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# Structural and functional differences between L-type calcium channels: crucial issues for future selective targeting

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**Within the family of voltage-gated calcium channels (VGCCs), L-type channels (L-VGCCs) represent a well-established therapeutic target for calcium channel blockers, which are widely used to treat hypertension and myocardial ischemia. L-VGCCs outside the cardiovascular system also control key physiological processes such as neuronal plasticity, sensory cell function (e.g. in the inner ear and retina) and endocrine function (e.g. in pancreatic beta cells and adrenal chromaffin cells). Research into L-VGCCs was stimulated by the discovery that the known L-VGCC isoforms ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$) possess different biophysical properties. However, no L-VGCC-isoform-selective drugs have yet been identified. In this review, we examine $Ca_V1.2$ and $Ca_V1.3$ isoforms at the level of genetic structure, splice variants, post-translational modifications and functional protein coupling. We discuss candidate $Ca_V1.2$- and $Ca_V1.3$-specific characteristics as future therapeutic targets in individual organs.**

## Introduction

Voltage-gated calcium channels (VGCCs) serve as key transducers coupling changes in cell surface membrane potential with local intracellular calcium ($Ca^{2+}$) pathways. Among the three families of VGCCs, L-type $Ca^{2+}$ channels (L-VGCCs), include four different isoforms of the $\alpha1$ pore-forming subunit ($Ca_V1.1$ to $Ca_V1.4$; see Glossary) that are uniquely sensitive to organic $Ca^{2+}$ channel blockers (Box 1).

Although expression of $Ca_V1.1$ and $Ca_V1.4$ is mainly restricted to skeletal muscle and the retina, respectively, the $Ca_V1.2$ and $Ca_V1.3$ isoforms display differing and shared expression patterns in many tissues, including neurons and other electrically excitable tissues (heart, smooth muscle), sensory (inner ear, retina) and endocrine (pancreatic beta, adrenal chromaffin) cells [1].

Because L-VGCCs are not all equally well blocked by dihydropyridines (DHPs, Box 1), no highly selective channel modulators are available at present [1]. $Ca_V1.2$ and $Ca_V1.3$ possess different biophysical properties but share significant sequence homology [1]; thus the identification of isoform-specific targets for drug development is

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### Glossary

**$Ca_V1$**: $\alpha1$ pore-forming subunit of L-type voltage gated $Ca^{2+}$ channels (L-VGCCs), encoded by *CACNA1*.
**Subthreshold voltage**: a voltage range that is more negative than the activation voltage for a predominant depolarizing mechanism.
**Alternative splicing**: mechanism that operates by combinatorial inclusion or exclusion of alternatively spliced exons, insertion of one or more base pairs or alternative 3′ and 5′ ends.
**$Ca^{2+}$-dependent inactivation (CDI)**: $Ca^{2+}$-dependent intrinsic feedback mechanism that limits its own $Ca^{2+}$ influx.
**Voltage dependent activation and inactivation**: L-VGCC mechanisms that respond to membrane depolarization by conformational changes, controlling channel opening and closing.
**C-terminal gating modulator (CTM)**: intrinsic gating modulator located in the C terminus of L-VGCCs. CTM prevents or reduces CDI and shifts the activation voltage range to more positive potentials.
**Spontaneous firing and pacemaking**: defined as the capability of a given cell type or tissue to generate action potentials by its own intrinsic electrical properties, without the need for an external physiological trigger.
**Upstate potential**: also known as upstate transition, indicates the period during which a neuron (or an excitable cell) can fire single or bursts of action potentials.
**Scaffold proteins**: although not strictly defined in function, they interact and/or bind with multiple members of a signaling pathway, tethering them into complexes.
**Single nucleotide polymorphism**: a DNA sequence variation occurring when a single nucleotide in the genome differs between members of a species or paired chromosomes in an individual.
**Inotropism**: the intrinsic property of the heart to develop a mechanical force, such as contraction. Drugs, neurotransmitters and ion channels can increase or decrease contractility, leading to positive or negative inotropism, respectively.

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**Box 1. The present state of calcium channel blockers pharmacology**

There are three principal classes of high-affinity $Ca^{2+}$ channel blockers targeting L-VGCCs: the 1,4-dihydropyridines (DHPs), such as nifedipine; the benzothiazepines such as diltiazem; and the phenylalkylamines, typified by verapamil [1,91].

Some of the isoform specificity of the DHPs is achieved by virtue of their selective binding to the inactivated state of L-VGCCs [92], which means that there is a greater blockade of channels in cells and tissues with a depolarized resting membrane potential (e.g. vascular smooth muscle), and for channel isoforms showing more rapid inactivation [92]. For this reason, DHPs are more effective at reducing vascular resistance than cardiac contractility and are thus extensively used for the treatment of hypertension. By contrast, $Ca^{2+}$ channel blocking by verapamil is less voltage dependent, making it relatively selective for myocardial $Ca^{2+}$ channels, and thus effective in the treatment of angina pectoris and as an anti-arrhythmic drug [92].

Several features of $Ca_V1$ channels explain why there are not more side effects of these drugs, despite the fact that all the isoforms can bind DHPs and given the essential roles of $Ca_V1.1$ in skeletal muscle contraction, $Ca_V1.3$ in hearing and $Ca_V1.4$ in vision [4]. $Ca_V1.3$ and $Ca_V1.4$ show a lower affinity for blocking by DHP blockers than does $Ca_V1.2$ [4]. Furthermore, $Ca_V1.1$ normally functions as a voltage sensor in skeletal muscle contraction (rather than conducting any significant amount of current), and voltage sensor movement in $Ca_V1$ channels has not been found to be affected by DHPs [93].

challenging. Recently, radioreceptor assays highlighted small differences in the binding pockets of $Ca_V1.2$ and $Ca_V1.3$ that could allow development of isoform-selective modulators [2]. L-VGCC blockers are currently used only in the treatment of cardiovascular diseases (Box 1). Studies of mouse models and genetic disorders have provided information on the essential roles of L-VGCCs in various organs [3,4]. This suggests that targeting L-VGCCs could be therapeutically valuable in a wide range of diseases. For example, L-VGCC genetic defects lead to structural aberrations within their pore-forming $\alpha1$ subunits, causing serious diseases (channelopathies) such as hypokalemic periodic paralysis and malignant hyperthermia sensitivity ($Ca_V1.1$), incomplete congenital stationary night blindness (CSNB2; $Ca_V1.4$), Timothy syndrome ($Ca_V1.2$, characterized by fatal arrhythmia and multiorgan dysfunction) [3,5], and sinoatrial node (SAN) dysfunction and deafness syndrome (SANDD, $Ca_V1.3$) [6].

