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Channelopathies in $Ca_v1.1$, $Ca_v1.3$, and $Ca_v1.4$ voltage-gated L-type Ca^{2+} channels

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Abstract Voltage-gated Ca^{2+} channels couple membrane depolarization to Ca^{2+}-dependent intracellular signaling events. This is achieved by mediating Ca^{2+} ion influx or by direct conformational coupling to intracellular Ca^{2+} release channels. The family of Ca_v1 channels, also termed L-type Ca^{2+} channels (LTCCs), is uniquely sensitive to organic Ca^{2+} channel blockers and expressed in many electrically excitable tissues. In this review, we summarize the role of LTCCs for human diseases caused by genetic Ca^{2+} channel defects (channelopathies). LTCC dysfunction can result from structural aberrations within their pore-forming $\alpha1$ subunits causing hypokalemic periodic paralysis and malignant hyperthermia sensitivity ($Ca_v1.1$ $\alpha1$), incomplete congenital stationary night blindness (CSNB2; $Ca_v1.4$ $\alpha1$), and Timothy syndrome ($Ca_v1.2$ $\alpha1$; reviewed separately in this issue). $Ca_v1.3$ $\alpha1$ mutations have not been reported yet in humans, but channel loss of function would likely affect sinoatrial node function and hearing. Studies in mice revealed that LTCCs indirectly also contribute to neurological symptoms in Ca^{2+} channelopathies affecting non-LTCCs, such as $Ca_v2.1$ $\alpha1$ in *tottering* mice. Ca^{2+} channelopathies provide exciting disease-related molecular detail that led to important novel insight not only into disease pathophysiology but also to mechanisms of channel function.

Keywords Channels · Channel gating · Channel activity · Neuronal excitability

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Introduction

Voltage-gated Ca^{2+} channels are Ca^{2+}-selective pores linked to voltage-sensing domains that couple membrane depolarization to intracellular signaling events. Among the three families of voltage-gated Ca^{2+} channels (VGCCs; Ca_v1, Ca_v2, and Ca_v3, [14]), the family of Ca_v1 channels, also termed L-type Ca^{2+} channels (LTCCs), is uniquely sensitive to organic Ca^{2+} channel blockers and expressed in many electrically excitable tissues. LTCCs were first described in heart and smooth muscle. Today, we know that these cardiovascular channels are almost exclusively of the $Ca_v1.2$ subtype and their block by clinically used Ca^{2+} channel blockers (such as nifedipine, amlodipine, verapamil, and diltiazem) explains most of their therapeutic effects, such as blood pressure lowering and cardiodepression. In addition to $Ca_v1.2$, three other isoforms ($Ca_v1.1$, $Ca_v1.3$, and $Ca_v1.4$) exist. $Ca_v1.3$ is expressed together with $Ca_v1.2$ in many tissues, such as the sinoatrial node and heart atria, neurons, chromaffin cells, and pancreatic islets. Available Ca^{2+} channel blockers inhibit both of these isoforms with similar affinities, such that their physiological roles could not be separated pharmacologically. This was possible by genetically modified mice revealing distinct functions of these two isoforms based on differences in their biophysical properties [62, 68]. In particular, $Ca_v1.3$ can serve pacemaker functions in neurons [57], the sinoatrial node [47], and in chromaffin

cells [49, 50]. In the brain, both isoforms couple neuronal activity to transcriptional events: $Ca_v1.2$ mediates long-term potentiation and spatial learning and memory in the hippocampus [55]. $Ca_v1.3$ mediates long-term potentiation in the amygdala and participates in the consolidation of fear memory [25].

$Ca_v1.1$ and $Ca_v1.4$ possess a much more restricted expression pattern, with expression almost exclusively in skeletal muscle and the retina, respectively. $Ca_v1.1$ channels (which also contain a γ-subunit) carry very slowly activating Ca^{2+} inward currents, too slow for providing Ca^{2+} to the contractile machinery in response to millisecond depolarizations eliciting muscle contraction. Although the fast conformational changes of their voltage-sensing domains induce pore opening very slowly, they are quickly transmitted to the sarcoplasmic reticulum (SR) ryanodine receptors (RyR1), thus serving as fast voltage sensors for SR Ca^{2+} release. This seems to be accomplished through a close physical association of $Ca_v1.1$ channels in the T-tubular membrane and RyR1 in the junctional SR of the skeletal muscle triads [45].

