5 Calcium Channel Splice Variants and Their Effects in Brain and Cardiovascular Function

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Abstract

Calcium ions serve as an important intracellular messenger in many diverse pathways, ranging from excitation coupling in muscles to neurotransmitter release in neurons. Physiologically, the concentration of free intracellular Ca^{2+} is up to 10,000 times less than that of the extracellular concentration, and increases of 10- to 100-fold in intracellular Ca^{2+} are observed during signaling events. Voltage-gated calcium channels (VGCCs) located on the plasma membrane serve as one of the main ways in which Ca^{2+} is able to enter the cell. Given that Ca^{2+} functions as a ubiquitous intracellular messenger, it is imperative that VGCCs are under tight regulation to ensure that intracellular Ca^{2+} concentration remains within the physiological range. In this chapter, we explore VGCCs' inherent control of Ca^{2+} entry as well as the effects of alternative splicing in $Ca_V2.1$ and posttranslational modifications of $Ca_V1.2/Ca_V1.3$ such as phosphorylation and ubiquitination. Deviation from this physiological range will result in deleterious effects known as calcium channelopathies, some of which will be explored in this chapter.

Keywords

Voltage-gated calcium channels · CACNA1C · CACNA1D · CACNA1A · Cav1.2 · Cav1.3 · Cav2.1 · Channelopathies · Splicing · Modulation

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5.1 Ion Channels in Biophysics and Physiology

5.1.1 Introduction to LTCC

L-type calcium channels (LTCC) are a subset of voltage-gated calcium channels (VGCC). LTCCs comprise calcium channel (Ca_v) isoforms $Ca_v1.1$–$Ca_v1.4$ and are encoded by the human genes *CACNA1S*, *CACNA1C*, *CACNA1D*, and *CACNA1F*, respectively. They are called

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"L-type" because of their long-lasting Ba^{2+} conductance, which is attributed to the slow inactivation kinetics of LTCCs (~500 ms). Unlike the other VGCCs, LTCCs are the only group that can bind to various 1,4-dihydropyridines (DHP) and are thus also called dihydropyridine receptors (DHPR).

LTCCs are transmembrane proteins found on the plasma membrane of excitable cells such as neurons, skeletal, cardiac, and smooth muscles [1, 2]. Similar to other VGCCs, LTCCs are voltage-gated and they open in response to membrane depolarization, thereby allowing for the selective influx of calcium ions (Ca^{2+}) into the cytoplasm. This Ca^{2+} influx regulates various downstream functions like synaptic vesicle anchorage and release, gene expression, and excitation–contraction (EC) coupling [3]. While the topological structure of LTCCs is generally similar across the tissues they are expressed in, their functions differ according to their tissue localization and biophysical properties. Further discussions will be focused on the cardiovascular system for the purpose of this review.

5.1.2 Structure and Localization of LTCC

LTCCs are transmembrane protein complexes made up of four to five subunits depending on where they are expressed—α_1-, β-, $a_2\delta$-, and γ-subunits [2]. In skeletal and cardiac muscles, the γ-subunit is not associated with $Ca_v1.2$ and $Ca_v1.3$ isoforms. The ~250 kDa α_1-subunit forms the main pore of the channel complex and is comprised of four repeat domains (DI–IV), with each domain made up of six transmembrane segments (S1–S6) that are linked by intracellular loops. To date, there are ten known mammalian isoforms of α_1-subunit of which 4 are LTCCs. The S4 of each domain acts as the voltage sensor for the channel due to the presence of positively charged amino acids, while the S5 and S6 segments form the activation gate of the α_1-subunit and the S5–S6 reentrant loop lines the selectivity filter. Upon membrane depolarization, the change in membrane potential is "sensed" by the four S4 segments, resulting in a conformational change from close to open state, thereby facilitating the flux of Ca^{2+} ions across the membrane.

The auxiliary β- and $a_2\delta$-subunits are necessary for the proper expression and insertion of the α_1-subunit into the plasma membrane. It was reported that the overexpression of either the β- or $a_2\delta$-subunits enhanced the current density of the α_1-subunit [4]. Conversely, the current density is reduced when either of these subunits was transiently knocked down. These data suggest that the function of the α_1-subunit is regulated by the coexpression of the auxiliary subunits.

Co-immunoprecipitation experiments revealed that the β-subunit physically associates with the α_1-subunit. More importantly, this association occurs within a region between DI and DII linker (I–II loop), named as the α_1-interacting domain (AID) [5]. Interestingly, in vitro experiments revealed a potential mechanism by which the binding of the β-subunit to the AID is important in regulating the expression of the α_1-subunit. Of note, disruption to this interaction was reported to have resulted in the poly-ubiquitination of the α_1-subunit, leading to proteasomal degradation and thus decreased trafficking of the α_1-subunit to the plasma membrane [6].

5.1.3 LTCC in the Cardiovascular System—Function

In the cardiovascular system (CVS), LTCC isoforms $Ca_v1.2$ and $Ca_v1.3$ are found to be expressed. $Ca_v1.2$ is the predominant isoform expressed in excitable cells [1]. Conversely, $Ca_v1.3$ is found to be dominantly expressed in the sinoatrial node (SAN) and the atrioventricular node (AVN) and is important for pacemaking activity [7].

$Ca_v1.2$ and $Ca_v1.3$ are responsible for the initiation of the excitation–contraction (EC) coupling [3], a process whereby an electrical signal is converted into mechanical signals. Macroscopically, this can be seen simply as "contraction" of the muscle cells. Microscopically, the depolarizing electrical signals are sensed by the

S4 "voltage sensors," and the ensuing protein conformational change leads to channel activation. The resultant Ca^{2+} influx causes a transient increase in intracellular Ca^{2+} concentration. The binding of Ca^{2+} by the ryanodine receptors (RyR) localized to the membrane of sarcoplasmic reticulum (SR) activates the RyRs to open to release Ca^{2+} within the intracellular store of the SR, and this process further increases cytoplasmic Ca^{2+} concentration. This process, also known as calcium-induced calcium release (CICR), triggers a cascade of downstream biochemical signals, ultimately allowing for the contraction of muscle fibers via activation of tropomyosin. Although the sliding motion of a few tropomyosin proteins may seem insignificant, nevertheless, the coordinated, simultaneous sliding of tropomyosin across the entire muscle fiber is sufficient to generate much mechanical force for various processes such as vasocontraction, contraction of the myocardium during systole, and concentric movements such as weightlifting.