In light of the distinct functions of $Ca_V1.2$ and $Ca_V1.3$ in different organs, we here summarize the currently known structural and functional variations of these two isoforms at the level of sequence information, alternative splicing, interacting proteins and signaling cascades. With this review, we aim to draw attention to putative isoform-selective drug targets.

## $Ca_V1.3$ contribution to spontaneous firing and pacemaking

L-type $Ca^{2+}$ currents have long been neglected as potential pacemaker currents because their threshold for activation was considered to be too positive to support spontaneous firing activity [7]. This paradigm changed with the finding that the voltage range for the activation and inactivation of $Ca_V1.3$ currents at physiological $Ca^{2+}$ concentrations occurs at relatively negative voltages [4]. Since then, the role of L-VGCCs in pacemaking and spontaneous activity has been investigated with increased interest. $Ca_V1.3$ has now been shown to drive pacemaking in dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) [8,9], SAN tissue [10], chromaffin cells [11] and immature inner hair cells [12]..

### *Genetic and molecular basis of the role of $Ca_V1.3$ in spontaneous firing and pacemaking*

Much effort has been invested into examining the differences in sequence information between $Ca_V1.2$ and $Ca_V1.3$ channels in attempts to determine the mechanisms underlying the more hyperpolarized activation voltage and the slower inactivation of $Ca_V1.3$. The isoforms share approximately 75% sequence homology, with higher variability in the II–III loop (~65% homology, Figure 2a) and in the C-terminal tail (~55% homology, Figure 2b). Singh *et al.* [13] described an alternatively spliced form of $Ca_V1.3$ that used either exon 42 to encode the full C terminus (long $Ca_V1.3_{42}$ splice variant) or exon 42A to encode a shorter stretch of the C terminus (short $Ca_V1.3_{42A}$ splice variant) (Figure 1b and 2b). The short $Ca_V1.3_{42A}$ splice variant lacks the C-terminal modulator domain (CTM, Figure 2b).

The CTM typically reduces $Ca^{2+}$-dependent inactivation (CDI) and shifts the activation voltage to more positive potentials [13]. Deletion of the CTM from the short $Ca_V1.3_{42A}$ splice variant results in more negative window currents [13] and might therefore contribute to $Ca^{2+}$ entry at (subthreshold) voltages even more negative than with the long splice variant. Whereas activation at more negative voltages of the less expressed $Ca_V1.3_{42A}$ could trigger pacemaking, faster CDI would limit $Ca^{2+}$ entry during action potentials and prolonged interspike depolarizations. This factor is important in cells that are especially susceptible to $Ca^{2+}$-induced toxicity, such as SNc neurons [8]. The long $Ca_V1.3_{42}$ splice variant, because of its slower CDI and greater expression *in vivo*, might be the main contributor to pacemaking or spontaneous firing activity, whereas the short $Ca_V1.3_{42A}$ splice variant might be important for the fine tuning of firing frequency [13]. However, the $Ca_V1.3_{42A}$ splice variant shows robust ionic currents, and comparative quantitative expression studies in single cells are necessary to support this hypothesis, in the context of future drug targeting.

The long $Ca_V1.3_{42}$ splice variant activates at more negative voltages and its voltage-dependent inactivation (VDI) is slower and less complete than that of $Ca_V1.2$, independent of auxiliary subunits [4]. This is considered to have important functions for pacemaking [14] and for the modulation of transcriptional or post-transcriptional events, as a result of the generation of different $Ca^{2+}$ microenvironments (see below). The molecular basis for differences in channel activation between $Ca^{2+}$ channel family members remains obscure, but some distinct splice events are regarded as candidate determinants of this peculiar gating property of the $Ca_V1.3$ long isoform in comparison to $Ca_V1.2$.

First, a splicing event in exon 9, present in the intracellular I–II loop of both isoforms (Figure 1) indicates sequence information for $Ca_V1.3$ activation at more negative voltages within this domain [15,16]. It was previously found that

