Splicing and Editing to Fine-Tune Activity of High Voltage-Activated Calcium Channels

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Abstract

The mRNA transcripts of voltage-gated calcium channels (VGCCs) are subjected to extensive alternative splicing giving rise to a plethora of channel variants with potentially altered biophysical properties to affect physiological functions. Notably, the discovery of *A*-to-*I* RNA editing within the IQ domain of Cav1.3 channel further expands on the post-transcriptional modification processes of VGCCs. Here, we highlight the functional influence alternative splicing and RNA editing has on fine-tuning the activity of High Voltage-Activated (HVA) calcium channels. We will discuss the limitations of employing heterologous expression systems and feature the extensive use of transgenic mouse models in the field, and suggest certain novel approaches that can be applied to further address the physiological consequences of these post-transcriptional modifications of HVA calcium channels.

Keywords

Calcium channel · Alternative splicing · RNA editing · Post-transcriptional modification · Calcium channelopathy

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Generation of mRNA Diversity by *A*-to-*I* RNA Editing and Alternative Splicing

The limited mammalian genome is diversified through dynamic and complex networks of post-transcriptional and post-translational modifications, giving rise to various mRNA transcripts and proteins with subtle to gross changes in structure to alter functions that are essential for adaptation and survival. The most prevalent form of RNA editing in mammals is mediated by ***A**denosine **D**eaminases **A**cting on **R**NA* (ADAR)

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that converts adenosine to inosine (*A*-to-*I*). Of note, *A*-to-*I* RNA editing that occurs within the coding sequence could potentially result in a pinpoint change of the coding of an amino acid in the final protein product. Alternative splicing, on the other hand, is a post-transcriptional process by which the exons of primary RNA transcripts are subjected to combinatorial reassembly resulting in the transcripts and final protein products with various sequences and diverse functions. It is a choreographed process that is catalyzed by a multistep assembly of the spliceosome machinery upon recognition of specific intronic or exonic cis-element within the pre-mRNA. Several mechanisms for alternative splicing exist and they include the utilization of the following: (i) cassette exon – an alternate exon could either be included or excluded; (ii) mutually exclusive exons – one or more adjacent exons are spliced such that only one exon is retained at a time; (iii) different 5′ or 3′ alternative splice acceptor or donor sites allowing for either the lengthening or shortening of a particular exon; (iv) intron retention where an intron is included in the mature mRNA; and (v) alternative promoters or polyadenylation sites.

Splice Variations in High Voltage-Activated Calcium Channels

Here, we will highlight splice variations that have been characterized and demonstrated to alter the electrophysiological or pharmacological properties of the channel or that may affect interaction with cytoplasmic proteins. We will feature splice loci by which alterations in or deregulation of splicing levels may affect physiology or are implicated in disease progression or pathogenesis.

$Ca_V1.1$ Splice Variant in Myotonic Dystrophy

Unlike other VGCCs that have multiple splice variants with diversified functions, $Ca_V1.1$ calcium channel has only one reported alternatively spliced site, and splice variants with either the inclusion or exclusion of exon 29 have been detected in rodent and human skeletal muscles (Tuluc et al., 2009). Exon 29 encodes 19 amino acids of the extracellular linker of domain IV transmembrane segments 3 and 4 (Bannister & Beam, 2013). Exon 29 deficiency in $Ca_V1.1$ channel was reported to induce a hyperpolarizing shift of 30 mV on voltage-dependent activation and an increase in open probability (P_o) as reflected by the increased slope in the plot of tail current amplitudes at the reversal potential against gating currents. As a result, the exclusion of exon 29 increases $Ca_V1.1$ current density by eightfold and triggers stronger skeletal muscle contraction in transfected myotubes of $Ca_V1.1$-deficient skeletal muscle cell line GLT (Tuluc et al., 2009). This finding was then further corroborated by two classical studies on $Ca_V1.1$ splicing: (1) In isolated flexor digitorum brevis (FDB) fibers from mice injected with an antisense oligonucleotide (ASO) that induced nearly complete exon 29 skipping, the voltage for half-maximal channel activation ($V_{0.5}$) had a dramatic 20 mV shift to hyperpolarizing direction and the peak current density was increased by about twofold (Tang et al., 2012). (2) In the FDB fibers isolated from exon 29-null mice, $V_{0.5}$ displayed an even larger shift of about 38.5 mV and the Ca^{2+} influx was also significantly increased during excitation–contraction coupling (Sultana et al., 2016).

Alternative splicing of exon 29 was found to be developmentally regulated. The transcripts of $Ca_V1.1_{\Delta e29}$ channels were reported to represent about 80% of total $Ca_V1.1$ expressed in myotubes, but was significantly shifted to exon 29 inclusion in late fetal and early postnatal development with 100% exon 29 inclusion at postnatal day 30 in mouse hind limb muscles (Tuluc et al., 2009; Flucher & Tuluc, 2011). These data suggest that larger Ca^{2+} currents from embryonic $Ca_V1.1_{\Delta e29}$ splice variant may be required for normal development of embryos and neonates. In contrast, patients with myotonic dystrophy type 1 exhibited the re-introduction of embryonic $Ca_V1.1_{\Delta e29}$ variant, with exon 29 inclusion level reduced to 30% in skeletal muscles from more

than 93% in healthy individuals (Tang et al., 2012). Mechanistically, this switch may be due to altered expression levels of two splicing factors: down-regulation of muscle blind-like 1 (MBNL1) that promotes exon 29 inclusion (Andre et al., 2019) and the up-regulation of CUG-binding protein 1 (CUGBP1) that increases exon 29 skipping (Tang et al., 2012). More importantly, exon 29 skipping induced by an ASO significantly aggravated muscle weakness in mice (Tang et al., 2012), indicating that altered $Ca_V1.1$ function in human myotonic dystrophy may contribute to the exacerbation of myopathy.

Recently another new $Ca_V1.1$ splice variant with exon 29 exclusion and substitution of exons 1 and 2 with five new N-terminal exons was identified in T cells (Matza et al., 2016). This T-cell $Ca_V1.1$ variant was reported to be constitutively open at resting potential in transfected HEK 293 cells (Matza et al., 2016), which may contribute to the death of $Ca_V1.1$-overexpressing cells. As $Ca_V1.1$ channels are essential for T-cell receptors (TCR)-induced Ca^{2+} entry, thus further studies are needed to investigate the electrophysiological properties of this T-cell splice variant and the expression level in TCR-stimulated T cells.

$Ca_V1.2$ Splice Variants in Health and Disease

Exon 9* in Cardiovascular Diseases

The alternatively spliced exon 9* consists of 75 nucleotides downstream of exon 9 and the amino acid sequences encoded by these two exons contribute in part to the intracellular I-II loop of $Ca_V1.2$ channel (Fig. 1) (Liao et al., 2004). Recent studies have shown that the level of exon 9* inclusion is regulated by Rbfox2 and is increased in smooth muscle of hypertensive rat arteries (Zhou et al., 2017) and in cardiac muscles of patients with end-stage heart failure (Papa et al., 2021). The $Ca_V1.2_{e9*}$ channels display altered electrophysiological properties (Liao et al., 2004; Papa et al., 2021) and specific responses to a calcium channel blocker (Zhang et al., 2010), diltiazem, and inclusion of exon 9* restricts the interaction of Galectin-1 with $Ca_V1.2$ channels (Hu et al., 2018; Wang et al., 2011).

Compared to $Ca_V1.2_{\Delta e9*}$ channels, whole-cell patch-clamp recordings demonstrated that human $Ca_V1.2_{e9*}$ channels displayed a hyperpolarized shift in voltage-dependent activation by 9 mV and *I–V* relationships by 11 mV in transfected HEK 293 cells (Liao et al., 2004). Moreover, the cardiomyocytes isolated from rabbit $Ca_V1.2_{e9*}$-expressing transgenic mice showed a significant increase in conductance density with more robust open probability (P_o) of calcium channel, as compared to wild-type cardiomyocytes (Papa et al., 2021). These results suggest that exon 9* inclusion may contribute to larger Ca^{2+} influx, which may explain that exon 9* inclusion level was increased to 11% in cardiac muscles of spontaneously hypertensive rats (SHRs) from 2% in Wistar Kyoto (WKY) rats (Tang et al., 1783), and was also up-regulated to 50.4% in mesenteric arteries of SHR from 40.1% in WKY arteries (Zhou et al., 2017). However, it is noteworthy that there is no direct correlation of mRNA–protein expressions of $Ca_V1.2$ channels in arteries of SHRs (Pratt et al., 2002), and the function of increased exon 9* in hypertensive arteries remains to be investigated. Although we do not know about the protein level of $Ca_V1.2_{e9*}$channels in arteries under hypertensive conditions, a study has reported that an ASO targeting$Ca_V1.2_{e9*}$ channels reduced the maximal contractility by 75% in rabbit cerebral arteries (Nystoriak et al., 2009), suggesting that $Ca_V1.2_{e9*}$ channels play a dominant role in artery.