In the heart, this coordinated EC coupling process and pumping action of the myocardium are essential for maintaining a constant blood supply throughout the body. In the vessels, $Ca_v1.2$ is essential for maintaining the basal vascular tone of blood vessels, contributing to the maintenance of blood pressure. In both events, mutations of $Ca_v1.2$ or $Ca_v1.3$ result in channelopathies and could lead to serious health complications such as those discussed subsequently.

5.1.4 Channelopathies in the CVS—$Ca_v1.2$

5.1.4.1 Timothy Syndrome

Timothy Syndrome (TS) was first discovered by Katherine W. Timothy and is a rare multi-organ disorder that affects not only the myocardium but also the brain. TS is associated with a gain-of-function mutation in the α_1-subunit of $Ca_v1.2$. There are two unique mutations categorized, G406R and G402S, which occur in the mutually exclusive exons 8 or 8a of the α_1-subunit [8, 9]. As such, TS can be further categorized as a classical type I TS or an atypical type II TS depending on which exon is affected. Of note, exon 8 and 8a are both found in the S6 segment of DI, which forms part of the activation gate. In type I TS, G406R mutation was observed in exon 8a, while in type II TS, either G406R or G402S can be found in exon 8. In both events, this gain-of-function mutation results in reduced voltage-dependent inactivation (VDI) of the $Ca_v1.2$ channel, thereby resulting in a sustained increase in Ca^{2+} influx, long QT syndrome (LQTS), and thus arrhythmias. In most cases, arrhythmias remain the primary underlying cause of death for patients presenting with TS. As TS is a multisystem disorder, it was observed that in addition to cardiac symptoms, patients with TS also present with extracardiac phenotypes. These typically include syndactyly, dysmorphic facial features, and mental retardation.

5.1.4.2 Brugada Syndrome

In contrast to TS, Brugada Syndrome (BS) occurs due to loss-of-function mutations in the α_1-subunit of $Ca_v1.2$. This results in shorter QT intervals and contributes to the manifestation of arrhythmias [10, 11]. While gain-of-function mutations within the α_1-subunit are responsible for TS, loss-of-function mutations in the β-subunit also contribute to the progression of BS. It has been reported that in Chinese hamster ovary (CHO) cells, the loss-of-function mutations in both α_1- and β-subunits led to decreased current density. Despite this, however, it is interesting to note the surface expression of $Ca_v1.2$ in BS was not affected when investigated in neuronal rat brain [11].

Despite the differences in mutations, these results, when taken together, strongly suggest that the function of $Ca_v1.2$ within the myocardium needs to be tightly regulated. As seen earlier, dysregulation of either the main pore-forming α_1-subunit or the auxiliary β-subunit could result in serious health complications.

5.1.5 Channelopathies in the CVS—$Ca_v1.3$

5.1.5.1 Cardiac Dysfunction/Arrhythmia

The $Ca_v1.3$ isoform is encoded by the *CACNA1D* gene, and like $Ca_v1.2$, channelopathies due to mutations in *CACNA1D* could result in abnormal heart functions. $Ca_v1.3$ is activated at a more hyperpolarizing potential relative to $Ca_v1.2$, enabling its contribution to pacemaking in the SAN and AVN [7]. In a study done to better understand the contributions of $Ca_v1.3$ mutations to cardiac symptoms, a family presenting with $Ca_v1.3$ mutations was recruited and their electrocardiogram (ECG) studied [12]. It was then reported that severe bradycardia was observed at rest between a 12- to 24-h period in individuals with $Ca_v1.3$ mutations but not in the healthy individuals. Yet, it is interesting to note that unlike $Ca_v1.2$ mutations, these affected individuals did not present with any abnormalities to either the QRS complex or the QT intervals, thereby indicating that this bradycardia was associated with the conductance of the nodes. Further screening and characterizing of mutations in this family revealed the insertion of a glycine residue in various positions of the α_1-subunit. Of note, the insertion of a glycine residue at position 404 was reported to be found only in the mutually exclusive exon 8b. Again, this exon is located in the S6 of DI, which is part of the activation gate. Functional studies using patch-clamp experiments into the differences of biophysical properties in tsA-203 cells between wild-type and mutant $Ca_v1.3$ revealed that the insertion of the glycine residue results in a nonconducting variant of $Ca_v1.3$. Moreover, reverse transcriptase-polymerase chain reaction (RT-PCR) results showed that exon 8b is predominant in the SAN and not in the ventricles of the myocardium. Thus, the cardiac symptom presented due to this $Ca_v1.3$ mutation is due to SAN dysfunction and arrhythmia and not due to ventricular dysfunctions.

5.1.6 Regulation of $Ca_v1.2$

As discussed earlier, it is imperative that the activity of Ca_v in the heart is tightly regulated to function within a specific narrow range, the deviation of which could lead to serious health implications. Therefore, it is only natural that various mechanisms, for example, posttranslational modifications (PTMs), are in place to properly regulate the function of these calcium channels. An in-depth review of $Ca_v1.2$ posttranslation modification has been recently published [13].

$Ca_v1.2$ is known to be phosphorylated by various protein kinases, namely, serine/threonine protein kinases A (PKA), C (PKC), G (PKG), and Ca2+/Calmodulin-dependent protein kinase II (CaMKII) [14]. It has been proposed that phosphorylation of serine residues in Ca_v is a mechanism that regulates protein–protein interactions. Of note, PKA phosphorylation of serine 1928 (S1928) was reported to transiently disrupt the interaction between $Ca_v1.2$ and the β_2-adrenergic receptor (β_2AR). Yet, despite being the most abundant phosphorylation site of $Ca_v1.2$ in the myocardium, S1928 was not involved in the β-adrenergic upregulation of $Ca_v1.2$ in the heart. Interestingly, S1928 can also be phosphorylated by PKC. However, unlike the prior PKA-dependent phosphorylation event, S1928 phosphorylation by PKC was reported to increase $Ca_v1.2$ current density. Furthermore, PKG was also reported to phosphorylate S1928. In this case, however, a reduced calcium current was reported in the transfected human embryonic kidney (HEK) 293 cells. Despite phosphorylation of the same serine residue within the full-length $Ca_v1.2$ channel, it is intriguing that phosphorylation by different protein kinases resulted in different downstream effects on the channel. From this, it can be seen that S1928 phosphorylation, while important, was not solely responsible for the changes in the function of $Ca_v1.2$. Nevertheless, it is imperative that S1928 phosphorylation be considered as part of the possible mechanisms for regulating $Ca_v1.2$ channel function.