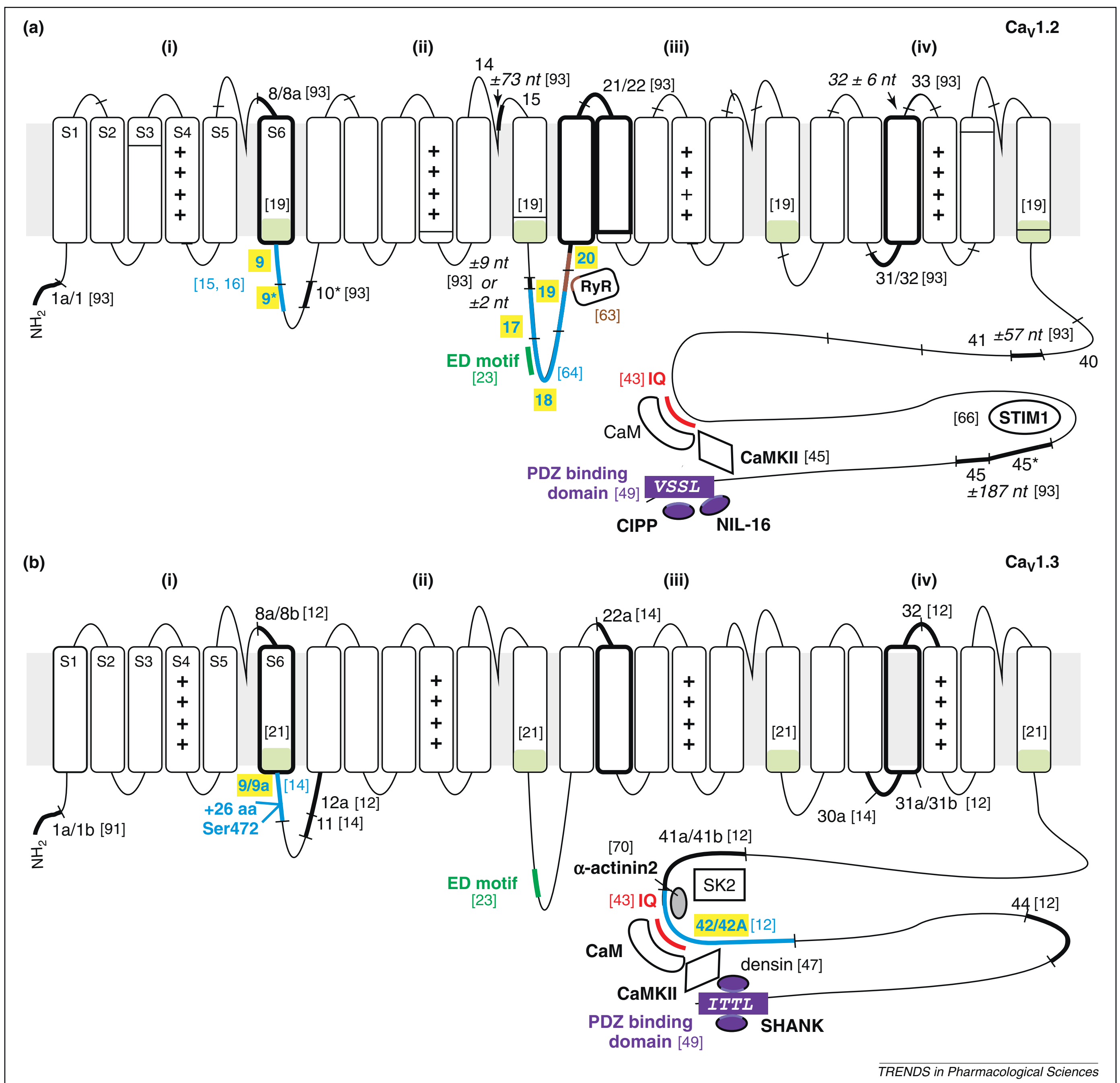

**Figure 1.** Comparison of $Ca_V1.2$ and $Ca_V1.3$ isoforms: from structure to function. Predicted membrane topology of the $\alpha1$ subunit of (**a**) $Ca_V1.2$ and (**b**) $Ca_V1.3$. The subunit comprises four homologous domains, I–IV, each containing six transmembrane segments (S1–6) and a pore region between segments S5 and S6; segment S4 in each domain has a net positive charge. Identified splice variants are shown in black or blue [94]. Variants described in this review that feature possible differences between $Ca_V1.2$ and $Ca_V1.3$ are highlighted in blue (with yellow boxes). Light-green boxes indicate voltage sensitive regions. Specific interaction partners of $Ca_V1.2$ and $Ca_V1.3$ are shown schematically.

deletion of exon 9 in $Ca_V1.2$ leads to a hyperpolarizing shift of voltage-dependent activation in smooth muscle [16]. $Ca_V1.3$ can also be spliced in exon 9, as shown in cochlear hair cells, in which a $Ca_V1.3$ variant contains a 26 residue insert in the I–II loop (exon 9a, Figure 1b) [15]. This insertion introduces at least one serine residue (Ser472) that is surrounded by four basic residues and is therefore a potential substrate for protein kinases. Given that the I–II loop binds to the auxiliary beta subunit, which can also modulate the voltage dependence of activation and inactivation [17], this splice form could be considered to be a $Ca_V1.3$-specific variant with altered channel gating properties.

Second, pore-forming S6 segments also contribute to channel activation and inactivation properties. Studies of several point mutations in the $Ca_V1.2$ S6 segments have been performed by Hering and colleagues to analyze the impact of structural changes in the pore region on steady state channel activation and kinetics [18]. Hydrophobic alterations in the region of pore-forming S6 segments often shift activation curves along the voltage axis in the hyperpolarization direction, approaching that of $Ca_V1.3$, and affect the kinetics of channel gating [18–20]. It was recently shown through a 'mutation correlation analysis' that there are significant links between activation gating and physi-