In addition to disease models, exon 9* was also developmentally regulated in mouse cortex by Rbfox1 and Rbfox2 proteins (Tang et al., 2009). The inclusion level dropped to 4% at embryonic day 18 from 25% at day 12 in mouse cortex, which was mediated by the up-regulated Rbfox1/2 protein as Rbfox1/2 knockdown significantly increased exon 9* inclusion level, while their overexpression suppressed exon 9* inclusion. This study indicates that Rbfox1 and Rbfox2 act as the splicing factors to inhibit exon 9* inclusion. Moreover, Rbfox2 was also reported

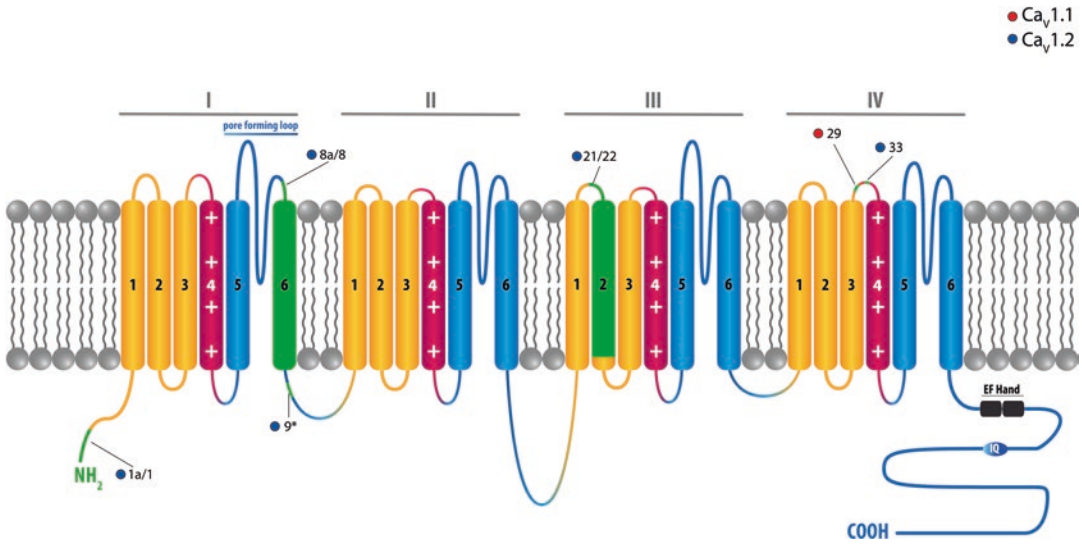

Fig. 1 Schematic diagram showing the positions of alternatively spliced exons in Ca$_V$1.1 and Ca$_V$1.2 channels discussed in this chapter. The only alternatively spliced exon 29 in Ca$_V$1.1 channel is marked as a red dot. Exon 29 exclusion induces a hyperpolarizing shift of 30 mV on voltage-dependent activation and an increase in open probability, and also contributes to the pathogenesis of myotonic dystrophy. The 5 functionally characterized and disease-associated mutually exclusive or alternatively spliced exons (exon 1a/1, 8a/8, 9*, 21/22, 33) of Ca$_V$1.2 channel are marked by blue dots

to regulate exon 9* inclusion in hypertensive arteries (Zhou et al., 2017). However, the up-regulated exon 9* inclusion was correlated to increased Rbfox2 protein in mesenteric arteries of SHR, which may be due to the increased expression of a dominant-negative Rbfox2 isoform lacking half of RNA recognition motif. As the inclusion level of exon 9* was increased in hypertensive arteries and failing hearts, this suggests that the reduced exon 9* level detected in certain rodent and human disease models may recapitulate the embryonal level. Potentially, Cav1.2$_{e9*}$ channels may play an essential role in the adaptation or pathogenesis of other diseases related to organs expressing high levels of Ca$_V$1.2.

Exon 9* is also able to mediate splice variant-selective modulation of Ca$_V$1.2 channel by its binding protein partner or a calcium channel blocker. Galectin-1, a member of β-galactoside-binding protein family (Camby et al., 2006), was reported to negatively modulate Ca$_V$1.2 channel function by interacting with the exon 9 fragment within the Ca$_V$1.2 I-II loop (Wang et al., 2011). However, inclusion of exon 9* completely abolishes the Ca$_V$1.2–Galectin-1 interaction and thereby the inhibitory effects of Galectin-1 on Ca$_V$1.2 channels (Wang et al., 2011). These results suggest that the inhibitory effects of Galectin-1 are specific to Ca$_V$1.2$_{Δe9*}$ channels. The positively charged amino acids in exon 9* could prevent Gal-1 binding to exon 9, or they could interact with the ER export signal that is composed of a few negatively charged residues, thereby attenuating the trafficking of the channels to the surface, mimicking the masking of the ER signal by Galectin-1. Additionally, diltiazem, a non-dihydropyridine calcium channel blocker, also displayed different inhibitory effects on Ca$_V$1.2$_{Δe9*}$ and Ca$_V$1.2$_{e9*}$ channels (Zhang et al., 2010). The IC$_{50}$ of diltiazem for Ca$_V$1.2$_{Δe9*}$ was increased twofold as compared to Ca$_V$1.2$_{e9*}$ channels, suggesting that exon 9* inclusion may contribute to the Ca$_V$1.2 splice isoform-specific effects of diltiazem.

Mutually Exclusive Exons 1a/1 and Exons 8a/8

The expression of N-terminal exons 1a/1 and exons 8a/8 encoding domain I S6 segment of the $Ca_V1.2$ channel displays strong tissue selectivity (Bartels et al., 2018). Exons 1 and 8 are predominantly expressed in smooth muscle, while exons 1a and 8a are the primary combination in cardiac muscle, representing about 80% of cardiac $Ca_V1.2$ mRNA (Hu et al., 2017; Splawski et al., 2005) and exons 1 and 8a are expressed as the major brain isoform (Bartels et al., 2018). A study on transfected HEK 293 cells reported that $Ca_V1.2_{e1}$ channels possessed larger unitary gating current and Ca^{2+} current density as recorded by cell-attached single-channel recording and whole-cell patch-clamp recording, respectively, and higher expression level at cell surface, although exon 1 increased the Ca^{2+}-dependent inactivation (CDI) by more than 70% triggered by local Ca^{2+} sensing by the N-lobe of calmodulin (CaM) (Bartels et al., 2018).

The most well-studied disease associated with mutually exclusive exons 8a/8 is Timothy syndrome (TS), a multisystem disorder with arrhythmia and autism caused by G406R and/or G402S mutations (Splawski et al., 2004, 2005). In the two seminal TS articles, exon 8a (GRCh38/hg38, chr12: 2504436-2504539), upstream of exon 8 (GRCh38/hg38, chr12: 2504842-2504945) in human genomic sequence, was labeled as exon 8, which was labeled differently from the rest of calcium channel community (Hu et al., 2017; Wang et al., 2006; Tang et al., 2011). To avoid confusion, their nomenclature for exon 8 and 8a is still used in this chapter when these papers are cited.

One de novo missense mutation G406R in exon 8a was reported to induce classical Timothy syndrome (TS1) by specific loss of voltage-dependent inactivation (VDI) and thereby sustained depolarization (Splawski et al., 2004). On the other hand, patients with mutations of G402S and G406R within exon 8, associated with atypical Timothy syndrome (TS2), have extreme long QT syndrome and severe mental retardation, and they do not have the cardinal feature of simple syndactyly as in TS1 patients (Splawski et al., 2005). However, a recent study showed that, in addition to VDI, G402S mutation also led to loss of CDI because of a decrease in F_{CDI} (a function of Ca^{2+}), while G406R mutation mainly resulted in a reduction of CDI_{max} (a function of channel gating) (Dick et al., 2016). Intriguingly, further studies in iPSC-derived TS human cardiomyocytes showed that only ventricular-like cardiomyocytes displayed prolonged action potential durations (APDs), whereas both ventricular- and atrial-like cardiomyocytes from patients with long QT syndrome type 1 (LQTS1) had long APD (Yazawa et al., 2011). Moreover, the arrhythmia and delayed depolarizations were spontaneously recorded in beating iPSC-TS cardiomyocytes without any stimulation (Yazawa et al., 2011), while LQTS1 cardiomyocytes needed isoproterenol stimulation. More importantly, roscovitine, a cyclin-dependent kinase inhibitor that promotes VDI in transfected HEK 293 cells, was applied to prevent the Long QT phenotype by shortening the APD in iPSC-derived TS human cardiomyocytes (Yazawa et al., 2011), which provides the possibility of rescuing the cardiac phenotypes of TS patients.

One point to note is that, in addition to the classical G402S and G406R mutations, there have been another 12 missense mutations identified to be related to Timothy Syndrome with highly variable phenotypes (Bauer et al., 2021). As there are hundreds of $Ca_V1.2$ splice isoforms due to various combinations of multiple alternatively spliced exons, some TS mutations may not fully reproduce the G406R phenotypes due to tissue-selective expression of certain $Ca_V1.2$ isoforms with more or less abundance.

Moreover, the $Ca_V1.2_{e8}$ splice variant exhibited higher sensitivity to dihydropyridine (DHP) calcium channel blockers (Lei et al., 2020), which may be the reason why the smooth muscle splice variant $Ca_V1.2b$-expressing exon 8 is more sensitive to inhibition by DHPs than the heart variant $Ca_V1.2a$ containing exon 8a (Liao et al., 2005, 2007).

Alternative splicing of exons 8a/8 has been shown to be regulated by the splicing factor polypyrimidine tract-binding protein 1 (PTBP1).

PTBP1 strongly repressed exon 8a inclusion and shift the spliced exon to exon 8 by direct interaction with the conserved sequence elements upstream of exon 8a in mouse cortex (Tang et al., 2011). Exon 8a was largely inhibited in mouse embryonic brains, whereas it was gradually up-regulated during neuronal development with PTBP1 depletion (Tang et al., 2011). Moreover, PTBP1 protein was highly expressed in embryonic hearts and then dramatically reduced during cardiac development in both rats and mice (Zhang et al., 2009; Martí-Gómez et al., 2020), which may be associated with the dominant $Ca_V1.2_{e8a}$ channels in heart (Liao et al., 2005). Interestingly, PTBP1 protein expression was highly induced in failing hearts from mice subjected to transverse aortic constriction (TAC) or myocardial infarction (Martí-Gómez et al., 2020), suggesting there may be a pattern shift of exon 8a and 8 inclusion in failing and normal hearts. In contrast, PTBP1 protein was significantly down-regulated in mesenteric arteries of SHR as compared to normotensive arteries (Lei et al., 2020), which may also result in a shift from exon 8 to 8a utilization in smooth muscle $Ca_V1.2$ channels under hypertension. These results suggest that the fetal splicing program of exons 8a/8 in both smooth muscles and cardiac muscles may be reactivated under cardiovascular disease conditions, which remains to be further validated in human models and other diseases in $Ca_V1.2$-abundant organs, such as the brain.