Ubiquitination is another common PTM known to regulate protein expression and turnover rates. Ret finger protein 2 (Rfp2), or tripartite motif-containing 13 (TRIM13), polyubiquitinates lysine residues located within the intracellular II–III loop, leading to proteasomal degradation of $Ca_v1.2$ [15]. However, it remains to be proven if Rfp2 physically associates with $Ca_v1.2$. Yet, when the β-subunit is co-expressed with the α_{1C}-subunit, there is a significant reduction in ubiquitination of the α_{1C}-subunit. Therefore, it can be hypothesized that the auxiliary β-subunit is necessary for the expression of $Ca_v1.2$. A recent study has shown that disruption of the interaction between the α_{1C}-subunit and the β-subunit by a novel binding partner, Galectin-1 (Gal-1), led to increased ubiquitination and turnover of $Ca_v1.2$ α_1-subunit protein [6]. More importantly, the study also revealed that overexpression of Gal-1 is associated with hypotension. Conversely, knockdown of Gal-1 protein via siRNAs led to increased blood pressure. It can thus be said that although the α_1-subunit is the main pore-forming subunit, its expression at the protein level is regulated tightly by auxiliary subunits like the β-subunit. Of interest, Gal-1 binding to the I–II loop is hugely reduced in the presence of alternative exon 9*, an exon that is selectively expressed in smooth but not cardiac muscle.

5.1.7 Conclusion

All in all, this section has discussed the importance of LTCC $Ca_v1.2$ and $Ca_v1.3$ in the CVS. Modulation of Ca_v function has to be tightly regulated such that its activity remains within a physiological range, outside of which could lead to the pathogenesis of cardiovascular diseases. Both de novo or familial genetic mutations of the *CACNA1C* gene are associated with a variety of diseases comprising cardiac and extracardiac symptoms. Thus, further studies to understand the regulation of $Ca_v1.2$ in the context of cardiovascular diseases are necessary to better treat cardiac diseases and improve the quality of life of patients.

5.2 $Ca_V2.1$

Much like the previous Ca_V1 channel family, channels in the Ca_V2 channel family comprise hetero-multimetric assemblies of a pore-forming $Ca_V\alpha_1$ subunit, auxiliary $Ca_V\beta$, and $Ca_V\alpha_2\delta$ subunits, with the α_1-subunit determining the channel subtype. There are three-channel subtypes in the Ca_V2 family, $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$, that are encoded by three genes *CACNA1A, CACNA1B*, and *CACNA1E*, respectively [16–18]. In this chapter, we will focus mainly on the history of $Ca_V2.1$ as well as the structure and function of this channel along with the various mechanisms that govern its modulation.

5.2.1 History of $Ca_V2.1$ Channel

$Ca_V2.1$ gives rise to both the P-type Ca^{2+} currents, aptly named P-type as it was first recorded in Purkinje neurons [19, 20], and Q-type Ca^{2+} currents that were first recorded in cerebellar granule cells [21]. Although both currents displayed very similar electrophysiological properties, they differed in their inactivation kinetics as well as their sensitivity to ω-Agatoxin IVA, with P-type Ca^{2+} channels exhibiting a higher sensitivity to ω-Agatoxin IVA [21]. Hence, it was initially thought that P-type and Q-type Ca^{2+} currents were generated by different α1 subunits [21], although both P-type and Q-type currents were found to be required for neurotransmitter release [22–25]. What eventually resolved this mystery was a combination of molecular cloning and electrophysiological methods.

The amino acid sequence of $Ca_V2.1$ was first obtained in 1991 from the cloned cDNA sequence of a rabbit brain calcium channel, then referred to as BI [16], or also as class A calcium channel by Snutch's group [26]. Northern blot analysis of the BI voltage-gated calcium (Ca_V) channel showed that it was highly expressed in the brain, particularly in the cerebellum, and at a far lower level in the heart. Using mutant mice models with

different patterns of cerebellar degeneration, it was deduced that BI calcium channels were highly expressed in both Purkinje cells and granule cells [16]. This was later confirmed in a later study where the spatial distribution of BI mRNA in the rat cerebellum (rbA for rat variant) showed a high expression of rbA mRNA in Purkinje cells and a lower expression of rbA mRNA in granule cells [27]. The electrophysiological and pharmacological properties as well as expression patterns were also similar to that observed of P-type channels in Purkinje cells, leading to postulations that BI/rbA Ca^{2+} channels correspond to the P-type Ca^{2+} channels [28], although there was initial caution to concluding that the BI channel was the same as the P-type Ca^{2+} channel due to certain differences in sensitivity to ω-Agatoxin IVA and inactivation kinetics [29].

Subsequently, there was also standardization of the calcium channel nomenclature, with class A calcium channel subunit (α_{1A}) gradually being the accepted name. Evidence for α_{1A} corresponding to P-type Ca^{2+} channel became more compelling when a study co-expressed different calcium channel β subunits with rat α_{1A} in *Xenopus* oocytes and was able to generate a current waveform similar to the P-type Ca^{2+} current observed in Purkinje cells when α_{1A} was expressed with β_{2a}. Interestingly, when α_{1A} was expressed with β_{1b} or β_3, current waveforms similar to the Q-type Ca^{2+} current observed in cerebellar granule cells were produced. A more native expression system involving rat neostriatal and cortical neurons further confirmed the effect of the β-subunit expression on α_{1A} channel kinetics [30]. Coupled with the in situ localization results showing that α_{1A} transcripts were also found to be highly expressed in Purkinje cells and granule cells, cells where P-type channels are highly expressed, it was highly conceivable that α_{1A} transcripts could correspond to P and Q-type Ca^{2+} channels [27]. The most convincing piece of evidence for α_{1A} giving rise to P and Q-type Ca^{2+} channels finally came when screening of a rat brain cDNA library was performed [31]. Three alternatively spliced α_{1A} isoforms were identified, with a valine insertion in the domain I–II linker (Val_{421}), an insertion of Asn and Pro (N_{1605}–P_{1606}), due to the use of an alternative 5′ splice donor site, in the extracellular linker between transmembrane segments S3 and S4 of domain IV, and 10 different amino acid residues in the carboxyl tail adjacent to domain IV S6 (also identified as EF hand-like motif). Expression of the different splice variants in *Xenopus* oocytes revealed alterations in channel properties. When α_{1A} was co-expressed with β_{2a}, there was slower inactivation kinetics similar to that observed in P-type Ca^{2+} channels. On the other hand, when α_{1A} was co-expressed with β_1, β_3, or β_4 subunits, faster inactivation kinetics similar to those observed in Q-type Ca^{2+} channels was seen. Sensitivity to ω-Aga-IVA was also found to be affected by alternative splicing. When the α_{1A} splice variant lacking N_{1605}–P_{1606} was expressed in HEK 293 cells, the sensitivity of α_{1A} to ω-Aga IVA was reduced, similar to native P-type channels. α_{1A} splice variants containing N_{1605}–P_{1606} was shown to be more sensitive to ω-Aga IVA, resembling Q-type channels. It was thus proposed that both P-type and Q-type are phenotypic variants arising from the alternative splicing of the α_{1A} transcript [31], hence solving the issue of the molecular origins of the P/Q-type Ca^{2+} channel.