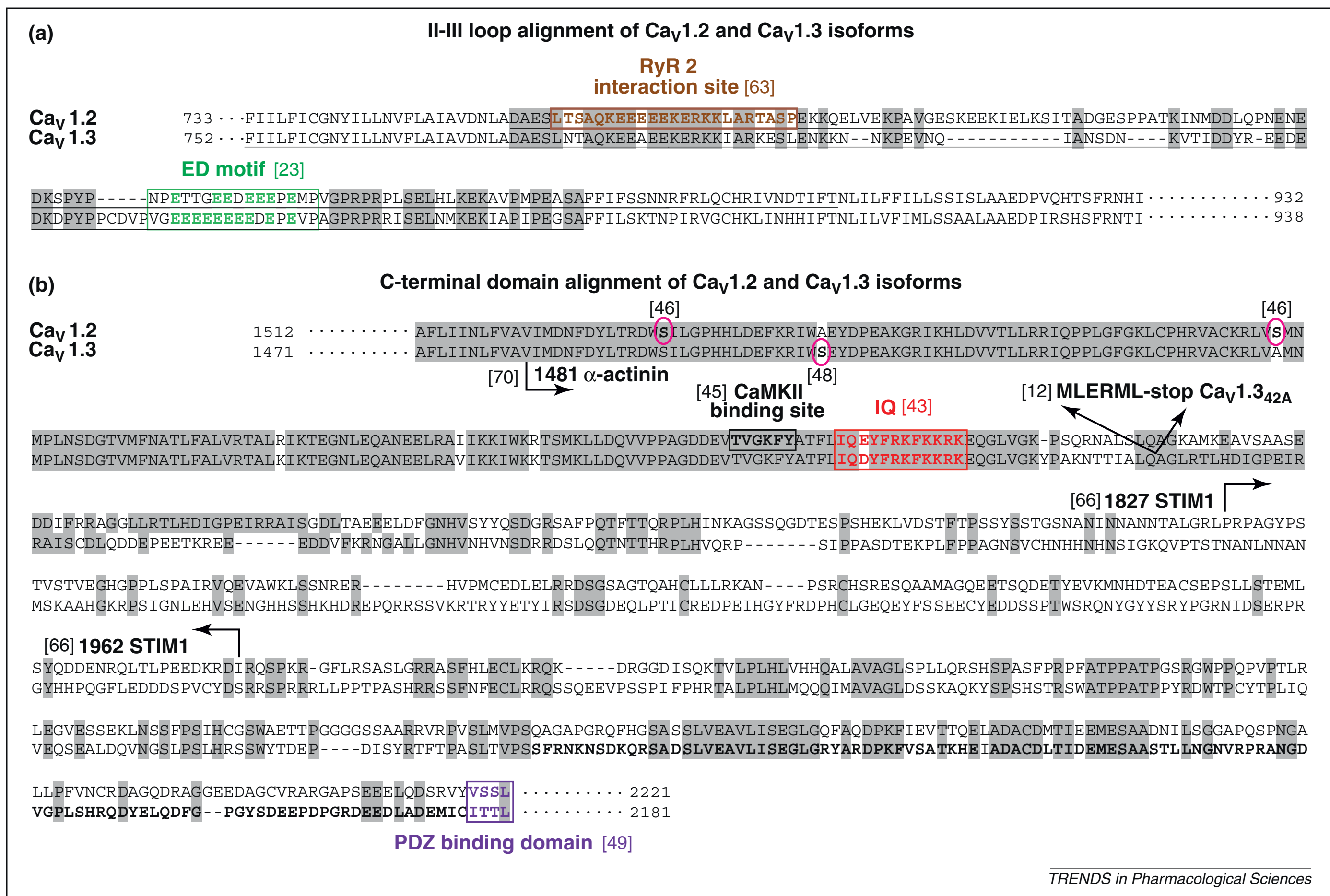


**Figure 2.** Alignment of highly variable sequences of $Ca_V1.2$ and $Ca_V1.3$ isoforms. (**a**) Sequence alignment of human $Ca_V1.2$ (UniProt accession number Q13936) and $Ca_V1.3$ (GenBankTM accession number NP_000711.1) II–III loops and (**b**) partial C-terminal domain. The position of the stop codon in the short $Ca_V1.3_{42A}$ splice variant and the putative α-actinin2 and STIM1 binding regions are indicated by black arrows. Gray box, sequence identity; -, gaps; brown box, RyR2 interaction site; green box, ED motif; purple circle, CaMKII phosphorylation residues; black box, CaMKII binding sites; red box, IQ domain; bold, CTM; violet box, PDZ binding domain motif (after [13]).

cochemical properties in positions Leu434 (IS6), Iso781 (IIS6) and Gly1193 (IIIS6) [20] (Figure 1a).

Third, how the sensor triggers channel inactivation remains under discussion. A number of pore mutations in S6 show similar strong effects on VDI and CDI in both isoforms [21,22]. It has been proposed for both isoforms that a 'hinged lid' mechanism, in which the loop between domains I and II plugs the channel thereby regulating VDI [22,23]. However, for $Ca_V1.3$, a hinged-lid scheme with an additional 'shield' that repels lid closure has been shown to be a unique specialization enabling $Ca_V1.3$ channels to remain open despite sustained activation [22].

A highly conserved poly ED motif (Glu/Asp cluster) among VGCCs has been shown to accelerate VDI if mutated in $Ca_V1.2$, thereby shifting channel inactivation to hyperpolarizing voltages [24]. This motif is located in both isoforms in the II–III loop (Figures 1 and 2a) but contains 10 negatively charged amino acids in $Ca_V1.3$ and only seven in $Ca_V1.2$. This sequence difference might have a functional impact on the slower inactivation kinetics of $Ca_V1.3$ compared to $Ca_V1.2$ [24].

In conclusion, $Ca_V1.3$-specific structural features defining channel gating properties should be investigated in more detail, especially in tissues where $Ca_V1.3$ is responsible for pacemaking and spontaneous firing.

### *$Ca_V1.3$ contribution to pacemaking in different tissues*

Pharmacological and molecular studies have shown that the regular pacemaker activity of DA neurons in the SNc depends on L-VGCCs [14]. It has been shown that micromolar concentrations of DHPs silence neuronal activity in SNc slice preparations [25,26], in which DA neurons express principally $Ca_V1.3$ [27]. However, direct evidence of $Ca_V1.3$ (rather than $Ca_V1.2$) involvement in pacemaking in SNc DA neurons was obtained only recently from studies using $Ca_V1.3$ knockout (KO) mice [8]. It is important to stress that the $Ca_V1.3$-dependent pacemaker activity of DA neurons has been linked to their vulnerability and cell death in Parkinson's disease [8]. A switch from exon 42 to 42A could result in a neuroprotective effect, because the 42A splice variant would accelerate the CDI and therefore limit $Ca^{2+}$ influx, with a consequent positive impact on $Ca^{2+}$ neurotoxicity. However, a drug able to switch the splice variant from 42 to 42A might cause bradycardia, because the 42A isoform is likely to initiate rather than sustain the pacemaker activity.

In the mammalian heart, three major structures possess automaticity and are able to drive the heartbeat: the SAN, which constitutes the physiological pacemaker, the atrioventricular node (AVN) and the Purkinje fibers [10]. Both $Ca_V1.3$ and $Ca_V1.2$ isoforms are expressed in SAN

and AVN and could participate in pacemaking by different mechanisms [10]. Pronounced bradycardia and SAN arrhythmia are seen in $Ca_V1.3$ KO mice, which can be considered a first genetic indication of the importance of $Ca_V1.3$ for pacemaker activity in the heart [10].