Exons 21/22

The mutually exclusive exons 21 or 22 encode a short fragment of the $Ca_V1.2$ domain III segment S2. A nearly complete switch of exon 21 to exon 22 was identified in carotid and femoral arteries of human patients with atherosclerosis (Tiwari et al., 2006), a cardiovascular disorder featured by inflammation-mediated endothelial perturbation in medium- and large-size arteries (Libby et al., 2002). Compared to $Ca_V1.2_{e21}$ channels, the $Ca_V1.2_{e22}$ channels showed hyperpolarized shifts of~15 mV in the *I–V* relationship and ~15 mV in the activation potential as recorded in *Xenopus* oocytes (Tiwari et al., 2006). This study suggests that human atherosclerotic smooth muscle cells may have larger Ca^{2+} influx, which may require Ca^{2+} imaging technique to further validate. However, the $Ca_V1.2$ channels used in this study do contain exon 9* which exists in about half of $Ca_V1.2$ channels expressed in smooth muscles. As such, a more comprehensive understanding of the role of alternative splicing of $Ca_V1.2$ channels in atherosclerosis remains to be investigated. In addition, the splicing factor responsible for this shift of exon 21 to exon 22 utilization has not been identified.

Additionally, a non-functional and developmentally regulated $Ca_V1.2$ splice variant containing both exon 21 and exon 22 in rodent and human hearts, $Ca_V1.2_{e21+22}$, was identified (Hu et al., 2016). Exon 21 + 22 inclusion level was reduced to 5.5% in adult heart from 14.3% in rat neonatal heart, which was then up-regulated 12.5-fold in adult mouse models of heart failure induced by TAC. This reactivation of the fetal splicing pattern is similar to that of exon 9* and exons 8a/8 in heart or smooth muscles. More importantly, co-expression of $Ca_V1.2_{e21+22}$ channels, which had a stronger interaction with β subunits, was able to promote the proteasomal degradation of wild-type $Ca_V1.2$ channels by competing for β-subunits.

Exon 33 and Exon 33L

Exon 33 is composed of 33 nucleotides encoding a portion of the extracellular loop linking domain IV segments 3 and 4. Loss of exon 33 led to hyperpolarized shifts for steady-state inactivation and activation potentials of $Ca_V1.2$ channel in transfected HEK 293 cells (Liao et al., 2009). Moreover, exon 33 inclusion level was reduced in the scar region of ischemic rat hearts subjected to myocardial infarction (Liao et al., 2009). More importantly, in exon 33-deficient mice cardiac contractility and output were significantly increased and ventricular tachyarrhythmia was also recorded due to larger Ca^{2+} influx in cardio-

myocytes (Li et al., 2017a). Additionally, exon 33 inclusion was remarkably up-regulated by about 20% in human failing hearts from patients with ischemic or dilated cardiomyopathy (Li et al., 2017a). Although the molecular mechanisms in mouse failing hearts have been delineated, the exact role that exon 33 plays in the pathogenesis of human heart failure remains unclear. Deleting exon 33 in human patient-specific iPSC-derived cardiomyocytes may be useful for modelling of exon 33-associated heart failure.

Rbfox1/2 were reported to act as the splicing factor to enhance exon 33 inclusion by binding to the downstream UGCAUG elements (Tang et al., 2009). In comparison with the non-failing heart, *Rbfox2* mRNA level displayed no significant differences in both dilated and ischemic human hearts, while *Rbfox1* mRNA levels in dilated cardiomyopathy were significantly reduced, and this was positively correlated with *CACNA1C* mRNA levels and negatively correlated with the exon 33 inclusion level (Wang et al., 2018). This finding was inconsistent with the Rbfox1/2-mediated up-regulation of exon 33 inclusion, and a possible explanation could be compensatory or adaptive responses to heart failure. Similarly, Rbfox2 was found to be up-regulated in hypertensive arteries, which was also negatively correlated with reduced exon 33 inclusion levels (Zhou et al., 2017). This inconsistency in hypertensive arteries may be attributed to the reported increase in expression of a dominant-negative Rbfox2 isoform (Zhou et al., 2017).

Recently, a 66-nucleotide extension downstream of exon 33 was identified, which was named exon 33L. Exon 33L led to a frame-shift, and the production of a non-functional $Ca_V1.2$ truncated short form that arose from premature termination (Liao et al., 2015). Interestingly, exon 33 L inclusion was reduced by more than twofold in left ventricles of adult rat hearts as compared to neonatal hearts. More importantly, exon 33L induced dominant-negative suppression of wild-type $Ca_V1.2$ channel function by increasing the proteasomal degradation of $Ca_V1.2$ channels, but not T-type $Ca_V3.2$ channels, indicating that the inhibitory effects of exon 33L on $Ca_V1.2$ channels may be β subunit dependent. By contrast, the human exon 33L only represents a single-nucleotide insertion, generating a functional full-length $Ca_V1.2$ channel with a much lower Ca^{2+}-conducting ability, which suggests that alternative splicing in exon 33L is species specific. Similar to exons 21/22, the splicing factor for exon 33L is still unknown.

As mentioned above, out of 50 exons in *CACNA1C* gene, in this chapter, 5 major alternative splicing loci (1a/1, 8a/8, 9*, 21/22 and 33) have been introduced, which have been reported to generate about 20 essential $Ca_V1.2$ splice variants with distinctive combinations in aorta or heart of rats. The combinatorial splicing patterns were determined by applying the transcript-scanning method on a mini library of full-length Cav1.2 cDNAs (Tang et al., 1783, 2007). However, a recent study on *CACNA1C* transcript profile of human brain identified 38 novel exons and 241 novel transcripts using long-range PCR and nanopore sequencing, which further substantiates the complexity of $Ca_V1.2$ alternative splicing (Clark et al., 2020). More importantly, the alternative splicing-mediated diversification of $Ca_V1.2$ function also displays a few unique features in tissue selectivity, DHP sensitivity, and reactivation under certain disease conditions. For example, the sensitivity of smooth muscle-specific $Ca_V1.2_{-1/8/9*/\Delta33}$ isoform to nifedipine was increased by 2.4-fold compared to the predominant smooth muscle $Ca_V1.2_{-1/8/9*/33}$ channel and by 9.3-fold compared to the cardiac isoform $Ca_V1.2_{-1a/8a/\Delta9*/33}$ (Liao et al., 2007), which is probably due to the large hyperpolarized shift of in $Ca_V1.2_{-1/8/9*/\Delta33}$ channel of VDI (Liao et al., 2007; Bean, 1984). This may help understand why smooth muscle $Ca_V1.2$ channels are particularly prone to nifedipine block, in addition to the more depolarized resting potential in smooth muscles and exon 8a/8 splicing-induced changes in VDI (Lei et al., 2020; Liao et al., 2007; Li et al., 2017a; Welling et al., 1997). Additionally, the effects of splice isoforms on the regulation of $Ca_V1.2$ channels by post-translational modifications and binding proteins remain to be comprehensively investigated (Loh et al., 2020).

$Ca_V1.3$ in Health and Disease

Among the four L-type channels, $Ca_V1.2$ and $Ca_V1.3$ are ubiquitously expressed in the central nervous system (CNS). The lack of selective blockers of $Ca_V1.3$ channels has hampered the understanding of the physiological roles of the channel. Nonetheless, extensive studies have suggested that as compared to $Ca_V1.2$, $Ca_V1.3$ channels play a significant role in gating low-threshold-activating Ca^{2+} currents that underlie neuronal pacemaking (Chan et al., 2007; Pennartz et al., 2002), excitation–transcription coupling (Wheeler et al., 2008; Zhang et al., 2005, 2006) normal synaptic function (Day et al., 2006; Sinnegger-Brauns et al., 2004), cardiac rhythm (Platzer et al., 2000), and hormone secretion (Marcantoni et al., 2007). It is widely expressed in the CNS, cochlea, sinoatrial node (SAN) of the heart, and in neuroendocrine tissues, including the beta cells of the pancreas and chromaffin cells of the adrenal gland.

The Pathophysiological Roles of $Ca_V1.3$

The $Ca_V1.3$ knockout mouse model discloses a plethora of phenotypic deficits as the $Ca_V1.3$ channels conduct significant inward current at the operating range of the hair cells of the cochlea and the pacemaking cells in SAN due to their low activation threshold (Koschak et al., 2001; Xu & Lipscombe, 2001). Correspondingly, deletion of $Ca_V1.3$ resulted in congenital deafness due to almost complete absence of calcium current (I_{Ca}) in the inner hair cells and degeneration of both outer and inner hair cells (Platzer et al., 2000). In addition, genetic knockout of $Ca_V1.3$ channels impairs the normal development of the auditory brain stem center. As the phenotype appears even before the onset of hearing (Hirtz et al., 2011; Satheesh et al., 2012), it is therefore suggestive that expression of $Ca_V1.3$ channels is essential for the development of both peripheral sensory cells and neurons. Furthermore, $Ca_V1.3^{-/-}$ mice exhibit bradycardia as a result of SAN dysfunction (Platzer et al., 2000). Moreover, in mouse chromaffin cells, the pacemaking $Ca_V1.3$ current drives downstream SK channels activation and allows for sustained action potential firing even with prolonged stressful stimuli (Vandael et al., 2012). Within the CNS, it was shown that $Ca_V1.3$ deletion impaired the consolidation of conditioned fear (McKinney & Murphy, 2006) due to compromised long-term potentiation of the amygdala (McKinney et al., 2009). Notably, by employment of DHP-insensitive $Ca_V1.2$ mice, it was uncovered that acute selective pharmacological activation of $Ca_V1.3$ on the other hand results in depressive-like behavior (Sinnegger-Brauns et al., 2004).