In the following sections, we will continue to explore alternative splicing and its possible physiological roles.

5.2.2 $Ca_V2.1$ Channel Diversity

Following the discovery of the three alternatively spliced loci in the human *CACNA1A*, further studies have revealed a total of seven and nine alternative splice sites in human (Fig. 5.1) and rat cerebellum, respectively [32, 33]. However, between human and rat cerebellum, there are only five common splice loci, some of which we will examine in greater detail.

5.2.3 Exon 31

Exon 31 encodes for the NP insert in the extracellular loop of $Ca_V2.1$ domain IV S3–S4 and

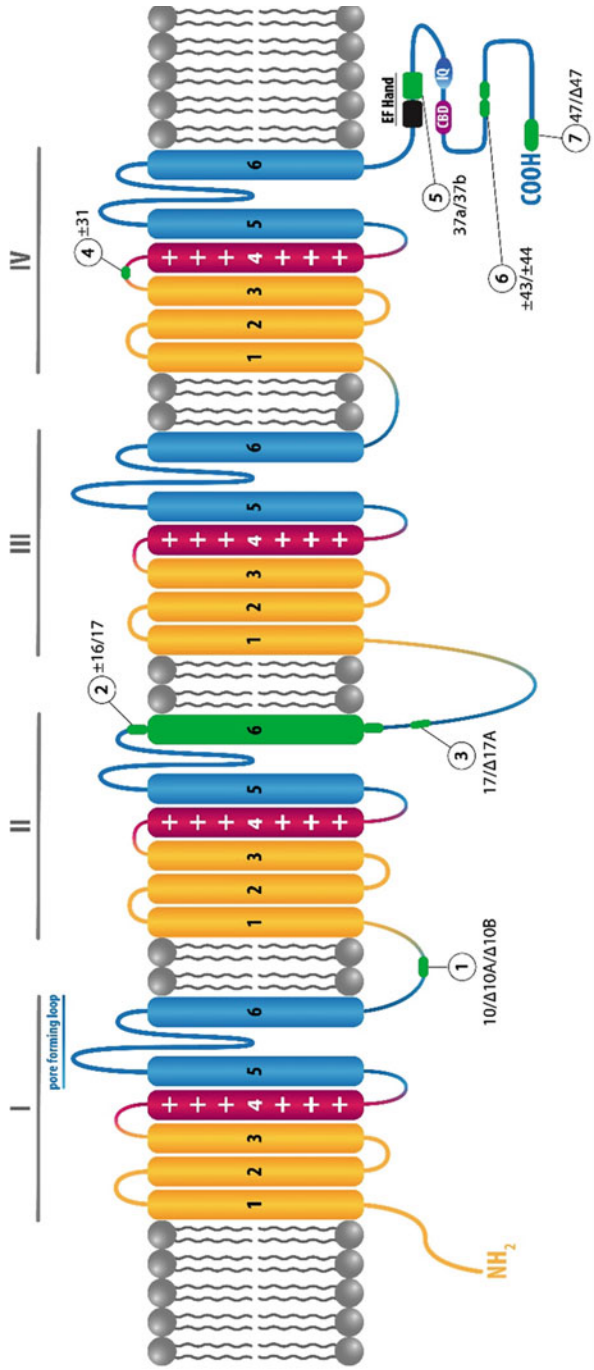

Fig. 5.1 Seven splice loci of human α_{1A} identified by transcript scanning

inclusion of NP decreases $Ca_V2.1$ affinity for ω-Aga-IVA as well as slowing channel activation and deactivation kinetics [31, 34]. It is worth noting that NP is encoded by a 6 nucleotide mini-exon (AATCCG) flanked by GT/AG acceptor-donor sites which are nested within intron 31 of humans, which is different from that observed in the rat variant where inclusion of NP was due to an alternative 5′ splice donor site located at the 3′ end of exon 31 [31]. When examining splice variant distribution in a human cerebellum cDNA library, 95% of the splice variants examined contained the +NP variant [31]. However, when single-cell RT-PCR of rat Purkinje cells was performed, the majority of the $Ca_V2.1$ transcripts were found to lack exon 31, which agrees with the required concentration of ω-Aga-IVA needed to almost completely block Ca^{2+} channel currents in rat Purkinje cells [24]. One possible explanation as to why the results from the human cerebellar cDNA library and the single-cell RT PCR of rat Purkinje cells do not match could be due to the proportion of Purkinje cells to granule cells in the cerebellum, where the granule cells can outnumber the Purkinje cells by 100–300 times [35, 36]. A human cDNA library constructed from bulk cerebellar tissue would hence have a higher proportion of granule cell transcripts, possibly masking the exact transcript expression profile in Purkinje cells.

