode variations outside of the major synaptic protein interaction regions that show altered biophysical properties,<sup>134-136</sup> alternate splicing of the II–III linkers and C-termini, notably by exons e18a and b respectively can add further functional diversity (Table 2).<sup>137</sup> P/Q-type splice variants lacking parts of the synprint region showed reduced current and a large (40 mV) rightward shift in inactivation,<sup>138</sup> while N-type variants with substantial deletions of the synprint motif show decreased ω-conotoxin MVIIA and GVIA sensitivity, depolarized voltage dependence of steady-state inactivation, and enhanced recovery from inactivation.<sup>139</sup> Another splice variant that encodes exon e18a is less sensitive to closed-state inactivation.<sup>140</sup> Finally, alternate splice isoforms lacking exon e19 that encodes part of the II–III linker have been reported for CaV2.3 that show increased sensitivity to calcium-dependent current enhancement.<sup>141</sup> C-terminal splicing of exon e37a results in selective targeting to small nociceptive neurons.<sup>142,143</sup>

CaV2.2 and CaV2.3 splice variants containing e18a appear to be both spatially and developmentally regulated, with increasing expression in CaV2.2 and decreasing expression in CaV2.3 over time.<sup>137</sup> Together, these studies suggest that such alterations in calcium channel sequence due to mRNA splicing that alter not only intrinsic channel properties but also the regulatory influences of certain
synaptic proteins, may provide an additional mechanism by which synaptic calcium entry, and thus neurotransmitter release, may be fine-tuned and adapted to specific physiological, and perhaps brain region specific requirements.

Surprisingly little is known about how synaptic protein isoforms contribute to calcium channel and exocytotic function through development. A number of studies however provide a good starting point from which to begin exploring this field. SNARE proteins synaptobrevin, SNAP-25 and syntaxin 1A are expressed in early development in hippocampal cultures, though more dominant isoforms may exist. For example, cleavage of the mature v-SNARE isoform synaptobrevin by tetanus toxin, completely blocks neurotransmission in mature terminals whereas new synapses express a tetanus toxin-resistant isoform of synaptobrevin. In rat brain, SNAP-25 expression switches from SNAP-25a during development, to SNAP-25b in mature terminals, which is purported to be controlled by developmental patterns of electrical activity (i.e., calcium influx). Finally, a splice isoform of syntaxin 1A, termed syntaxin 1C, has been reported. This isoform lacks the membrane insertion domain, hinting at a very different cellular function of this variant. Its consequences on N-type channel regulation have only recently been investigated, showing that this isoform is unable to modulate N-type channel inactivation. Overall, the regulatory influences of alternate splicing of synaptic proteins are largely unknown, though it seems likely that these processes will ultimately turn out to play a critical role in synaptic transmission.

Concluding Remarks

Synaptic proteins are able to exert a fine degree of control over the biophysical properties of individual channels that have critical implications for the nature of synaptic transmission, going beyond the simple formation of SNARE complexes. SNARE and other synaptic protein families contain many isoforms, and similarly undergo extensive alternative splicing that yields a vast array of proteins that can differentially regulate calcium channel function. However, several areas of these regulatory processes require further research.

First, it has been shown that PQ-type calcium channels can induce calcium dependent gene transcription of synaptic proteins such as syntaxin 1A. The molecular mechanism of this negative feedback regulation needs to be explored further, as does a possible role of other CaV2 channel family members. There is also emerging evidence that synaptic proteins may be able to regulate exocytosis by controlling the distance between the SNARE complex and associated calcium channel(s). This points to an additional mechanism by which SNARE function can be controlled, although it remains to be seen which proteins underlie this association, and how it occurs.

Finally, the question of stoichiometry between calcium channel and vesicle apparatus is intriguing. Can one SNARE complex physically associate with multiple calcium channels, and is this stoichiometry optimized over time? Or can multiple SNARE complexes—each with their own single calcium channel—share the same overlapping calcium microdomain? Clearly, our understanding of the intricate interactions between calcium channels and the synaptic release machinery is in its infancy. A great deal of work, especially in native neuronal settings, remains to be done in order to unravel the intricacies of synaptic protein regulation on calcium channel physiology.

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