In line with the findings in $Ca_V1.3^{-/-}$ mice, a loss-of-function mutation of human $Ca_V1.3$ was reported in two consanguineous Pakistani families (Baig et al., 2011). The mutation resulted in production of non-conducting $Ca_V1.3$ channels and expectedly subjects homozygous for such mutations suffered from **s**ino**a**trial **n**ode **d**ysfunction and **d**eafness (SANDD) syndrome (Baig et al., 2011), consistent with the phenotypes of $Ca_V1.3^{-/-}$ mice. While the neurological phenotypes of SANDD patients were not known, gain of function mutations of $Ca_V1.3$ channels in humans are associated with autism spectrum disorder (ASD) (Pinggera et al., 2015), aldosterone-producing adrenal adenomas (APAs) (Azizan et al., 2013), and primary aldosteronism, seizures, and neurological abnormalities (PASNA) (Scholl et al., 2013).

The Unique Biophysical and Pharmacological Properties of $Ca_V1.3$ Channels and Modulation

The property of the $Ca_V1.3$ channel is defined by its gating mechanisms. While the low activation threshold appears to be an intrinsic property of $Ca_V1.3$ channels, which is only starting to be understood, a variety of feedback mechanisms that inactivate the channel in response to either voltage-induced conformational change (VDI) or elevation of intracellular [Ca^{2+}] (CDI) have been well characterized. The process of VDI is initiated by the voltage-dependent conformational

rearrangement of voltage-sensing domain comprising S1-to-S4 segments (Swartz, 2008) leading to subsequent opening of the S6 gate (Liu et al., 1997; Xie et al., 2005) and finally the occlusion of the gate by the I-II loop in a "hinge lid" mechanism. Interestingly, a "shield" that repels the closure of the channel gate by the I-II loop "lid" appears to be a unique feature of the $Ca_V1.3$ channel (Tadross et al., 2008) allowing the channel to remain open despite prolonged activation.

In comparison, CDI (calcium-dependent inactivation) is a negative feedback mechanism arising from the influx of Ca^{2+}ions. Calcium ions activate the bi-lobe calcium sensor, CaM, that is pre-associated with the preIQ-IQ domain of the C-terminus of the channel, subsequently triggering a series of conformational changes which lead eventually to channel inactivation (Dick et al., 2008; Erickson et al., 2003; Mori et al., 2004; Peterson et al., 1999; Pitt et al., 2001; Zuhlke et al., 1999). With a combination of technologies, such as patch-clamp electrophysiology and live-cell FRET (live-cell Foerster resonance energy transfer), conformational intermediates of CDI have gradually been elucidated. It is now clear that rather than acting as an effector site of CDI, the preIQ-IQ domain functions as a pre-association domain of apo-calmodulin. Activated CaM upon Ca^{2+} binding switches its binding partners, with its N-lobe binding to an N-terminal spatial Ca^{2+} transforming element (NSCaTE) module on the channel amino terminus and the C-lobe binding to the EF-hand domain on the carboxyl terminus (Ben Johny et al., 2013). Beyond regulating channel inactivation or calmodulation (Ben-Johny & Yue, 2014), pre-association of apo-calmodulin primary to the IQ domain enhances single-channel open probability of $Ca_V1.3$ in response to voltage stimuli, thus explaining the low threshold of activation of the short-form $Ca_V1.3$. Overexpression of CaM in dopaminergic neurons led to the broadening of action potential (Adams et al., 2014). Lastly, strong affinity of CaM to the IQ domain was found to be associated with attenuated sensitivity of the channels towards dihydropyridine (Huang et al., 2013).

Fitting with the diverse functional roles of the channel, the gating of $Ca_V1.3$ channel is often differentially modulated in a tissue-specific manner. The native $Ca_V1.3$ currents in pancreatic β-cells and SAN cells display substantial inactivation (Mangoni et al., 2003; Plant, 1988) matching the profile of $Ca_V1.3$ channels characterized in heterologous systems (Xu & Lipscombe, 2001; Song et al., 2003). In contrast, I_{Ca} recorded from hair cells in cochlea shows little inactivation (Platzer et al., 2000; Song et al., 2003) suitably allowing for persistent cellular activity even in the presence of the prolonged sound stimulus (Shen et al., 2006; Yang et al., 2006). Several mechanisms have been proposed to explain the tissue-specific specialization of $Ca_V1.3$ channels. Taking the cochlea as an example, selective co-localizations of $Ca_V1.3$ channels with various proteins, such as syntaxin, CaBP (calcium-binding protein), and Rab3-interacting molecule (RIM), were observed and co-expression of such proteins with $Ca_V1.3$ heterologous system were shown to slow channel inactivation (Song et al., 2003; Yang et al., 2006; Gebhart et al., 2010). Furthermore, it was shown that PDZ domain-containing protein, erbin, enhanced the $Ca_V1.3$ channel function via direct interaction with the C-terminus of the long-form channel (Calin-Jageman et al., 2007). Tissue-selective manipulation of these protein binding partners in the native system would provide more conclusive evidence of such regulation.

Regulation of α_{1D} Transcripts by Alternative Splicing and A-to-I RNA Editing

The $Ca_V1.3$ channels are subject to extensive alternative splicing, and a total of 16 exons have been reported to be alternatively spliced and some of them show tissue- and even species-specific distribution (Fig. 2). Despite the rich assortment of channel isoforms with possibly different functional characteristics, the functional impact of alternative splicing of the α_{1D} transcript is only partially understood.

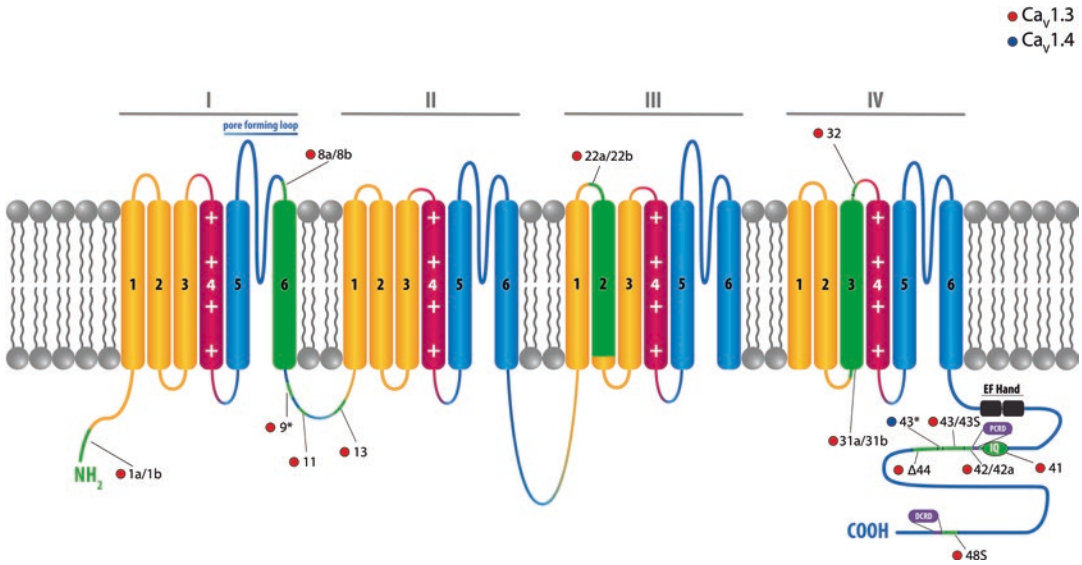

Fig. 2 Alternative splice loci of the $Ca_V1.3$ and $Ca_V1.4$. Segments of the channel colored in green denote the relative location of alternative splice sites in the α1 subunit. Red circles are used to indicate $Ca_V1.3$ splice loci; blue circles are used to indicate $Ca_V1.4$ splice loci

(a) *Alternative Splicing Pattern Within N-terminal Domain*

Alternative splicing of the amino terminus (N-terminus) was known to affect the current density of $Ca_V1.3$ channels (Klugbauer et al., 2002; Xu et al., 2003). Inclusion of either exon 1a (Hui et al., 1991; Seino et al., 1992; Williams et al., 1992a) or 1b (Klugbauer et al., 2002) has been reported in mouse. Exon 1b appears to be mouse specific, while in rat and human, exon 1a is constitutively expressed. Although both splice variants support functional currents with similar gating properties in heterologous expression system, exon 1a confers much larger current density as compared to exon 1b (Klugbauer et al., 2002; Xu et al., 2003).

(b) *Alternative Splicing Pattern Within the Repeat Domains*

The IS6, IIIS2, and IVS3 segments of $Ca_V1.3$ are encoded by three pairs of mutually exclusive exons, including exons 8a/8b, 22a/22b, and 31a/31b, respectively. Interestingly, $Ca_V1.2$ channels display the same splicing patterns in the abovementioned regions, and relatively high sequence conservation was observed between $Ca_V1.3$ and $Ca_V1.2$ channels in these three pairs of mutually exclusive exons (Tang et al., 2004). It is noteworthy that the exon nomenclature employed by many labs are different and this often causes confusion in comparing results from various papers. For instance, exon 8a occurs upstream of exon 8b and was named as such in papers from Joerg Striessnig's group (Koschak et al., 2001; Baig et al., 2011; Pinggera et al., 2015; Pinggera & Striessnig, 2016), while they were named as 8a and 8, respectively, in papers from Tuck Wah Soong's group (Liao et al., 2005; Huang et al., 2013; Tang et al., 2004). For the sake of consistency, they are termed 8a and 8b in the current discussion.