5.2.4 Exon 37

Exon 37, together with exon 36, encodes for a Ca^{2+} binding site, known as an EF-hand-like motif, comprising 29 amino acids [31, 37, 38]. The general EF-hand motif consists of an α-helix E, a linker region, and an α-helix F [39]. Exon 37 encodes for part of the linker region as well as the α-helix F region. Mutually exclusive splicing of exon 37 results in variants EFa and EFb [31, 33]. Both splice variants are highly similar, with only a substitution of 10 amino acids out of a region of 30 amino acids [31]. Electrophysiological characterization involving transient overexpression of splice variants in HEK 293 cells revealed that both splice variants possessed almost similar properties, with the exception of EFa being able to undergo Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI) (both processes will be explored in greater detail later), while EFb was only able to undergo CDI [40]. Subsequent experiments involving single-channel recordings of the two splice variants showed that EFa, when in the CDF mode, had an increase in channel open probability as well as an increase in channel open duration. On the other hand, EFb was found to be locked in a normal gating mode [41]. How the differences in amino acid sequences between the two splice variants determine whether the channel is able to undergo CDF is still unknown, but chimeric channel analysis involving the EF-hand region and the pre-IQ-IQ domain of $Ca_V2.1$ and $Ca_V1.3$ as well as $Ca_V2.2$ showed that both the EF-hand region and the pre-IQ-IQ domain of $Ca_V2.1$ are required for eliciting CDF [42, 43].

While the physiological significance for the splice variants is not known, an unequal distribution of splice variants has been observed in the cerebellum, with EFa being the dominant splice variant expressed in adulthood [44, 45]. However, it was also observed in mice where EFb was the dominant variant from the embryonic stage up till P5, after which there was a developmental switch from P7 onward to an EFa dominant expression pattern in the cerebellum [45]. Coincidentally, the developmental switch observed in the cerebellum between P5 and P7 coincides with a time period where a climbing fiber (CF) is selectively strengthened, a process that is critical for proper cerebellar circuitry formation [46], hence suggesting that the developmental switch of the splice variants could be required for CF strengthening and proper formation of cerebellar circuitry.

Chronic reduction of network activity in rat primary hippocampal neuron cultures via toxin application resulted in the upregulation of EFa but not EFb mRNA, with the greatest increase observed at 24 hr. An increase in $Ca_V2.1$ protein expression was also observed following network deprivation, which was blocked when EFa was knocked down with miRNA, suggesting that

neurons are able to control EFa and EFb splice variant levels in a homeostatic manner [47].

While the verdict is still open on the physiological significance of exon 37 splice variants, the evidence presented earlier seems to suggest that modulation of splice variant expression is activity dependent. To date, the splicing factors governing the expression of exon 37 splice variants are still unknown.

5.2.5 Exon 43/44

Alternative splicing of exons 43 and 44 gives rise to combinations where both exons can be present or absent in four combinations (±43/±44). Transcript scanning of a human cerebellum cDNA library showed that $43^+/44^+$ splice variant was the most abundant (80–90% of total colonies counted), followed by $43^+/44^-$, $43^-/44^+$ and $43^-/44^-$ [33, 48]. Overexpression of the different splice variants in HEK 293 cells followed by electrophysiological characterization revealed that alternative splicing at exons 43 and 44 did not affect CDF. However, Ca^{2+} current amplitude and CDI were shown to be affected. CDI was the greatest in $43^-/44^-$, while the other splice variant combinations had similar CDI values. $43^-/44^-$ splice variant had a Ca^{2+} current amplitude that was twofold larger than that of the $43^+/44^+$ splice variant [33]. Given the increases in current amplitude and CDI, with CDI being affected by global increases in Ca^{2+}, it was postulated that the various splice variants at this particular locus affect surface channel expression. Furthermore, the presence of exon 44 was required for binding to Rab3-interacting molecules (RIM), scaffolding proteins important for the anchoring of $Ca_V2.1$ to synaptic vesicles at the active zone. Disrupting binding of $Ca_V2.1$ to α-RIMs was found to result in a release of suppression of voltage-dependent inactivation (VDI), and hence, $Ca_V2.1$ splice variants lacking exon 44 were found to have a stronger VDI [48].

5.2.6 Exon 47

Addition of a pentanucleotide GGCAG at the start of exon 47 results in an in-frame translation of exon 47 to produce a long version of the C terminus (termed as 47), allowing for the insertion of a polyglutamine (polyQ) tract. On the other hand, omission of GGCAG in splice variants causes a frameshift, resulting in the generation of a stop codon near the beginning of exon 47, thus producing a shorter variant of the channel (Δ47) [33, 49]. Studies involving knock-in mouse models of the human splice variants at this particular locus show no change in the intrinsic electrophysiological properties of the calcium channel [50–52], although studies utilizing heterologous transient overexpression systems provided conflicting evidence [53–55]. This further highlights the need to study the effects of calcium channel mutations, specifically polyQ expansion discussed here, in a more physiological setting. While the basic properties of the calcium channel are not altered by alternative splicing, it must be noted that the longer splice variant containing an expanded polyglutamine tract is an underlying cause for spinocerebellar ataxia 6 (SCA6), which will be covered in greater detail in the later section subsequently concerning $Ca_V2.1$ channelopathies.

5.2.7 Modulation of Calcium Channels

5.2.7.1 Voltage-Dependent Inactivation (VDI)

VGCCs open in response to membrane depolarization, allowing for the influx of Ca^{2+} ions. However, as Ca^{2+} is an important second messenger for many molecular pathways in the cell, there exist feedback mechanisms such as VDI and Ca^{2+}-dependent inactivation (CDI) to prevent unregulated and excessive Ca^{2+} entry.

VDI is common across all VGCC subtypes, but the degree of VDI varies for individual subtypes and is greatly modulated in high voltage-activated (HVA) channels, such as Ca_V1

and Ca_V2, by $Ca_V\beta$ subunits [56, 57]. VDI has both fast and slow components to it, with fast inactivation occurring on a timescale of milliseconds, while slow inactivation is generally observed over a more prolonged membrane depolarization time frame (~1 min) and is not as well understood [58, 59]. Hence for this section, we will be focusing on fast VDI.

Generally, when HVA channels are co-expressed with β_{1b} or β_3 subunits, VDI is observed to be accelerated, while β_4 is observed to not affect VDI by much and the inactivation kinetics are like that of α_1 alone. However, when expressed with β_{2a}, inactivation kinetics is significantly slowed down [27, 60].