A human genetic mutation, in a highly conserved, alternatively spliced region near the channel pore (IS6) of $Ca_V1.3$, was identified recently [6]. This mutation generates a non-conducting $Ca^{2+}$ channel with abnormal voltage-dependent gating associated with pronounced SAN dysfunction [6].

Further investigation of splice variant expression in the SAN, AVN and myocytes could improve future pharmacological targeting of those channels. This would be supported by a recent study describing alternatively spliced $Ca_V1.2$ channels that can selectively modify diltiazem sensitivity in the heart and blood vessels [28].

Adrenal chromaffin cells fire spontaneously, and their activity is associated with the basal release of catecholamines. Although both $Ca_V1.2$ and $Ca_V1.3$ are expressed in rodent chromaffin cells, genetic deletion of $Ca_V1.3$ decreases the number of firing cells, reinforcing the crucial role of $Ca_V1.3$ for pacemaking [11]. Expression of the long $Ca_V1.3_{42}$ splice variant has been shown to prevail over that of $Ca_V1.3_{42A}$. Because of its slower CDI, the long $Ca_V1.3_{42}$ splice variant is thought to remain open for prolonged periods and contribute significantly to the long lasting subthreshold depolarization, bringing the cell to the spike voltage threshold [11].

In the mammalian cochlea, the inner hair cells (IHCs) are the primary sensory receptors [12]. In the immature pre-hearing stage, $Ca_V1.3$ is responsible for the generation of spontaneous action potentials in IHCs, which are thought to control the remodeling of immature synaptic connections within the cochlea [12]. Transient spiking before the onset of hearing and the ongoing expression of presynaptic $Ca_V1.3$ for the release of glutamate from IHCs in adults probably require differential expression of $Ca_V1.3$ splice variants, which need to be elucidated.

$Ca_V1.3$ is reported to be necessary for postnatal beta cell mass expansion in mice [29], but spiking has not been investigated in this process. Although often underestimated, pancreatic beta cells also fire action potentials when the blood glucose load is low; that is, at glucose concentrations that match the physiological resting (or preprandial) condition [30]. Given that human beta cells never rest (baseline potential –55 to –50 mV) and fire spontaneously in this state, $Ca_V1.3$ might be the most probable candidate for those events [31], considering its predominant expression in human beta cells [32]. Beta cells express different subtypes of VGCCs in a species-dependent manner [33], which is an issue to be considered regarding drug safety. Using an array of different KO mouse models, several studies have shown that insulin release in the murine beta cell depends principally on $Ca_V1.2$ [4,34], whereas studies undertaken in rats suggest that $Ca_V1.3$ is the major L-VGCC involved [35,36], similar to what is proposed for humans [31,32]. The activity of $Ca_V1.3$, if it is involved in the spontaneous firing of preprandial human beta cells, remains to be investigated.

For cells undergoing high rates of vesicle fusion and hormone/neurotransmitter release, it is important to maintain a constant surface area through membrane retrieval (endocytosis) [37]. Normally, two mechanisms for membrane internalization coexist, but only the fast/compensatory mechanism is $Ca^{2+}$ dependent [37]. Recent studies demonstrated L-VGCC-mediated endocytosis in chromaffin cells [38] and IHCs [39]. Because $Ca_V1.3$ is the predominant isoform in those tissues [4,11], it might be the most appropriate candidate isoform for a fast endocytotic process [40]. However, experiments in $Ca_V1.3$ KO models should be performed to confirm this hypothesis.

In future investigations, it will be important to elucidate the regulatory mechanisms behind tissue-specific $Ca_V1.3$ exons usage during alternative splicing events [41], in relation to its pacemaking function.

## Regulation of $Ca_V1.2$ and $Ca_V1.3$ by protein–protein interactions: implications for cellular responses and gene transcription

Physiological functions shared by $Ca_V1.2$ and $Ca_V1.3$ might be best described in the central nervous system (CNS). $Ca_V1.2$ accounts for approximately 80% of $Ca_V1$ channels in the CNS, whereas $Ca_V1.3$ appears to be restricted to a much smaller subset of neurons [42]. L-VGCCs in general are not involved in neurotransmitter release because they are predominantly localized in postsynaptic structures [42]. However, several studies suggest the possibility of presynaptic localization [43,44], where L-VGCCs are an alternate $Ca^{2+}$ source during high frequency stimulation [45]. At postsynaptic sites, $Ca^{2+}$ influx through both $Ca_V1.2$ and $Ca_V1.3$ has been shown to trigger transcriptional and post-transcriptional events [46]. These events are modulated by scaffold proteins that bind to the C-terminal tail of $Ca_V1.2$ and $Ca_V1.3$, a region with only 55% sequence homology (Figure 2b). Three important processes can be linked to this interesting domain.

The first is CDI, which is mediated by a stable association with calmodulin (CaM) via the isoleucine–glutamine (IQ) domain in both isoforms (Figures 1 and 2) [46].

The second process is $Ca^{2+}$-dependent facilitation (CDF), in which calmodulin-dependent kinase II (CaMKII), upon CaM-mediated activation, phosphorylates the C-terminal tail and increases the probability that the channel is open during repeated or prolonged activation [47]. Distinctions between this process in $Ca_V1.2$ and $Ca_V1.3$ have been identified at the molecular level. CaMKII interacts directly with $Ca_V1.2$ in a region close to the IQ domain (TVGKFY, Figures 1a and 2b) [48], leading to the phosphorylation of two C-terminal sites in the channel (Ser1512 and 1570) (Figure 2b) with a subsequent increase of $Ca^{2+}$ influx [49]. In $Ca_V1.3$, the interaction with CaMKII requires the scaffold protein densin, which binds to the postsynaptic density-95/discs large/zona occludens-1 binding-domain (PDZ) (Figures 1b and 2b) and clusters $Ca_V1.3$ close to CaMKII [50]. Thus, CaMKII is able to phosphorylate Ser1486 in the $Ca_V1.3$ C-terminal tail (Figure 2b) [51], which, interestingly, is not conserved in $Ca_V1.2$.