Alternative splicing in IS6, IIIS2 and IVS3 segments of $Ca_V1.2$ were known to alter the sensitivity of the channels towards DHP inhibition with exons 8, 22, and 31, conferring higher drug sensitivity (Liao et al., 2005). In contrast, mutually exclusive splice variants 8a/8b and 31a/31b do not dramatically alter the pharmacological properties of $Ca_V1.3$ channels (data no shown) when expressed in HEK293FT cells. In addition, exon 22b of $Ca_V1.3$ appeared to be expressed specifically in the rat organ of Corti with unknown

functional role, while exon 22a is constitutively expressed in other tissues (Ramakrishnan et al., 2002). The alternate exon 32 encodes part of the extracellular loop between IVS3 and IVS4. Inclusion or exclusion of exon 32 in $Ca_V1.3$ channels has no effect on the gating properties of the channel and neither was sensitivity towards nitrendipine significantly changed (Xu & Lipscombe, 2001).

Notably, a number of disease-associated mutations were found to occur either in exon 8a or 8b. For example, the insertional mutation that results in loss of function of human $Ca_V1.3$ channel is located in exon 8b[61].The ASD-linked mutation G407R only occurs in exon 8a[62], while G403D was identified in either exons 8a or 8b in APAs but was present only in exon 8b in PASNA (Azizan et al., 2013; Scholl et al., 2013). While dominant in heart tissue, exon 8b is included in almost 60% of rat brain (unpublished data). Therefore, understanding the tissue-specific distribution of exon 8a and 8b in different brain tissues could have profound implications for prognosis and possible target treatment of any neurophysiological disorders for patient suffering from mutations within this pair of mutually exclusive exons.

Given that $Ca_V1.3$ splice variants containing either exon 8a or 8b do not display distinct biophysical and pharmacological properties, it is therefore conceivable that understanding how exons 8a and 8b are regulated could contribute towards designing novel therapeutic strategy of treating the abovementioned diseases arising from mutations in either exon 8a or exon 8b. Interestingly, binding of splicing factor polypyrimidine tract-binding protein (PTB) in the intronic regions upstream of exon 8a was found to cause skipping of exon 8a of $Ca_V1.2$ (Tang et al., 2011). Of note, siRNA-mediated knockout of PTB leads to inclusion of exon 8a and developmental down-regulation of PTB correlated with up-regulation of exon 8a. Discovery of a similar mechanism that regulates the alternative splicing of 8a/8b in $Ca_V1.3$ would be highly relevant. Masking the binding of the splicing factors by using ASO could be employed to manipulate the level of alternative splicing of exon 8a/8b relative to each other and therefore restore the normal channel function in the case of gain of function mutation or rescue of loss-of-function mutation in SANDD.

(c) *Alternative Splicing Pattern Within the I-II Loop*

The I-II loop of $Ca_V1.3$ contains three splice variations, including alternate exon 9*, 11, and 13. Exon 9* (Ramakrishnan et al., 2002) and 13 (Ihara et al., 1995) were identified in the rat organ of Corti and pancreas, respectively, with uncharacterized functional impact. On the other hand, exon 11 is more ubiquitously expressed in brain and pancreas and deletion of exon 11 was found not to affect the channel gating of $Ca_V1.3$ (Xu & Lipscombe, 2001). Inclusion of exon 9* introduces 26 amino acids into the I-II loop of the Cav1.3 channels. Sequence of exon 9* in chicken $Ca_V1.3$ contains a consensus sequence of serine surrounded by four basic amino acid residues and is therefore a potential substrate for protein kinase (Ramakrishnan et al., 2002). In contrast, no such consensus site was found in exon 9* of rat or human $Ca_V1.3$ (Ramakrishnan et al., 2002).

(d) *Alternative Splicing and A-to-I RNA Editing Within the C-terminus*

The carboxyl-terminus (C-terminus) of $Ca_V1.3$ represents another hotspot of alternative splicing that has been more extensively characterized. Of note, exon 41 encodes the IQ domain and truncation of exon 41 due to the alternative use of splice acceptor site in exon 41 results in complete removal of the IQ domain and early termination of the C-terminus (Shen et al., 2006). Although functional currents could still be recorded when this splice variant ($Cav1.3_{\Delta IQ}$) was expressed in HEK293 cells, deletion of IQ domain resulted in complete elimination of CDI (Shen et al., 2006). Selective localization of $Cav1.3_{\Delta IQ}$ channels in cochlear outer hair cell (Shen et al., 2006) was hypothesized to partially underlie the previous observation of slowly inactivating native $Ca_V1.3$ currents recorded in hair cells. Again, this result highlights the

tissue-specific role of such splice isoforms in supporting normal function, in this case, of the cochlea. Moreover, exon 41 could also behave as a cassette exon. $Ca_V1.3$ transcript without the entire exon 41 has been reported in both rat and human brain (Bock et al., 2011; Tan et al., 2011). Deletion of exon 41 results in complete elimination of the IQ domain, leading to frame-shifting and early truncation of the C-terminus. Expectedly, $Ca_V1.3_{\Delta 41}$ shows much lower current density and much slowing of CDI (Tan et al., 2011) given the important role of IQ domain as a pre-association site for apo-calmodulin. Notably, the expression level of $Ca_V1.3_{\Delta 41}$ was very low as detected by RT-PCR assay (Bock et al., 2011; Tan et al., 2011).

Interestingly, a more recent study identified three closely spaced *A*-to-*I* RNA editing sites in the mRNA sequence which codes for tetrapeptide "IQDY" in the IQ domain (Huang et al., 2012). The editing is mediated by ADAR2, an isoform of the family of enzymes known as ADARs. Codon changes from ATA to ATG, CAG to CGG, and TAC to TGC result in amino acid changes from I to M, Q to R, and Y to C, respectively, giving rise to a total of 8 possible combinations of editing in the IQ domain. Peptide variants containing various edited amino acids in IQDY could also be detected by mass spectrometry. Biophysically, IQ→MR change arising from RNA editing resulted in the edited $Cav1.3_{MR}$ channels exhibiting slowed CDI, owing to the decreased binding affinity of apo-calmodulin. Expectedly, overexpression of wild-type CaM in substantia nigra pars compacta (SNc) neurons could effectively compensate for this decreased in binding affinity by mass effect to up-regulate CDI from a modest CDI recorded in native SNc neurons (Bazzazi et al., 2013). Physiologically, editing in the IQ domain is shown to regulate normal rhythmic firing activity of neurons in the suprachiasmatic nucleus, a hypothalamic region well known for its role as the master control of biological clock in mammalian system. Most importantly, RNA editing in the IQ domain is restricted to the CNS and is evolutionarily conserved across species from mouse and rat to human (Huang et al., 2012). More recently, the molecular mechanism of *A*-to-*I* editing of the exon 41 has been elucidated whereby exon 41 was found to form a RNA duplex structure with an upstream intronic sequence termed Editing site Complementary Sequence (ECS). Notably, editing can be repressed by a splicing factor, Serine and Arginine-Rich Splicing Factor 9 (SRSF9). Down-regulation of SRSF9 in neurons appeared to correlate with tissue-selective editing of $Ca_V1.3$ in various CNS tissues (Huang et al., 2018).

Further downstream, alternative use of either exon 42 or 42a gives rise to long-form (LF) or short-form (SF) $Ca_V1.3$ channels, respectively (Singh et al., 2008). The stop codon in exon 42a results in expression of only 6 amino acids immediately after exon 41 and therefore the early termination of C-terminus. Although both variants are ubiquitously expressed in the brain, the LF $Ca_V1.3_{e42}$ channels display distinctive properties, such as more depolarized shift in window current, higher expression, lower current density, and significantly diminished CDI (Singh et al., 2008). The attenuated CDI in the long form was later explained by the presence of the distal C-terminal autoinhibitory domain (DCRD) at the distal carboxyl terminal which interacts intramolecularly to the proximal C-terminal autoinhibitory domain (PCRD) and leads to displacement of apo-calmodulin from binding to the IQ domain (Singh et al., 2008; Liu et al., 2010). The weakened binding between apo-calmodulin and $Ca_V1.3_{e42}$ channel therefore results in much slower channel inactivation, while the absence of the PCRD domain in the SF$Ca_V1.3_{e42a}$ variant due to the early termination of C-terminus leads to fast CDI and much larger current density (Adams et al., 2014).

Moreover, half truncation of exon 43 due to the alternative use of splice site within exon 43 results in a frame-shift and early termination of the C-terminus (Seino et al., 1992; Bock et al., 2011; Tan et al., 2011; Williams et al., 1992b). The $Ca_V1.3_{e43s}$variant represents the most dominant C-terminal splice variant and could be detected in diverse brain regions in human (Bock et al., 2011). As expected, the exclusion of PCRD domain in such a splice isoform would support

rapid CDI similar to that of short-form $Ca_V1.3_{e42a}$ channels. However, it is worth mentioning that splice variants with premature stop codons are often considered faulty and subjected to degradation via mRNA surveillance mechanisms, such as nonsense-mediated mRNA decay (NMD). Briefly, the exon junctions of the mature mRNA are decorated with a protein complex known as Exon Junction Complex (EJC) after alternative splicing. During the first-round translation, ribosomes scan the mRNA and remove the EJC and the process continues towards the last exon. However, the presence of premature stop codon arrests the ribosome and if the unremoved EJC is located 50 nt downstream of the ribosome, the cell recognizes this transcript as aberrant and removes it via NMD (Jopling, 2014). By coupling alternative splicing to NMD, it is possible that a cell could functionally down-regulate expression of that gene under desired conditions by mediating alternative splicing. However, it remains to be tested if manipulation of exon 43 splicing could affect the expression level of $Ca_V1.3$ and in turn affect neuronal activity in a native system.