To study VDI on its own, Ba^{2+} is used as the charge carrier instead of Ca^{2+} to avoid evoking Ca^{2+}-dependent inactivation [61]. The earliest attempt to uncover structural determinants of calcium channel inactivation involved chimeric channels produced from rat $Ca_V2.1$ and marine ray $Ca_V2.3$. Subsequent electrophysiological analysis of the chimeric channel showed that differences in domain IS6 of $Ca_V2.1$ and $Ca_V2.3$ were the reason for the difference in inactivation kinetics between the two Ca_V2 subtypes [62]. Further evidence for the importance of S6 segment to VDI came from studies where the S6 segments of domain II or III of slowly inactivating $Ca_V1.2$ were swapped with the corresponding S6 segments of rapidly inactivating $Ca_V2.3$, transforming $Ca_V1.2$ to a rapidly inactivating phenotype [63]. Additionally, mutations in IIS6 and IVS6 regions of $Ca_V2.1$ in familial hemiplegic migraine 1 (FHM1) patients resulted in altered inactivation kinetics [64, 65]. Introduced point mutations in IIS6, IIIS6, and IVS6 of L-type calcium channels were also found to underlie an increase in inactivation kinetics [66–68]. One other compelling piece of evidence for S6 to be critical for VDI comes from the G406R mutation in TS patients, as mentioned in the earlier section. G406 is at the end of IS6 segment, and mutation to the G406R results in a near-total abolishment of VDI [69]. These studies thus support the notion that all four S6 regions of HVA channels are involved in VDI.

Apart from S6 segments of HVA channels being implicated in VDI, the intracellular loop linking domain I–II has been implicated in VDI [64, 68, 70, 71], leading some to propose the hinged-lid model as a molecular basis for VDI [72]. An alternative theory based on current kinetics and steady-state activation of $Ca_V1.2$ proposed that the calcium channel can exist in four different states, a closed resting state where the pore is closed and the voltage sensors lock the pore (R), an activated closed state where the pore is closed but the voltage sensors are in their "up" position (A), an activated open state where the pore is open and the voltage sensors are in their "up" position (O), and a deactivated open state where the pore is open but the voltage sensors are in their down position [73]. A recent review by Hering et al. [74] discusses this model in greater detail along with the recently discovered crystal structure of $Ca_V1.1$ [75, 76].

5.2.7.2 Ca^{2+}-Dependent Inactivation (CDI)

CDI serves as a form of negative feedback regulation for VGCCs and was first discovered in Paramecium where it was observed that inactivation of VGCCs occurred at a faster rate in solutions containing Ca^{2+} than solutions containing Ba^{2+}, and increased buffering of intracellular Ca^{2+} resulted in decreased CDI, suggesting that accumulation of intracellular Ca^{2+} is required for CDI [77, 78]. This was also supported by the observation that CDI has a U-shaped dependency on voltage, a very telling sign of a Ca^{2+}-regulated process [79]. However, the extent to which Ca^{2+} chelators affect CDI differs between $Ca_V\alpha$ subunits and will be explored further subsequently.

To study CDI, a double-voltage pulse protocol is used where a prepulse voltage step to various potentials is delivered, and a test pulse of a fixed voltage is delivered after a brief pause [77]. The difference between the remaining current of Ba^{2+} and Ca^{2+} after a stipulated duration is taken to be a measure of pure CDI. [80] It must however be noted that for channels exhibiting faster inactivation kinetics such as Ca_V1 channels, shorter

depolarizing pulses, and consequently a shorter index r will be used [81].

Advances in experimental techniques allowed for the isolation and recording of Ca^{2+} currents in both multicellular and single myocytes, revealing the existence of CDI [82–84]. Single-channel recordings of an L-type calcium channel from an adult rat cardiac myocyte revealed that Ca^{2+} influx of a single channel was sufficient for CDI to occur [80]. However, while it was agreed that Ca^{2+} influx through the calcium channel was responsible for providing negative feedback, the mechanism by which it exerted its effects was then still largely unknown. Identification of an EF-hand Ca^{2+} binding motif in the C-terminal region of $Ca_V1.2$ [38] gave rise to the possibility that the binding of Ca^{2+} to this region could be responsible for the initiation of CDI. Chimeric channel studies involving $Ca_V1.2$ (observed to have strong CDI) and $Ca_V2.3$ (observed to have little CDI) helped to provide the first evidence for the EF-hand motif to be involved in CDI. When the C-terminal region of $Ca_V2.3$ and $Ca_V1.2$ were swapped, it resulted in $Ca_V1.2$ exhibiting weak CDI and $Ca_V2.3$ exhibiting strong CDI [85]. These results implied that the proximal third of the carboxyl terminal (CI region) as well as the EF-hand motif within this region were integral to CDI. Subsequent studies utilizing point mutations in the critical residues within the EF-hand region required for Ca^{2+} binding were unable to completely eliminate CDI, suggesting that the Ca^{2+} sensor may reside somewhere else within the CI region [86, 87].

Calmodulin (CaM), a Ca^{2+} binding protein, is a ubiquitously expressed cytoplasmic protein and is involved in many Ca^{2+}-related processes. Downstream of the EF-hand region is an IQ CaM-binding motif that was shown to be essential for CDI [88]. Coexpression of mutant CaM that was unable to bind Ca^{2+} along with $Ca_V1.2$ resulted in ablation of CDI, confirming the role of CaM as a Ca^{2+}-sensor in initiating CDI of L-type calcium channels. Glutathione S-transferase (GST) pulldown experiments involving GST fusion proteins with various regions of $Ca_V1.2$ CI region confirmed that the IQ domain in $Ca_V1.2$ is the site for CaM interaction [89]. GST pulldown experiments also demonstrated that the IQ domains of Ca_V2 family were able to interact with CaM in a Ca^{2+}-dependent manner. Further evidence for CaM being the Ca^{2+} sensor for VGCCs came from studies where Ca^{2+}-free CaM (apoCaM) being pre-associated with the α_1-subunit were reported [90–92].

Using the C-terminus of the rat $Ca_V2.1$ α_1-subunit as bait, a yeast two-hybrid screen of a rat brain cDNA library identified CaM to be interacting with the C-terminus. When low Ca^{2+} buffering conditions were used (0.5 mM EGTA), CDI of $Ca_V2.1$ channels was observed [93]. On the other hand, under high Ca^{2+} buffering conditions (10 mM EGTA), CDI was not observed in transfected cells [81, 94], suggesting that CDI in $Ca_V2.1$ is dependent on global increases in Ca^{2+}. This hypothesis was later confirmed with recombinant mutant CaM co-expression with $Ca_V2.1$ in HEK 293 cells, where it was observed that Ca^{2+} binding to the N-lobe of CaM (sensitive to global increases in Ca^{2+}) was responsible for CDI [81, 95]. This is in contrast to CDI of $Ca_V1.2$, where CDI was observed even in high Ca^{2+} buffering conditions and even at single-channel level [96], demonstrating that CDI in $Ca_V1.2$ is dependent on local increases in Ca^{2+} near the channel pore. In this initial study demonstrating the importance of CaM to Ca^{2+} regulation of $Ca_V2.1$ channels, a site downstream of the IQ-like motif, calmodulin-binding domain (CBD), was thought to be vital for CaM binding and consequently Ca^{2+} regulation of the channel [93, 94, 97]. However, subsequent studies have called the importance of this binding site into question, where deletion of CBD was found to have no effect on CDI [44, 81]. However, there is consensus for the importance of the IQ-like motif for CDI of $Ca_V2.1$ channels to occur [43, 81, 97, 98].