The third process involves transcriptional and post-transcriptional events. The specific $Ca_V1.3$ PDZ-binding domain (ITTL, Figures 1b and 2b) interacts with the

scaffold protein SHANK, whereas the $Ca_V1.2$ PDZ-binding domain (VSNL, Figures 1a and 2b) interacts with neuronalinterleukin-16 and channel-interacting PDZ domain protein [52]. Through this CaM/CaMKII/scaffold protein complex, $Ca_V1.2$ and $Ca_V1.3$ can influence differently the signaling to the nucleus [52]. This process involves Ras/MAPK and protein kinase A signaling, and culminates in the activation of cAMP response element-binding protein and myocyte enhancer factor 2 transcription factors, and thus in activity-dependent gene expression [46].

In summary, because of differing biophysical properties and scaffold proteins, $Ca_V1.2$ and $Ca_V1.3$ can generate diverse subcellular $Ca^{2+}$ microdomains that, even within a single cell, can differentially influence signaling to the nucleus. It has been shown that $Ca_V1.3$ is more effective at low levels of activity (during interspike intervals in slow firing cells) and $Ca_V1.2$ more efficient at high levels of activity (during spikes in rapidly firing cells) [53]. Based on these differences, a rational therapeutic approach could be developed, targeting specific interacting proteins selectively to modulate one isoform.

## Is there differential coupling of $Ca_V1.2$ or $Ca_V1.3$ to intracellular $Ca^{2+}$ stores?

It is widely accepted that the influx of $Ca^{2+}$ ions through sarcolemmal L-VGCCs locally controls the opening of clusters of neighboring ryanodine receptors (RyRs), allowing the release of $Ca^{2+}$ ions from the sarcoplasmatic reticulum in cardiac myocytes [54].

Also in neurons, there is evidence for coupling of L-VGCCs to intracellular $Ca^{2+}$ stores for both $Ca_V1.2$ [55] and $Ca_V1.3$ [56,57]. Furthermore, data confirm that L-VGCCs contribute to the asynchronous release of neurotrophins and peptides from individual vesicles [55,58]. This is possible only through massive $Ca^{2+}$ availability, and this can be satisfied uniquely by $Ca^{2+}$ release from internal stores [59]. Therefore, the association between L-VGCCs and internal $Ca^{2+}$ stores might be relevant in other cells besides muscle. In the brain, coupling of L-VGCCs to intracellular $Ca^{2+}$ stores is probably related to $Ca_V1.2$, which is the L-VGCC isoform with the widest expression [4,42]. L-VGCC-dependent neurotrophin release in the hippocampus [55,60] is most probably $Ca_V1.2$ dependent, which is crucial for long term potentiation [61] and for the acquisition of fear memory [62]. It has also been shown that insulin release from murine beta cells is dependent on $Ca_V1.2$ [4]. This neuroendocrine secretion has been suggested to rely on IP3-dependent $Ca^{2+}$-induced $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores [63], similar to cardiac excitation–contraction coupling [64]. Studies have demonstrated a functional and physical interaction between $Ca_V1.2$ and RyR2 in myocytes [65], which has been confirmed by immunocytochemical evidence [64]. *In vitro* studies show that the interaction occurs with the II–III loop of $Ca_V1.2$ (Figures 1a and 2a) and is facilitated by additional binding proteins and kinases [66]. Splicing events in exons 17–20 (Figure 1a), encoding the $Ca_V1.2$ II–III loop, predict the formation of truncated $Ca_V1.2$ hemi-channels (RH-1 and RH-2) in cardiac myocytes and neurons [67], leading to the crucial issue of whether these proteins can still couple to RyR2 and regulate the release of intracellular $Ca^{2+}$.

Recently, a direct and specific interaction was described between the C terminus of $Ca_V1.2$ and stromal interaction molecule 1 (STIM1), a $Ca^{2+}$ sensor of the endoplasmic reticulum lumen that controls the mechanism of store-operated $Ca^{2+}$ entry [68,69]. This interaction is a new and additional mechanism to regulate refilling of $Ca^{2+}$ stores. STIM1 is the main activator of $Ca^{2+}$ release-activated $Ca^{2+}$ channels after the depletion of the internal $Ca^{2+}$ stores, and interacts with $Ca_V1.2$, resulting in a decrease in $Ca_V1.2$-mediated current of approximately 85% and triggering chronic $Ca_V1.2$ internalization [69]. This mechanism would prevent any release of $Ca^{2+}$ as a consequence of $Ca_V1.2$ activation during refilling of the endoplasmic reticulum. Interestingly, STIM1 interacts with the $Ca_V1.2$ C-terminal region (Figure 1a), which is not conserved in $Ca_V1.3$ (Figure 2b), suggesting a typical specific interaction of STIM1 with $Ca_V1.2$ [69].

A juxtaposed localization of $Ca_V1.2$ and RyR [70] and a physical interaction between $Ca_V1.2$ and RyR1, and between $Ca_V1.3$ and RyR2 [57], has also been proposed in the brain. By contrast, a physical interaction between RyR and $Ca_V1.2$ remains elusive in pancreatic beta cells, in which RyRs are highly expressed and have been located to insulin-containing dense-core vesicles beneath the plasma membrane [31]. Because insulin-containing vesicles have been found to be tightly associated with $Ca_V1.2$ in mouse beta cells [58], it can be hypothesized that RyR might be an integral part of the beta cell exocytotic microdomain.

Taken together, these findings do not demonstrate preferential coupling of one of the two channel isoforms to intracellular $Ca^{2+}$ stores. It is unclear whether $Ca_V1.2$ is typically coupled to intracellular stores in all $Ca_V1.2$-expressing cells. It is also important to understand whether there is isoform-selective usage of interacting proteins similar to the STIM1 mechanism. This could be studied with new interactome mapping techniques [71] in cells known to express $Ca_V1$ functionally coupled to intracellular $Ca^{2+}$ stores. This would be an essential first step in elucidating the presumptive selectivity of isoform-specific interacting proteins to intracellular $Ca^{2+}$ stores.