Lastly, deletion of exon 44 and use of a splice acceptor site within exon 48 result in shortening of C-terminus but do not result in early truncation of the channel. Interestingly, both $Ca_V1.3_{\Delta e44}$ and $Ca_V1.3_{e48s}$ variants display only slightly slower CDI as compared the channel with intact C-terminus (Tan et al., 2011).

Apart from regulation of biophysical and pharmacological properties, truncations within the C-terminus in variants, such as $Ca_V1.3_{\Delta e41}$, $Ca_V1.3_{e42a}$, and $Ca_V1.3_{e43s,}$ have additional functional implications. Firstly, early truncation of the C-terminus effectively excludes two consensus sites for PKA activity. The two sites, identified using mass spectrometry, include serine 1743 and serine 1816 located on exon 43 (Ramadan et al., 2009). Phosphorylation of $Ca_V1.3$ channels by PKA is known to substantially increase $Ca_V1.3$ current which potentially contributes to the sympathetic control of heart rate (Qu et al., 2005). The C-terminal alternative splicing of the α_{1D} transcripts, particularly in SAN, could therefore regulate the responsiveness of heart rate to the regulation by activation of β-adrenergic receptors via cAMP-dependent PKA. Secondly shortening of $Ca_V1.3$ C-terminus omits C-terminal **S**rc **h**omology **3** (SH3) domain-binding motifs and **p**ostsynaptic **d**ensity-95/discs large/**z**ona occludens-1 (PDZ) binding motif which is crucial for interaction with the scaffold protein Shank (Zhang et al., 2005). Such interaction results in postsynaptic clustering of long-form $Ca_V1.3$ channels and was later found to be important for processes, such as $Ca_V1.3$-dependent phosphorylated cAMP response element-binding protein (pCREB) signaling (Zhang et al., 2005) and G-protein modulation of $Ca_V1.3$ channels by D2 dopaminergic and M1 muscarinic receptors (Ohlson et al., 2007). In addition, the PDZ binding motif of the $Ca_V1.3$ channel is also known to interact with PDZ domain-containing protein, erbin. The association of erbin with long-form $Ca_V1.3$ results in voltage-dependent facilitation of the current (Calin-Jageman et al., 2007).

$Ca_V1.4$ in Health and Disease

Among the four L-type channels, expression of $Ca_V1.4$ encoded by the gene *CACNA1F* is restricted to the photoreceptor of the eye whereby its current supports the release of glutamate from photoreceptor synaptic terminals in darkness, a process that is critical for vision. Antibodies to $Ca_V1.4$ label dominantly mammalian rod terminals, suggesting that $Ca_V1.4$ is the principal subtype in rods. Mutations in the channel were found to be associated with X-linked incomplete congenital stationary night blindness (xlCSNB) (Strom et al., 1998).

Structure–Function Relationship Learnt from Human Mutations and Alternative Splicing Patterns

In terms of biophysical properties, $Ca_V1.4$ channels display low ionic conductance and slow channel inactivation (Singh et al., 2006). Truncation of the entire C-terminus downstream

of IQ domain in the xICSNB-linked K1591X mutation leads to enhanced CDI and hyperpolarizing shift in activation. The enhanced channel inactivation was deemed ill-suited for the role of $Ca_V1.4$ current to support tonic release of neurotransmitter in the photoreceptor and was thought of as the basis of pathogenesis in such xICSNB patients (Singh et al., 2006).

Patch-clamp electrophysiological characterization of K1591X was critical in the identification of C-terminal regulatory domain (CTM) that regulated many biophysical properties, such as channel open probability, channel inactivation kinetics, and voltage gating of the channels (Singh et al., 2006). A similar finding was published by Wahl-Schott et al., in the same year and the similar sequence was named as ICDI in their paper (Wahl-Schott et al., 2006). Subsequently, the molecular mechanism was further dissected to reveal that the interaction of DCRD with PCRD leads to the displacement of apocalmodulin from the IQ domain (Singh et al., 2006, 2008; Liu et al., 2010). Curiously, such a mechanism appeared to be highly conserved among the three L-type channels inclusive of $Ca_V1.2$, $Ca_V1.3$, and $Ca_V1.4$ owing to high sequence conservation among the three channel subtypes.

The transcript of $Ca_V1.4$ was reported to undergo extensive alternative splicing (Haeseleer et al., 2016; Tan et al., 2012) (Fig. 2). Notably, inclusion of novel exon 43*in between exon 43 and exon 44 leads to the truncation of C-terminal after PCRD domain as a result of a stop codon within the exon 43*. Remarkably, $Ca_V1.4_{e43*}$ occurs at frequency of 13.6% among all the $Ca_V1.4$ transcripts expressed in human retina. Expectedly, the $Ca_V1.4_{e43*}$ variant displays hyperpolarizing shift inactivation, enhanced CDI, and increased current density, hallmarks of lack of CTM. Of interest, co-expression of the ICDI peptide that spans the DCRD domain suppressed CDI and caused a depolarizing shift in the activation potential (Tan et al., 2012). Additional splice variants of $Ca_V1.4$ have been reported. For example, the exclusion of exon 47 leads to in-frame deletion of 66 amino acid sequence in the C-terminus which overlaps with the proximal portion of the DCRD domain. Remarkably the $Ca_V1.4_{\Delta e47}$ variant displays hyperpolarizing shift in the voltage dependence of activation, enhanced CDI, and increased current density, suggestive that the integrity of the DCRD domain is essential for its function.

The Ca_V2 Channel Family

$Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$ are VGCCs, encoded by *CACNA1A*, *CACNA1B*, and *CACNA1E*, respectively, belonging to the Ca_V2 channel family. They are highly expressed in the presynaptic terminals of the central nervous system where they play a major role in neurotransmitter release (Gasparini et al., 2001; Mintz et al., 1995; Wu & Saggau, 1994). When an action potential invades the presynaptic terminal, Ca_V2 VGCCs open in response to membrane depolarization, allowing for Ca^{2+} influx which eventually results in the release of neurotransmitters across the synaptic cleft.

Pathophysiological Roles of Ca_V2 Channel Family

One can gain a deeper understanding of the function of each Ca_V2 channel isoform by examining Ca_V2 channel knockout mouse models. For example, $Ca_V2.1$-null mice display ataxia, and they exhibit absence seizures and die within 4 weeks after birth (Jun et al., 1999). Although $Ca_V2.1$ is the major Ca_V2 channel isoform at the neuromuscular junction, $Ca_V2.1$-null mice were not paralyzed presumably due to compensation by $Ca_V2.2$ and $Ca_V2.3$ (Jun et al., 1999; Urbano et al., 2003). Purkinje cell-specific postnatal conditional knockout of $Ca_V2.1$ (PC-$Ca_V2.1$ KO) recapitulated the ataxic phenotype observed in $Ca_V2.1$-null mice, but PC-$Ca_V2.1$ KO mice survived past 4 weeks (Todorov et al., 2012), which is not entirely unexpected given the cerebellum's role in motor control and $Ca_V2.1$ being the major

Ca_V2 channel isoform expressed in PCs (Stea et al., 1994).

In contrast to $Ca_V2.1$-null mice, $Ca_V2.2$-null mice have a normal life span albeit being accompanied by impairments in blood pressure control (Ino et al., 2001), are less anxious, and have decreased hyperalgesia and allodynia in an inflammatory context (Saegusa et al., 2001). The resultant phenotypes due to $Ca_V2.2$ deficiency could be attributed to $Ca_V2.2$ being highly expressed in the glomerular primary afferent terminals of the spinal dorsal horn, an important component of the pain pathway, where $Ca_V2.2$ is vital for neurotransmitter release (Nieto-Rostro et al., 2018).

$Ca_V2.3$-null mice are also viable and have decreased responses to inflammatory pain (Saegusa et al., 2000), and have deficits in second-phase insulin release (Jing et al., 2005). Chemical induction of seizures in $Ca_V2.3$-null mice revealed a decreased susceptibility to hippocampal seizures (Weiergraber et al., 2007). Ablation of $Ca_V2.3$ in mice also resulted in altered hippocampal theta oscillations (Muller et al., 2012).

Alternative Splicing in Ca_V2 Channel Family

Much like the Ca_V1 channel family, alternative splicing occurs extensively in the Ca_V2 channel family, generating various Ca_V2 variants that differ in channel electrophysiological and/or pharmacological properties as well as localization and expression (Fig. 3).

Of the different Ca_V2 channel isoforms, $Ca_V2.1$ has the most reported splice loci, seven in humans and 10 in mice (Bourinet et al., 1999; Soong et al., 2002; Allen et al., 2010). A total of eight splice loci were identified in rodent $Ca_V2.2$ (Lipscombe et al., 2002), and three splice loci were identified in rodent $Ca_V2.3$ (Fig. 3) (Schneider et al., 2020). As the functional consequences of each splice locus are not fully understood, only splice loci that have known functional consequences will be discussed in the following sections.

(a) *Alternative Splicing Within II-III Loop*

Exon 18a of $Ca_V2.2$ is a cassette exon nested between exons 18 and 19 which encodes 21 amino acids within the cytoplasmic II-III loop (Fig. 3). Alternative splicing of exon 18a results in the inclusion or exclusion of 18a in $Ca_V2.2$ mRNA (Pan & Lipscombe, 2000). Expression levels of e18a-containing $Ca_V2.2$ mRNAs vary in different parts of the CNS and is more highly expressed in adult rat tissue as compared to newborn rats (Pan & Lipscombe, 2000; Gray et al., 2007). When $Ca_V2.2_{e18a}$ channels were co-expressed with β_1 and β_4 subunits, they were observed to be protected from entering into closed-state inactivation (Pan & Lipscombe, 2000; Thaler et al., 2004).