While the abovementioned lines of evidence present a common mechanism by which VGCCs are Ca^{2+} regulated, the IQ domain, discovery of another site for CaM binding in $Ca_V1.2/1.3$ but not Ca_V2 channels adds another layer of control to Ca^{2+} regulation of VGCCs. Given that C-lobe of CaM has a stronger binding affinity to Ca^{2+}, it has always been traditionally viewed that local

increases in Ca^{2+} drive CaM C-lobe mediated processes, while global increases in Ca^{2+} were thought to only drive CaM N-lobe mediated processes [95, 99]. A chimeric channel consisting of the N-terminus of $Ca_V1.2$ and $Ca_V2.2$ was found to demonstrate strong CDI even in the presence of strong Ca^{2+} buffering. Likewise, when the N-terminus of $Ca_V1.2$ was substituted in $Ca_V2.1$, CDI was observed under strong buffering conditions, demonstrating CDI driven by local Ca^{2+} increase, unlike the usual CDI driven by the global increase in Ca^{2+} in Ca_V2 channels [81, 100]. Subsequent alanine point mutations of the N-terminus combined with in situ FRET as well as electrophysiological experiments confirmed the existence of a CaM-binding element in the N-terminus of $Ca_V1.2/1.3$ which was subsequently named as NSCaTE [100]. NSCaTE was shown to bind to the N-lobe of Ca^{2+}/CaM with a local Ca^{2+} sensitivity, resulting in CDI.

5.2.7.3 Ca^{2+}-Dependent Facilitation (CDF)

On the flipside of CDI is Ca^{2+}-dependent facilitation (CDF), a positive feedback regulatory mechanism of VGCCs where further Ca^{2+} entry is increased due to enhanced channel opening. CDF has been observed in $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, and Ca_V3 channels.

The common mechanism underlying CDF in $Ca_V1.2/1.3$ is through the actions of CaMKII [101, 102]. In $Ca_V1.2$, CaMKII becomes tethered to the α1 subunit near the IQ domain, where it is then able to be activated by Ca^{2+}/CaM and subsequently phosphorylating the channel and inducing CDF [103, 104]. CaMKII also induces CDF in $Ca_V1.2$ by phosphorylating β_{2A}, which when bound to $Ca_V1.2$ increases channel open probability, resulting in CDF [105–107].

Like $Ca_V1.2$, CDF of $Ca_V1.3$ is also dependent on CaMKII. However, when $Ca_V1.3$ and CaMKII were co-expressed in HEK 293T cells, no CDF was observed. Only when $Ca_V1.3$, CaMKII, and densin-180, a scaffolding protein that is highly expressed at excitatory synapses and CaMKII-interacting, was CDF elicited [108, 109]. In hippocampal neurons, a long duration form of CDF was found to be dependent on CaMKII and densin-180, and also functionally coupled to $K_{Ca}3.1$, an intermediate-conductance Ca^{2+}-gated potassium channel that is thought to be important in regulating neuronal excitability [110].

CDF of $Ca_V2.1$ is widely studied in the Calyx of Held synapse, where Ca^{2+} currents are largely mediated by $Ca_V2.1$ channels [111]. At this synapse, when high-frequency depolarizations are applied, CDF followed by CDI is observed in $Ca_V2.1$ channel recordings [112]. Efforts to elucidate the molecular mechanisms underlying CDF came in the form of studies overexpressing recombinant $Ca_V2.1$ where it was discovered that like CDI, CDF is also dependent on CaM binding to the IQ domain of $Ca_V2.1$ [81, 93, 94]. As mentioned earlier, the N-lobe of CaM detects increases in global Ca^{2+} and induces CDI in $Ca_V2.1$ channels. The binding of Ca^{2+} to the C-lobe of CaM is largely triggered by local Ca^{2+} increase and results in CDF [81], with single-channel recordings confirming the local Ca^{2+} sensitivity of CaM in inducing CDF [41]. In L-type channels, binding of Ca^{2+} to the C-lobe of CaM results in CDI while in $Ca_V2.1$, CDF is evoked. How is it possible that binding to the same C-lobe results in opposite Ca^{2+} regulation? Crystal structures of Ca^{2+}/CaM bound to the IQ peptide of $Ca_V2.1$ channels revealed that CaM is able to exist in both parallel and antiparallel conformations [43, 98]. The ability of the C-lobe of CaM to bind in an antiparallel conformation upstream of the IQ domain in $Ca_V2.1$, as opposed to a parallel conformation for L-type channels, led to the proposition that this difference in binding conformation underlies the difference in CDF or CDI [98]. Structural analysis of the Ca^{2+}/CaM-$Ca_V2.1$ complex along with alanine scanning mutagenesis and chimeric channel experiments suggested that differences in CaM C-lobe mediated effects could be due to differences in multiple interactions with binding sites within and upstream of the IQ domain. Chimeric channel experiments pointed to an important role for the EF-hand domain, preIQ-domain,

and the IQ domain for CDF to occur, as substitution of any of the abovementioned regions with the corresponding region from $Ca_V1.3$ resulted in a decrease in facilitation [43]. Coincidentally, a recent chimeric channel experiment involving $Ca_V2.1$ and $Ca_V2.2$ also demonstrated the importance of these three domains [42]. Furthermore, alternative splicing of exon 37 of $Ca_V2.1$ results in mutually exclusive splice variants, of which one preserves CDF while the other splice variant does not support CDF [40].

These studies, while mainly centered on the IQ-domain as being critical to Ca^{2+}/CaM regulation of VGCC, also demonstrate the importance of other regions in the vicinity of the IQ-domain, which will be important to consider when investigating the effects of Ca^{2+}/CaM-mediated regulation.