Transcripts for all four LTCC α1 subunit isoforms and accessory β3- and β4-subunits are also present in immune cells [2, 36]. Although reduced expression of $Ca_v1.1$, β3, or β4 was each associated with reduced Ca^{2+} influx after T-cell receptor cross-linking in T-cells [52], the exact role of LTCCs for T-cell signaling remains unknown.

Here, we summarize the role of LTCCs for human diseases caused by genetic Ca^{2+} channel defects (channelopathies) in Ca^{2+} channel α1 subunits. LTCC dysfunction can result from structural aberrations within their pore-forming α1 subunit (L-type Ca^{2+} channelopathies), such as in retinal $Ca_v1.4$ α1 found in patients with incomplete congenital stationary night blindness (CSNB2), or in skeletal muscle $Ca_v1.1$ α1 found in patients with hypokalemic periodic paralysis (HPP) or malignant hyperthermia susceptibility (MHS). However, LTCC dysfunction can also occur in Ca^{2+} channelopathies with structural aberrations in the α1 subunit of non-LTCCs [13] (non-L-type Ca^{2+} channelopathies), such as $Ca_v2.1$ α1 mutations in *tottering* mice. Ca^{2+} channelopathies involving defects of auxiliary subunits (which may not selectively affect only LTCCs) will not be discussed in this review.

$Ca_v1.1$ channelopathies (*CACNA1S* gene)

Hypokalemic periodic paralysis type 1

Familial HPP is an autosomal dominant disorder caused by mutations in the pore-forming $Ca_v1.1$ α1-(hypokalemic periodic paralysis type 1,HPP-1) or Na^+-channel α-subunit ($Na_v1.4$, *SCN4A* gene; HPP-2; see chapter on skeletal muscle Na^+-channel channelopathies in this issue). *CACNA1S* mutations are found in about 75% of patients and SCN4A mutations in about 15% [41]. HPP symptoms generally manifest around the second decade of life and are characterized by hypotonia and attacks of local or generalized skeletal muscle weakness or paralysis. The frequency of the attacks is variable. A lower penetrance often occurs in females. Attacks are accompanied by hypokalemia, and therapeutic potassium supplementation relieves symptoms. Precipitating factors are high-carbohydrate meals, insulin intake, acute stress, sudden exposure to heat or cold, and sudden rest after exercise. The long-term prognosis is generally good, and crises may decrease in midlife. However, severely affected families were reported, and involvement of respiratory muscles may lead to death [7]. The discovery of single missense *CACNA1S* mutations in humans with HPP-1 which still allow expression of a full-length $Ca_v1.1$ α1 subunit protein suggested that changes in channel gating or channel expression on the cell surface may account for altered skeletal muscle function. The most frequent mutations affect arginine residues in two of the channel's voltage sensors (R528, R1239; Fig. 1). In contrast to skeletal muscle Na^+-channels, $Ca_v1.1$ channels are difficult to express in heterologous systems [56]. Results from such studies, and even from recordings of mutant Ca^{2+} currents from myotubes cultured from affected patient muscle [69], were rather controversial and did not reveal a clear unifying picture of how the reported biophysical changes may explain the episodic failure of muscle excitability in association with a decrease in serum potassium.

A fresh perspective for a unified hypothesis for HPP pathophysiology came from several independent observations.