A 57-nucleotide exon was discovered to be nested within a 6221 bp intron of human $Ca_V2.3$ genomic DNA, which was also previously identified from previous studies involving $Ca_V2.3$ mRNA (Gray et al., 2007; Pereverzev et al., 1998; Williams et al., 1994). Although similar in size and position to e18a of $Ca_V2.2$, e18a of $Ca_V2.3$ encodes a distinct nucleotide sequence, with only 25% homology to $Ca_V2.2_{e18a}$[132]. The $Ca_V2.3_{e18a}$ channels were found to be more sensitive to Ca^{2+}-dependent modulation, where accumulation of $[Ca^{2+}]_i$ resulted in increased current density, slowed inactivation, and increased recovery from inactivation (Leroy et al., 2003; Pereverzev et al., 2002). $Ca_V2.3$ channels that include e18a were shown to be more stimulated by phorbol esters (Klockner et al., 2004).

The $Ca_V2.2_{e18a}$ mRNAs were found to be more highly expressed in the adult brain, spinal cord, and peripheral ganglia (Pan & Lipscombe, 2000; Gray et al., 2007), while $Ca_V2.3_{e18a}$ mRNAs were more highly expressed in fetal brains (Gray et al., 2007). What this reciprocal expression pattern means physiologically remains to be discovered as the effects of $Ca_V2.2_{e18}$ are dependent on $Ca_V\beta$ subunit expression. To better understand this,

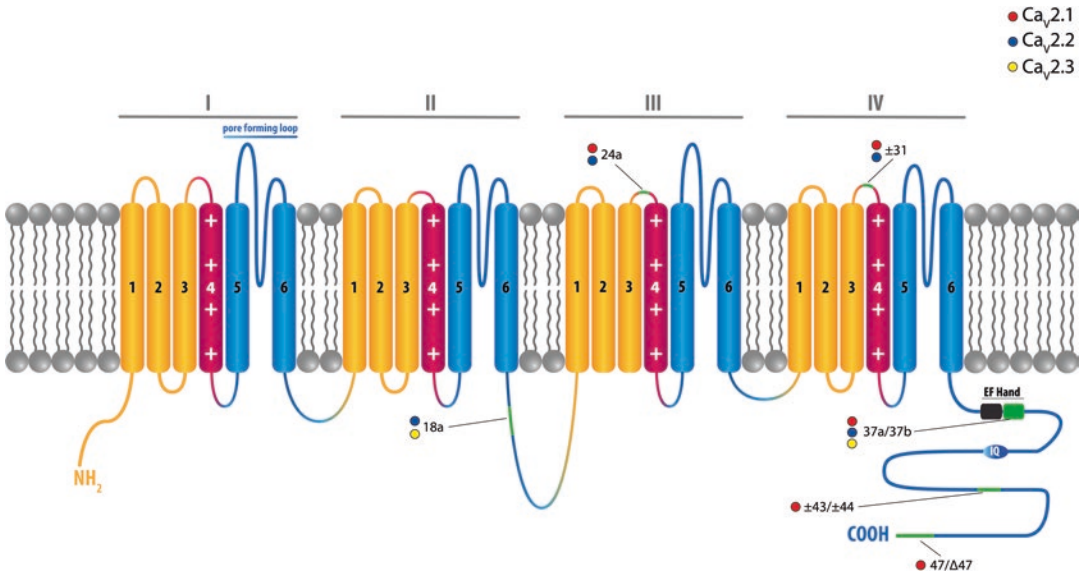

Fig. 3 Alternative splice loci of the Ca_V2 family. Segments of the channel colored in green denote the relative location of alternative splice sites in the α1 subunit. Red circles are used to indicate $Ca_V2.1$ splice loci, blue circles are used to indicate $Ca_V2.2$ splice loci, and yellow circles are used to indicate $Ca_V2.3$ splice loci

future studies will need to move away from bulk tissue expression and adopt a more targeted cell-specific expression study, such as single-cell RNA-seq. Previous studies involving the use of genome-wide analysis identified a Rbfox2 binding site upstream of e18a in *Cacna1b* (Weyn-Vanhentenryck et al., 2014; Gehman et al., 2012). This was confirmed by Lipscombe and colleagues where they showed that Rbfox2 represses e18a inclusion in $Ca_V2.2$ mRNAs (Allen et al., 2017).

(b) *Alternative Splicing Within Extracellular Linkers of III and IV*

E24a was first identified in *Cacna1b* cDNA obtained from rat sympathetic ganglia where it was found to encode four amino acids (SFMG) in the IIIS3-IIIS4 extracellular linker (Lin et al., 1997). Subsequent efforts to identify the functional consequence of e24a inclusion seem to suggest that e24a inclusion has minimal influence on $Ca_V2.2$ channel properties (Allen et al., 2010). In comparison to $Ca_V2.2$, $Ca_V2.1_{e24a}$ was discovered much later by Lipscombe and colleagues where it was also found to encode four amino acids (SSTR) in the IIIS3-IIIS4 extracellular linker (Fig. 3). Given its relatively recent identification, the effect of e24a inclusion on $Ca_V2.1$ channel properties remains to be determined (Allen et al., 2010).

Exon 31 in both $Ca_V2.1$ and $Ca_V2.2$ (e31a for $Ca_V2.2$) encode two amino acids, NP in $Ca_V2.1$ [126] and ET in $Ca_V2.2$ (Lin et al., 1999), in the IVS3-IVS4 extracellular linker. Inclusion of NP in $Ca_V2.1$ results in decreased affinity for ω-Aga-IVA and the slowing of channel activation and deactivation kinetics (Bourinet et al., 1999; Soong et al., 2002). While both human and rat $Ca_V2.1$ splice variants have NP inclusion, the mechanism by which NP is included or excluded is different. In the human variant, NP is encoded by a mini-exon of six nucleotides (AATCCG) in the middle of GT/AG acceptor–donor sites within intron 31. On the other hand, the presence of an alternative 5′ splice donor site at the 3′ end of exon 31 allows for the inclusion of NP in the rat variant (Bourinet et al., 1999). Interestingly, the inclusion of ET in $Ca_V2.2$ in the rat variant is similar to how NP is included in the human $Ca_V2.1$ isoform. In rat *Cacna1b*, a six-base cassette exon (GAAACG) flanked by GT/AG acceptor–donor sites is located within intron 31 (Lin

et al., 1999). Insertion of ET in IVS3-IVS4 of $Ca_V2.2$ causes the channel to activate at a slower rate (Lin et al., 1999).

The $Ca_V2.1$ mRNA transcripts containing e31 have been shown to be expressed in many regions of the brain and are the predominant variant in the cerebellum (Bourinet et al., 1999; Soong et al., 2002). On the other hand, $Ca_V2.2$ mRNA transcripts containing e31a were shown to be expressed at very low levels in the brain, but more highly expressed in the spinal cord (Lin et al., 1999).

Darnell, Lipscombe, and colleagues have identified Nova-2 to be the splice factor that regulates alternative splicing of e24 and e31 in *Cacna1a* and *Cacna1b*. In the context of e24, Nova-2 binds to the YCAY motif in introns downstream of e24 Ca_V2 pre-mRNA to enhance its inclusion, while binding of Nova-2 to the YCAY motif in introns upstream of e31 Ca_V2 pre-mRNA promotes the exclusion of e31 (Allen et al., 2010).

(c) *Alternative Splicing in C-terminal Region*

Exon 37

Exons 36 and 37 code for an EF-hand-like motif in the C-terminal region of Ca_V2 channels (Fig. 3). Alternative splicing of exon 37 is conserved in $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ generating mutually exclusive splice variants, EFa and EFb (Gray et al., 2007).

Electrophysiological characterization of $Ca_V2.1$ EFa and EFb splice variants in a heterologous overexpression system showed that while both splice variants had similar channel kinetics, only $Ca_V2.1$ EFa splice variants exhibited Ca^{2+}-dependent facilitation (CDF). Subsequent single-channel recording experiments demonstrated CDF of EFa splice variants at a single-channel level where it was shown to be due to an increase in channel open probability and channel open duration, while EFb splice variants were shown to be locked in a normal gating mode (Chaudhuri et al., 2007). While the critical amino acids required for CDF have not been identified, chimeric channel experiments comprising $Ca_V2.1$ EF-hand region and the pre-IQ-IQ domain of $Ca_V1.3$ and $Ca_V2.2$ demonstrated the necessity of both the EF-hand region and the pre-IQ-IQ domain of $Ca_V2.1$ for CDF to occur (Thomas et al., 2018; Mori et al., 2008).

While the physiological significance for EFa/EFb splice variants remains unknown, a reciprocal expression pattern of EFa/EFb splice variants in the developing rodent cerebellum has been observed. In the developing cerebellum, from the embryonic stage to P5, EFb was the dominantly expressed in the cerebellum. A developmental switch to an EFa dominant expression pattern was subsequently observed from P7 onwards (Chang et al., 2007; Chaudhuri et al., 2004), while recent RNA-seq data demonstrated that this switch occurred between P1 and P10 (Farini et al., 2020). Interestingly, this switch overlaps with the maturation timeline of the cerebellum, suggesting that the switch in splice variant expression could be vital for proper cerebellar circuitry maturation (Kano et al., 2018).

The $Ca_V2.1$ EFa/EFb splice variants have been found to play a role in homeostatic regulation of presynaptic plasticity in hippocampal neurons. Upon application of toxins or drugs to reduce hippocampal network activity, hippocampal neurons were found to selectively up-regulate $Ca_V2.1$ EFa splice variants, the isoform with higher synaptic efficacy, allowing for the regulation of presynaptic homeostatic plasticity (Thalhammer et al., 2017).