5.2.7.4 $Ca_V2.1$ Channelopathies

Human mutations in $Ca_V2.1$ α_1-subunit result in several neurological disorders in an autosomal-dominant inheritance fashion, such as episodic ataxia type 2 (EA2), familial hemiplegic migraine type 1 (FHM1), and spinocerebellar ataxia type 6 (SCA6) [49, 113]. It must be noted that with advances in RNA sequencing, changes in alternative splicing of $Ca_V2.1$ have been implicated in psychiatric disorders such as autism spectrum disorder (ASD) [114, 115].

5.2.8 Episodic Ataxia Type 2 (EA2)

EA2 is a neurological disorder that commonly affects patients in their childhood and is characterized by paroxysmal attacks of ataxia, vertigo, nausea, and may be accompanied by migraine. Symptoms may last from minutes to days. Emotional and physical stress as well as caffeine and alcohol may trigger attacks. In between attacks, EA2 patients may exhibit a progressive cerebellar syndrome with nystagmus as well as cerebellar atrophy in the vermis [116, 117]. Most of the EA2-related *CACNA1A* mutations reported disrupting the open reading frame resulting in a truncated channel. The EA2 mutations that do not affect the open reading frame are mainly located in the pore-forming loops of the channel [117, 118].

5.2.9 Familial Hemiplegic Migraine Type 1 (FHM1)

Clinically, patients with FHM1 exhibit hemiplegic migraines with an aura that might be accompanied by other symptoms such as nausea and ataxia and may last for hours to weeks. Some FHM1 patients exhibit permanent cerebellar symptoms comprising progressive cerebellar ataxia that might be accompanied by nystagmus [119, 120]. Mutations in *CACNA1A* have been identified as the underlying genetic cause of FHM1 [113]. The majority of the FHM1 mutations in *CACNA1A* affect conserved amino acid residues in critical functional regions of $Ca_V2.1$ such as the pore-lining loop as well as the S4 voltage sensors (see [121, 122] for reviews and references). The functional changes brought about by the FHM1 mutations have been probed in heterologous expression systems and reveal that the mutations bring about a gain-of-function where there is increased Ca^{2+} influx as a result of increased channel open probability and a decrease in voltage activation of the channel [123–125]. A similar result was also observed in knock-in mouse models of R192Q and S218L mice, where there was a larger $Ca_V.2.1$ current density in cerebellar granule cells despite no change in membrane expression of functional $Ca_V2.1$ channels [126, 127].

Interestingly, alternative splicing of *CACNA1A* at exon 47 affects the impact of FHM1 mutations on the channel kinetics. Using three FHM1 mutations, R192Q, S218L, and K1336E, expressed in a $Ca_V2.1$ (+47) and $Ca_V2.1$ (Δ47) background, it was shown that the FHM1 mutations result in different biophysical changes in channel properties depending on which splice variant the mutations are expressed in. Depending on the proportion of splice variants expressed in various neuronal subtypes, the same FHM1 mutations could result in varying degrees of symptoms [128].

5.2.10 Spinocerebellar Ataxia Type 6 (SCA6)

SCA6 is a polyglutamine disease caused by expansion of the trinucleotide (CAG) repeat in exon 47 of the *CACNA1A* gene, with longer expansions resulting in earlier onset of the disease [129]. SCA6 patients have a late onset of mild but slowly progressive cerebellar ataxia affecting the limbs and gait as well as slight vibratory and proprioceptive sensory loss and may be accompanied by nystagmus and dysarthria. Post-mortem examination of the cerebellum revealed a severe loss of Purkinje cells particularly in the vermis, moderate loss of granule cells, dentate nucleus neurons as well as neuronal loss in the inferior olive [49, 130]. In situ hybridization with probes for detecting exons 46–47 of human *CACNA1A* mRNA as well as RT-PCR showed that Purkinje cells had the highest expression of $Ca_V2.1$ protein with an expanded polyglutamine tract and partially explains the selective neurodegeneration observed in the cerebellum of SCA6 patients [131]. Unlike most polyglutamine diseases, SCA6 rarely results in intranuclear inclusions but instead, cytoplasmic aggregates are more commonly observed and were found to be sufficient to result in cell death [132, 133]. It was initially thought that the polyglutamine expansion of $Ca_V2.1$ would affect channel function and subsequently result in cell death [53–55]. However, as discussed earlier, the knockin mouse models of SCA6 showed no differences in $Ca_V2.1$ channel kinetics in cerebellar Purkinje cells [50–52].

Apart from its channel function, another interesting role for $Ca_V2.1$ was discovered. Similar to the C-terminal fragment of L-type channels translocating to the nucleus and functioning as a transcription factor [134], a 75 kDA C-terminal fragment of $Ca_V2.1$ (α1ACT) was found in the nucleus of Purkinje cells and was found to be toxic when the fragment contained the SCA6 polyglutamine expansion [135]. *CACNA1A* was later found to be bicistronic, with an internal ribosomal entry site (IRES) encoding for α1ACT that also served as a transcription factor for a number of genes found to be important for proper Purkinje cell and neural development. When α1ACT contained the SCA6 polyglutamine expansion, its normal gene expression regulation was abolished and resulted in increased cell death [136].

5.2.11 Psychiatric Disorders

Genome-wide association studies (GWAS) have uncovered a strong link between SNPs in *CACNA1C* and psychiatric disorders such as schizophrenia (SCZ), bipolar disorder, and major depressive disorder, which is not entirely unexpected given its high expression in affected brain regions such as the hippocampus [114, 137–141]. $Ca_V2.1$ was however not shown to be linked to any psychiatric disorders in the GWAS studies although some FHM1 and EA2 patients also presented with SCZ symptoms, difficulties in learning, and attention deficit disorder [142]. Interestingly, analysis of transcript isoforms from the PsychENCODE study showed changes in alternative splicing of *CACNA1A* in ASD [114].

5.3 Conclusion

In this chapter, we presented the varied roles in the body that VGCCs are involved in, from the heart to skeletal muscles and the brain. Given its importance, many mechanisms exist to modulate channel function, preventing excitotoxicity due to Ca^{2+} overload. Changes in splice isoforms have also been shown to alter channel kinetics and function and may be a way in which the cell modulates Ca^{2+} entry. Much is still unknown about the combinatorial effects of the splice variants as well as the splice factors responsible for the different splice variant expressions. More work will definitely be required if we wish to modulate VGCCs for therapeutic treatments.

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