First, even normal skeletal muscle cells are known to show a bistable membrane behavior. Initial lowering of extracellular K^+ (K_{ex}) hyperpolarizes, but further lowering (usually to below 1 mM in normal muscle) then abruptly (and paradoxically) depolarizes the sarcolemmal membrane to about -50 to -60 mV [79]. This behavior reflects the existence of two stable resting membrane potentials (V_R), one near the K^+-equilibrium potential (around -80 mV) and one around -50 to -60 mV resulting from two opposing conductances: a Ba^{2+}-sensitive inward rectifier K^+-current (which determines the more negative V_R) and a linear, non-selective leak inward current. With decreasing K_{ex}, first hyperpolarization occurs as expected from the Nernst equation, but with the inward rectifier conductance declining the hyperpolarizing K^+-current will become smaller than the depolarizing leak current with decreasing K_{ex}. V_R is then uncoupled from the K^+-equilibrium potential and becomes more depolarized. Accordingly, the sensitivity of this paradoxical depolarization to K_{ex}-lowering (i.e., a shift to higher K_{ex}) is increased by either blocking the inward rectifier K^+-current (e.g., by Ba^{2+}) or by enhancing the depolarizing leak currents. Indeed, HPP muscle fibers are

more susceptible to K^+-lowering than normal muscle [41]. Since K^+-channels are not mutated in HPP-1 or HPP-2, the only possibility is that mutations observed in the pore-forming subunits of $Ca_v1.1$ α1 or $Na_v1.4$ α somehow increase leak current.

Second, a large number of $Na_v1.4$ α-subunit point mutations, also outside of the S4 helices, are known to cause different muscle channelopathies (for review, see [39]) but as in $Ca_v1.1$ α1 for HPP-1, only neutralizing mutations in S4 arginines cause HPP-2. This strongly pointed to a specific role of these residues but it was unclear how the voltage-sensing domains of two different ion channels with different ion selectivity could account for the paradoxical depolarization associated with low K_{ex}.

The third and intriguing finding was that mutations of S4 arginines in Shaker K^+-channels can create a pore in the voltage-sensing domain independent of the main K^+-selective pore. This new pore can selectively conduct protons when mutated to histidine [73] or other cations when mutated to non-charged amino acids [81]. It was termed ω-current or gating pore current. Gating of this pore is voltage-dependent because the position of the S4 arginines strongly depends on the position of the S4 helix which moves during gating (Fig. 2). Mutating the outermost arginine appears to create a pore in the closed state (Fig. 2a, b) that gets plugged by an inner arginine [74], once the S4 moves outward and tilts upon depolarization (Fig. 2c). An opposite voltage dependence would be expected for a mutation of arginines further inside S4, such as arginine in position 3 (Fig. 2d). The finding that a single residue could transform the voltage-sensing domain into a pore was further strengthened by the fact that the voltage-gated proton channel Hv1 contains the typical four transmembrane segments S1–S4 of a voltage-sensing domain but lacks the two transmembrane segments that form the classical pore domain in other voltage-gated channels [82]. Together, these observations paved the way for studies on HPP-2 and HPP-1, demonstrating that these mutations indeed induced a gating pore current which represents the depolarizing conductance predicted from the susceptibility to "paradoxical" depolarization. For $Na_v1.4$ mutations, this could be directly shown from recordings in heterologous expression systems [70]. As mentioned above, heterologous expression is more difficult with $Ca_v1.1$. However, in a series of elegant experiments in myofibers from HPP-1 patients with R528H and R1239H $Ca_v1.1$ mutations, Jurkat-Rott and colleagues [41] measured a non-selective cation leak of 12–19.5 μS/cm from steady-state current density–voltage relationships, consistent with the assumption that the $Ca_v1.1$ α1 mutations also induce gating pore currents. This may also explain the high intracellular Na^+ concentrations found in the muscle of these patients in vivo and in vitro [41]. However, these experiments do not allow predictions about the cation selectivity of the $Ca_v1.1$ α1 mutations, especially because Cav1.1 α1 mutations to histidines are expected to conduct only protons, as shown for corresponding arginine mutations in $Na_v1.4$ and Shaker K^+ channels.