## $Ca_V1.2$ and $Ca_V1.3$ show differential tissue-specific coupling with $Ca^{2+}$-activated potassium channels

Proteins involved in $Ca^{2+}$-dependent processes will become activated in $Ca^{2+}$ nano- or microdomains in the vicinity of the $Ca_V$ pores, according to their affinity for $Ca^{2+}$ [72]. Coupling to L-VGCCs has been demonstrated for $Ca^{2+}$-activated potassium channels of both the big conductance (BK) and the small conductance (SK) type, in neurons, cochlear hair cells, heart and endocrine tissue [72]. Robust BK activation is thought to occur in the immediate vicinity (~10 nm) of an active $Ca^{2+}$ source (affinity > 10 μM), and an interaction between L-VGCC and BK channels has been demonstrated. By contrast, SK channels, which are more sensitive to $Ca^{2+}$ (affinity ~1 μM), do not have to reside in close proximity to the $Ca_V$ pores (20–100 nm), and therefore might require scaffold proteins for anchoring and spacing [72].

In cardiac myocytes, a C-terminal region of $Ca_V1.3$ (in the human sequence: amino acids 1481–2218) displays a

strong interaction with α-actinin2 (Figures 1b and 2b), an F-actin cross-linking protein that could directly bridge to the C-terminal part of SK2 [73]. Indeed, genetic deletion of $Ca_V1.3$ leads to a lack of SK2 currents in cardiac myocytes [73]. Although the evidence is not conclusive, the loss of an interacting binding protein could also explain the reduction of BK currents in IHCs [74] and in chromaffin cells [11,14] after genetic deletion of $Ca_V1.3$.

In rat CA1 hippocampal pyramidal neurons, SK channels also appear to be functionally coupled to $Ca_V1.2$ channels, which gives rise to the slow after-hyperpolarization in these neurons [72]. Furthermore, L-VGCCs affect the firing pattern of suprachiasmatic nucleus neurons, according to diurnal rhythms, as a result of their activation of BK currents [75]. The identification of *CACNA1C* (the gene encoding $Ca_V1.2$) as one of the genes that follow a circadian expression pattern (see http://expression.gnf.org/circadian) might indicate the preferential coupling of $Ca_V1.2$ with BK in this context.

The $Ca_V1.3$ C-terminal tail, where α-actinin2 is likely to interact, shows low sequence homology with the same region in $Ca_V1.2$ (Figure 2b). Do these differences reflect coupling with SK or BK through different interacting proteins? This is a crucial question if this domain is to be considered as a future pharmacological target.

### Polymorphisms and individual pharmacotherapy

Understanding of distinct single nucleotide polymorphisms (SNPs) in $Ca_V1.2$ and $Ca_V1.3$ genes might aid population-adapted therapies and also create opportunities for future individual pharmacotherapy.

$Ca_V1.2$ is encoded by *CACNA1C* and $Ca_V1.3$ by *CACNA1D*, which map to human chromosome 12 band p13.3 [76] and chromosome 3 band p14.3 [77], respectively. The two genes differ considerably in their length (~650 kbp for *CACNA1C* and 320 kbp for *CACNA1D*), but the resulting full-length proteins contain about the same number of amino acids (~2100, see http://www.ncbi.nlm.nih.gov/protein). Furthermore, both have the same SNP frequency of about 1 in 130 bp (see http://www.ncbi.nlm.nih.gov/snp). To date, 4681 SNPs ('markers') are listed for *CACNA1C* and 2537 for *CACNA1D*, and many have been shown to associate with disease. A striking example is marker rs79891110 in *CACNA1C*, in which a guanidine to adenine exchange causes a missense mutation that leads to a faulty channel protein [78]. This impairs voltage-dependent inactivation of $Ca_V1.2$, triggering potentially lethal cardiac arrhythmias and other serious consequences, known as Timothy syndrome [3,5]. Interestingly, the mutation also severely affects the DHP sensitivity of the channel, with an almost fourfold increase of the $IC_{50}$ for nisoldipine [78]. A more severe cardiac phenotype distinct from Timothy syndrome was reported in patients carrying a similar mutation in another splice variant in which $Ca_V1.2$ exon 8a is substituted with exon 8 [79].

Several genome-wide association studies have highlighted SNPs in non-coding (intronic) regions of *CACNA1C* that alter DHP sensitivity, thereby impacting the therapeutic outcome of pharmacological hypertension therapy. An example is the marker rs1051375 in *CACNA1C*, which was associated with hypertension treatment response in cohorts comprising up to 22 000 individuals [80,81]. Depending on the genotype and resulting drug sensitivity, either $Ca^{2+}$ channel or β-blockers were identified as the best drugs for genetically distinct groups of hypertension patients. Two other markers in *CACNA1C*, rs2239128 and rs2239050 [82,83], have been shown to increase the efficiency of nimodipine in hypertensive C (rs2239128) and G (rs2239050) allele carriers. Therefore, genotypes might predict the optimal treatment, reducing the risk of fatal outcomes such as cardiac infarction [80]. Importantly, in rs2239128 the allele frequencies for the major/minor ('good'/'bad') allele are almost reciprocal between Caucasians and Chinese; the best treatment option is therefore reversed between these two populations [82]. Other studies have revealed polymorphisms in *CACNA1D* with statistically stronger associations than *CACNA1C* to the outcome of blood pressure treatment [84].The risk of bipolar disorder, schizophrenia or recurrent major depression might also be associated with SNPs in *CACNA1C* [85,86], although there are discrepancies among different genome-wide association studies [87].

Collectively, these studies suggest that it will be beneficial to further individualize pharmacotherapy based on genetic sequencing of L-VGCCs. Matching phenotypically/clinically associated SNPs with known splice variants and their suggested effects on L-VGCC pharmacology now appears worthwhile. This might be a shortcut to identifying clinically relevant splice variants and potentially testable new targets.