Similar to $Ca_V2.1$, alternative splicing of e37 of $Ca_V2.2$ mRNA results in the generation of mutually exclusive splice variants, e37a and e37b. Splicing of $Ca_V2.2_{e37}$ was first identified in various regions of the adult rat nervous system and it was later shown that $Ca_V2.2_{e37a}$ mRNA was only selectively expressed in the dorsal root ganglion, and preferentially within a subset of capsaicin-responsive neurons, while $Ca_V2.2_{e37b}$ mRNA was widely expressed throughout the CNS (Bell et al., 2004). Subsequent electrophysiological characterization of the $Ca_V2.2_{e37a}$ and $Ca_V2.2_{e37b}$ splice variants showed that the $Ca_V2.2_{e37a}$ variant had a higher membrane expression level, opened at slightly more hyperpolarized membrane potentials, had a longer open time, and is slower to deactivate and inactivate,

resulting in a larger macroscopic Ca^{2+} current when compared to cells that only expressed $Ca_V2.2_{e37b}$ variant (Bell et al., 2004; Castiglioni et al., 2006). Differences in AP-1 binding motifs, [DE]xxxL[LI] and Yxxφ, between $Ca_V2.2_{e37a}$ and $Ca_V2.2_{e37b}$ splice variants, were demonstrated to underlie the difference in membrane trafficking of the e37 splice variants, with $Ca_V2.2_{e37a}$ being the more efficiently trafficked splice variant (Macabuag & Dolphin, 2015). Given that there is a difference in channel property among $Ca_V2.2$ e37a/e37b, and that $Ca_V2.2_{e37a}$ is differentially expressed in neurons involved in the pain pathway, functional consequences relating to pain processing have been demonstrated.

Exon 43/44

Alternative splicing of exons 43 and 44 of $Ca_V2.1$ results in a combination of splice variants where either exon can be present or absent, giving rise to four possible variants (±43/±44) (Fig. 3). Electrophysiological characterization of these splice variants revealed changes in CDI and Ca^{2+} current amplitude. The $43^-/44^-$ splice variant was shown to have the greatest degree of CDI and also produced the largest Ca^{2+} current amplitude (Soong et al., 2002). As CDI is affected by global increases in Ca^{2+}, it was not surprising that $43^-/44^-$ splice variant had the greatest CDI due to it having the largest Ca^{2+} current amplitude.

Exon 47

Insertion of GGCAG, a pentanucleotide, at the beginning of $Ca_V2.1$ exon 47 causes an in-frame translation of exon 47, producing a long variant of the C-terminus that contains a polyglutamine (polyQ) tract (Fig. 3). Absence of GGCAG in splice variants results in a frameshift, generating a stop codon near the beginning of exon 47, resulting in a shorter isoform of the channel (Δ47) (Soong et al., 2002; Zhuchenko et al., 1997). While $Ca_V2.1$ variants containing the polyQ tract do not have any changes in channel properties, the long form of $Ca_V2.1$ with the polyQ tract has been linked to spinocerebellar ataxia type 6 (SCA6), with longer repeats of the polyQ tract found in patients with an earlier onset of the disease (Ishikawa et al., 1997). Knock-in animal models of SCA6 mutants also demonstrated no observable differences in $Ca_V2.1$ expressed in Purkinje cells (Aikawa et al., 2017; Saegusa et al., 2007; Watase et al., 2008).

In healthy control brains, it was demonstrated via in situ hybridization for the long variant of $Ca_V2.1$ mRNA containing the CAG repeat that $Ca_V2.1_{e47}$ was most highly expressed in Purkinje cells as compared to other brain regions (Ishikawa et al., 1999). This would partially explain why selective degeneration of the cerebellum is observed in SCA6 patients and not other brain regions.

It was first discovered that the C-terminus of α1A subunit (α1ACT) contained the polyQ tract and could exist as a stable fragment of 75kDA in cultured cells and Purkinje cells (Kubodera et al., 2003; Kordasiewicz et al., 2006). The α1ACT fragment was identified to possess nuclear localization signals, allowing it to be enriched in Purkinje cell nuclei (Kordasiewicz et al., 2006). α1ACT fragments containing the expanded polyQ tract were found to be toxic when localized to the nuclei of cultured cells and neurons (Kubodera et al., 2003; Kordasiewicz et al., 2006; Ishiguro et al., 2010).

The discovery of an internal ribosomal entry site (IRES) in the *CACNA1A* mRNA was later found to be responsible for the generation of α1ACT from full-length α1A mRNA (Du et al., 2013). α1ACT was also shown to be a transcription factor with the start of its amino acid sequence within the IQ-like domain of $Ca_V2.1$ (Du et al., 2013). Normal WT α1ACT is non-pathological and is in fact required for proper development and survival of cerebellar Purkinje cells (Du et al., 2019). Only when α1ACT contains exon 47 with a pathological expansion of the polyQ tract that the fragment becomes deleterious (Kordasiewicz et al., 2006).

Novel Approaches to Uncover the Pathophysiological Consequences of Alternative Splicing or Gene Mutations of Ion Channels: Moving Away from Heterologous Systems

Ca^{2+}ions are second messengers that are involved in many signaling pathways in the cell. Hence,

tight coupling of Ca_V2 channels to neurotransmitter release machinery has been observed at the presynaptic terminal (Eggermann et al., 2011). Alternative splicing generates many different variants of Ca_V2 channels that affect channel kinetics and possibly neurotransmitter release. It will thus be beneficial to know the subcellular localization of each splice variant and determine how they affect neurotransmitter release. However, given that certain splice variants, such as $Ca_V2.1$ EFa/EFb, can be highly similar in sequence, generating antibodies that are able to distinguish the two isoforms can be extremely challenging. Nanobodies generated by commercial companies have been shown to be specific for the detection of a single amino acid change between two proteins and could possibly be one solution to detecting highly similar splice variants. Newer techniques, such as single-cell labeling of endogenous proteins by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-mediated homology-directed repair (SLENDR), could be used to label a protein of interest with an epitope tag (Mikuni et al., 2016). This could be further combined with knockout of a specific splice variant and by comparing the difference between the wild-type and splice variant knockout, one could deduce the localization of the deleted splice variant.

In addition, many of the ion channel mutations and alternative splice variants discussed thus far have been studied in heterologous overexpression systems, such as in HEK293 cells, and expressing only specific cDNA clones of the channel. Although valuable knowledge regarding the structure–function relationship could be gleaned from such analysis, many aspects of RNA regulation were often missed or ignored. Taking $Ca_V1.3_{e43s}$ and $Ca_V1.4_{e43*}$ variants as examples, both transcripts contain the premature stop codon which could subject the mRNA transcripts to be degraded by NMD as discussed previously. If indeed true, such variants would not be expressed as functional proteins in native cells, such as neurons or photoreceptors, respectively, but instead serve to tune the expression level of the WT channels under certain physiological conditions. This also applies to nonsense mutation K1519X of $Ca_V1.4$. While the enhanced CDI of the mutant was proposed to explain the functional deficit of the channel in photoreceptors, it awaits to be tested if aberrant transcripts containing such mutation are simply degraded resulting in the deficiency of $Ca_V1.4$ channel protein in the presynaptic terminal. Such information would be highly relevant to design targeted treatments for xICSNB; rather than focusing on slowing down the inactivation kinetics of the channel, overexpression of the WT channel would potentially help ameliorate the symptoms.

To overcome such shortcomings, many approaches could be taken involving transgenic mouse models whereby cassette exons could be deleted (Andrade et al., 2010; Li et al., 2017b) or mutations could be knocked in (Wu et al., 2011). Alternatively, human-induced Pluripotent Stem Cells (hiPSCs) could be engineered by CRISPR-Cas9-mediated genome editing and subsequently differentiated to either cardiomyocytes or neurons to study their physiological roles. It is noted that while such approaches provide a clean system for the functional role of alternative splicing to be studied, alternative splicing and RNA editing are often developmentally and tissue-selectively regulated events. Complete abolishment of such RNA regulation may result in unwanted side effects and exaggerate the interpretation the physiological consequences of such RNA processing. To further address such issues, RNA-binding ASOs could be employed to either disrupt formation of RNA duplex structure to reversibly abolish A-to-I RNA editing mediated by ADAR2 or mask the binding of splicing factor to its cis-element within pre-mRNA sequence so as to cause splice switching. Such ASO could be modified at the backbone, replacing the phosphodiester bond with phosphorothioate (PS) linkages to be nuclease resistance and at the base with 2'-O-methoxyethyl (2'-MOE) 2'-O-methyl (2'OMe) to prevent the activation of RNase H (Roberts et al., 2020). Overcoming the challenges of effective and cell-specific delivery of oligonucleotides may eventually provide a better approach to facilitate the analysis of many of these RNA

processing mechanisms, such as A-to-I RNA and alternative splicing discussed here.

Summary

Alternative splicing and RNA editing are exquisite post-transcriptional mechanisms to expand calcium channel structures to potentially optimize function in development and for tissue-selective purposes. In this regard, careful consideration of combinatorial assortment of the various splice sites and the cellular context of the expression of the splice variations in health and disease may contribute towards better understanding of the genotype–phenotype relationship, especially with regard to mutation-causing disorders. As alternative splicing and RNA editing could be dynamically regulated, the search for the cues and upstream signals that govern these processes will be of scientific and clinical interests. With the advent of sophisticated technologies to visualize cellular localization of transcripts, to interrogate protein–protein interactions and to analyze massive single-cell transcriptomics and proteomics, the possibility to identify splice variant-selective interactors in specific cellular milieu in health and disease, together with the knowledge of altered channel biophysical properties, will probably help answer the question on why a cell expresses so many splice variants.

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