The HPP-1 mutations currently known are illustrated in Fig. 1. Two additional mutations affecting the first and

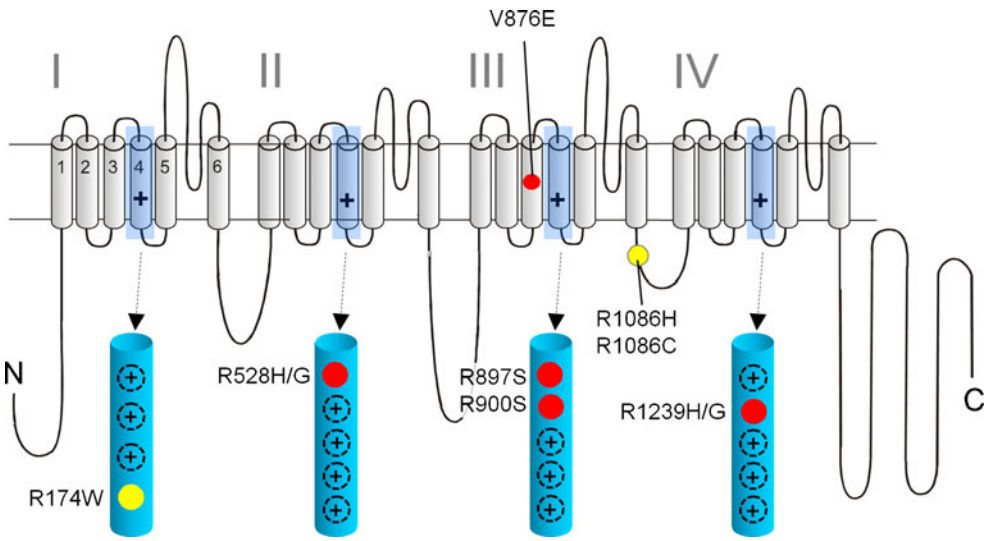

Fig. 1 Mutations in Ca^{2+} channel $Ca_v1.1$ α1 subunits identified in patients with HPP-1 and MHS: a folding model of α1-subunits based on hydrophobicity analysis is shown. *Plus sign* indicates several positive charges in the transmembrane S4 helices within the hydrophobic repeats I–IV. S4 helices and their positively charged residues are shown in the enlarged structures. Together with S1, S2, and S3 helices, they form the four voltage-sensing domains of the channel controlling the opening and closing of a single pore domain formed by S5 and S6 helices together with the connecting linkers. HPP-1 mutations are indicated in *red*; MHS mutations are shown in *yellow*. The location of other positive charges in the S4 domains is indicated as *black circles* (*plus sign*)

second arginine in S4 of domain III (R897S, R900S) were discovered more recently [51] and are in agreement with the gating pore current theory. The first mutation not affecting a S4 arginine, V876E, was reported in a HPP-1 family in South America [42]. V876E is located within the transmembrane helix S3 and replaces a hydrophobic residue by a negative charge. S3 helices are located close to the S4 helix in different models of voltage-gated cation channels [90] and help to stabilize the S4 helix. Upon activation, the S4 helix moves outward, rotates clockwise, and its extracellular end tilts away from the pore axis (Fig. 2). Although the relative movements of the adjacent S1–S3 helices with respect to S4 are a matter of debate [90], the negative charges in these helices (including S3) were shown to form salt bridges with the S4 positive charges, and these interactions change dynamically upon gating-induced S4 movements (as shown, e.g., for a "sliding helix model" [90]). Therefore, a negative charge in the S3 helix is likely to disturb this delicate network of charges. It is possible that this leads to conformational changes that create an ion pore within the voltage sensor. Although this hypothesis needs to be addressed in future studies, the location of this mutation outside S4 is not a priori contradicting the gating pore concept underlying HPP pathophysiology.