### Concluding remarks: considerations for isoform-selective drugs

Because $Ca_V1.2$ and $Ca_V1.3$ channels have diverse and essential functions (often in the same organ), the development of an isoform-selective activator or blocker could be therapeutically valuable in a wide range of diseases. Novel studies describe $Ca_V1.3$ as a determinant of heart rate but not of ventricular excitation–contraction coupling [6]. Accordingly, the development of $Ca_V1.3$-selective blockers might be an attractive possibility for managing cardiac ischemia and other coronary artery diseases, without the concomitant negative inotropism due to $Ca_V1.2$ channel inhibition in the working myocardium. A $Ca_V1.3$-selective inhibitor would potentially be important in the neuroprotection of SNc DA neurons, neuronal loss in Parkinson's disease [8] and the age-dependent decrease of cognitive function [4].

Because Timothy syndrome (which results from gain-of-function mutations in the gene encoding $Ca_V1.2$) is also characterized by neurological disorders such as autism and mental retardation [5], these disorders might benefit from a $Ca_V1.2$-selective modulator.

Besides selective targeting of $Ca_V1.2$ and $Ca_V1.3$, targeting cellular function as a system rather than on the level of a single target, would significantly increase the number of drug treatable targets, especially in the treatment of complex disorders (e.g. mental disorders) [88]. In this context, analysis of cell-specific spliceosomes (the ribonucleoprotein assemblies that perform splicing [89]) and protein interactomes [90] in either $Ca_V1.2$ or $Ca_V1.3$ should

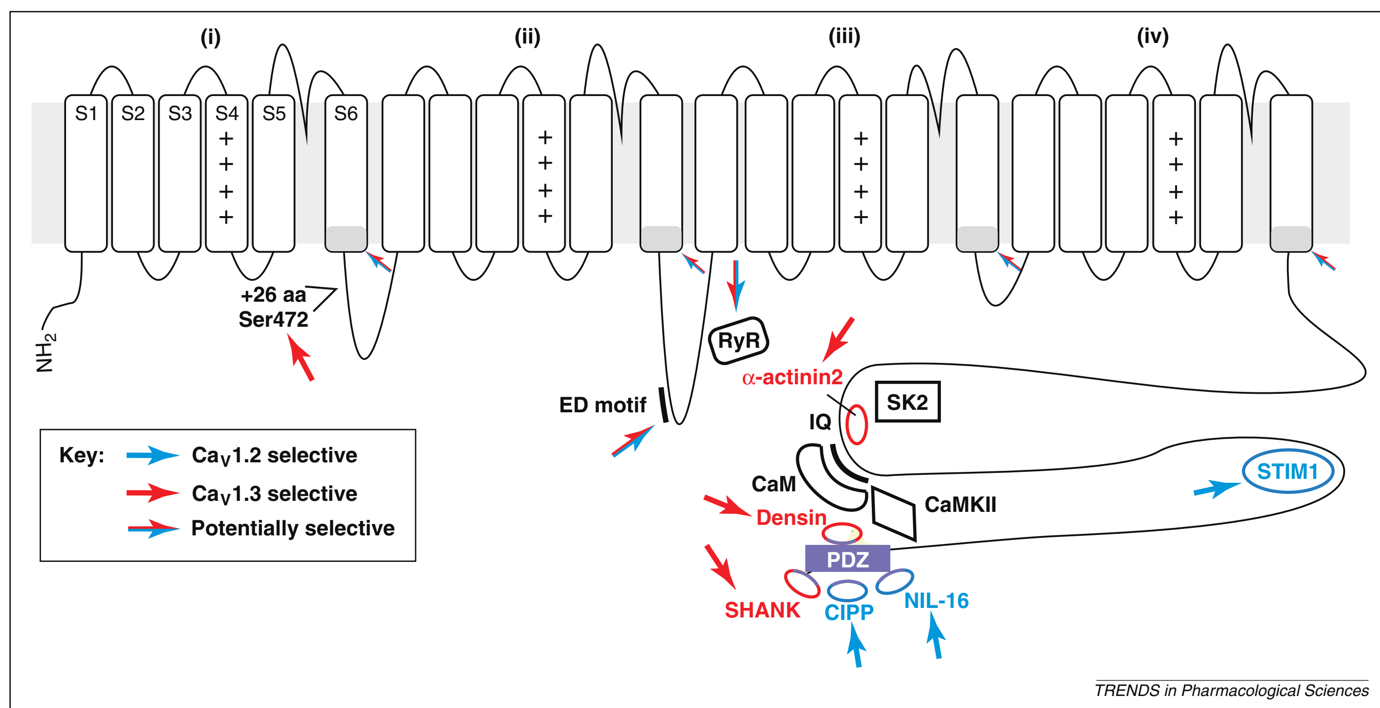


**Figure 3**. Potential targets for isoform selective drugs. Schematic representation of L-VGCCs. Potential sites and/or interacting proteins for isoform selective drugs are indicated in blue for $Ca_V1.2$ and in red for $Ca_V1.3$. CIPP, channel-interacting PDZ domain protein; NIL-16, neuronalinterleukin-16.

be conducted as a next step, particularly regarding tissues of high therapeutic value.

In this review, we have highlighted isoform-specific differences that might be considered and eventually exploited in the process of isoform-selective drug design. A fruitful experimental approach to the development of isoform-selective lead molecules should include (Figure 3):

(i) the correlation between sequence composition and gating state and the 3D conformation of the drug-binding site;
(ii) gating state modulation (CDI and CDF) by protein interaction;
(iii) the interaction with intracellular $Ca^{2+}$ stores;
(iv) PDZ binding domains and their interaction with scaffold proteins;
(v) SNPs and individual pharmacotherapy.

However, because the expression and function of each isoform is not restricted to one tissue or cell type, we must be aware of potential side effects, such as pro-arrhythmic effects and hypertension with $Ca_V1.2$ activators.


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