Malignant hyperthermia susceptibility

Malignant hyperthermia (MH) is a potentially lethal autosomal dominant disorder with susceptibility of otherwise healthy individuals to severe adverse reactions to volatile anesthetics (e.g., halothane) or depolarizing muscle relaxants. Exposure to these drugs can quickly lead to skeletal muscle hypermetabolism resulting from an uncontrolled increase in the concentration of free myoplasmic Ca^{2+} released from the SR Ca^{2+} stores [40]. This state results in skeletal muscle contractures with adenosine

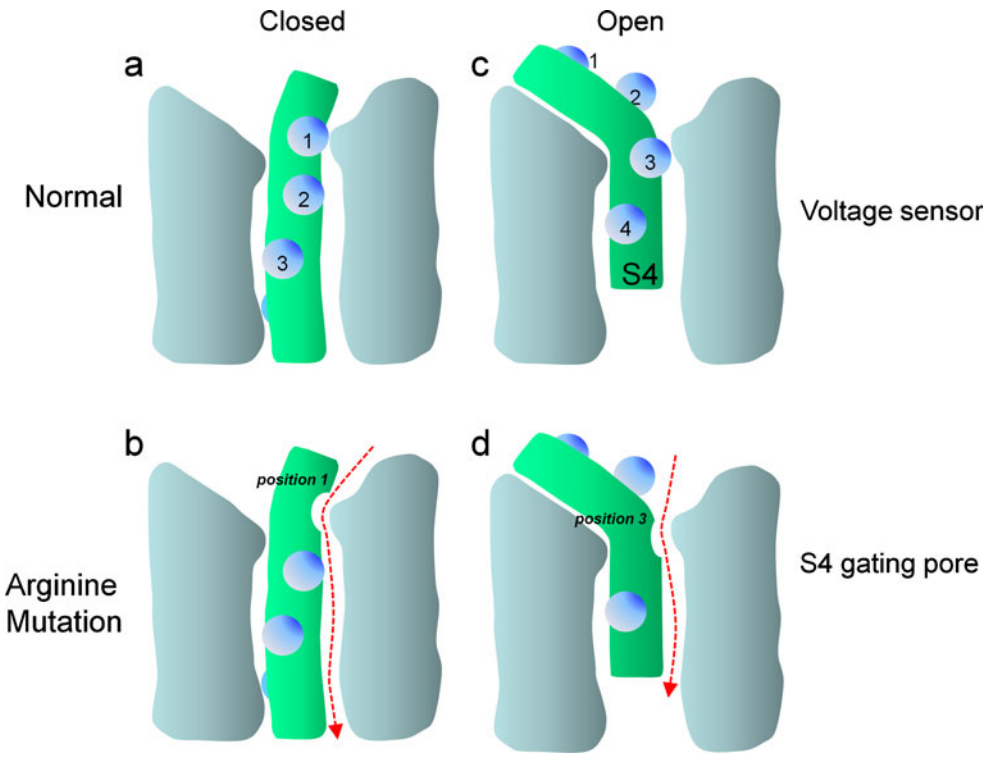

Fig. 2 Simplified scheme illustrating the membrane potential-dependent conformations of the voltage sensor: only one of the four voltage-sensing domains is illustrated. S4 helices are shown in *green*, positively charged residues (mostly arginines) as *blue spheres*. In the closed state, the positively charged S4 helix is pulled inside by the negative resting potential. The outermost arginine residue (*1*) interacts with residues of other helices forming the voltage-sensing domain (e.g., a key negative charge in S2; [70]) (**a**). In Shaker K^+, $Ca_v1.1$, or $Na_v1.4$ channels, a mutation of arginine in position 1 (*1*) to an uncharged residue (e.g., serine or glycine) opens a new permeation pathway (*arrow*) as long as the channel is in the closed state (**b**). Upon depolarization, the S4 helix is driven outward, rotates, and its extracellular portion tilts (**c**). This movement shifts the arginine in position (*3*) outward and would close the gating pore induced by a mutation in position 1. The mechanism can account for the depolarizing current observed in muscle cells from HPP-1 patients carrying the $Ca_v1.1$ $\alpha1$ subunit mutations in S4 helices illustrated in Fig. 1 (HPP-1) or analogous mutations in $Na_v1.4$ (HPP-2, not illustrated, [51]). Conversely, whenever the sensor is in the open state, mutation of an arginine in position 3 (*3*) would enable a gating pore current (**d**), which would be closed upon repolarization by inward movement of arginine 1. Such a mechanism can explain the depolarization-activated gating pore current conducted by mutant $Na_v1.4$ channels in potassium-sensitive normokalemic periodic paralysis [70]

triphosphate-depletion, excessive activation of glycogenolysis and cell metabolism, hypercapnia, hypoxemia and lactic acid acidosis, and an increase in body temperature. Rhabdomyolysis occurs with subsequent creatine kinase elevation, hyperkalemia, cardiac arrhythmias, myoglobinuria, and the possibility of renal failure. Treatment of a crisis by early administration of dantrolene, an inhibitor of SR Ca^{2+} release, substantially reduces mortality. Anesthesia-induced MH incidence is estimated to about 1:10,000. However, the true prevalence must be higher because the clinical penetrance is low. The skeletal muscle ryanodine receptor RyR1 gene (RYR1) has been identified as the primary MHS locus and there are about 180 missense mutations described across RYR1 that co-segregate with MHS [12]. Several alternative loci have also been proposed, but so far, only the $Ca_v1.1$ α1 subunit gene (*CACNA1S*) has been identified as an additional causative gene. HPP-1 and MHS can therefore be considered allelic diseases. The $Ca_v1.1$ α1 mutations associated with MHS are located in the cytoplasmic linker between repeats III and IV (R1086H, R1086C [54]) or replace the innermost arginine in S4 of repeat I (Fig. 1). Because $Ca_v1.1$ mainly serves as the voltage sensor of RyR1 rather than a Ca^{2+} channel (see above), these mutations may alter the voltage-dependent signaling between these two Ca^{2+} channels. In a porcine model of MHS (RyR1 point mutation), the typical increased sensitivity to a broad range of pharmacological stimuli was accompanied by a lower threshold for SR Ca^{2+} release and contraction [24]. The fast depolarization-induced conformational changes of $Ca_v1.1$ α1 subunits (also termed dihydropyridine receptors, DHPRs, in muscle) mechanically activate RyR1 and elicit SR Ca^{2+} release. In addition to this orthograde coupling, there is also a retrograde signaling because the activity of DHPRs is strongly influenced by its RyR1 interaction. Both forms of coupling are mediated through a "critical domain" in the cytoplasmic II–III linker [26]. Obviously, measurements of MHS mutation-induced effects on $Ca_v1.1$-mediated ion currents appear of limited value. Instead, the functional coupling needs to be studied, which requires introduction of the mutated channels into a skeletal muscle environment. This can either be achieved by homologous expression of mutant constructs in cultured muscle cells devoid of $Ca_v1.1$ α1 subunits or by engineering of MHS mutations into the CACNA1S gene in mice. Muscle cells can then be isolated to monitor changes of $Ca_v1.1$-mediated excitation–contraction coupling. $Ca_v1.1$-deficient skeletal muscle myotubes were successfully used to demonstrate that the $Ca_v1.1$ α1 R1086H mutation lowers the half-maximal voltage required for the induction of SR Ca^{2+} release by about 5 mV and enhances the sensitivity of SR release to caffeine [24], a drug that is used as a primary diagnostic measure for MHS. This finding is compatible with a mutation-induced facilitation of SR Ca^{2+} release by both pharmacologic (caffeine) and endogenous (voltage sensor) activators. Notably, a lower activation threshold for Ca^{2+} release was also found for RyR1 mutations, including a heterozygous RyR1 mutation in a MHS mouse model. Sensitization of Ca^{2+} release therefore appears as the unifying principle underlying susceptibility to MH. Given the strategically important location of the voltage sensor arginine, it is quite possible that the novel mutation R174W acts through the same pathophysiological mechanism.

$Ca_v1.3$ channelopathies (*CACNA1D* gene)

So far, no human diseases resulting from mutations in the *CACNA1D* gene encoding the $Ca_v1.3$ α1 subunit have been reported. This could be due to the fact that loss-of-function mutations cause no phenotype in the heterozygous state (as in mice) but are lethal in the homozygous state. However, spontaneous gain-of-function mutations may cause a clinical syndrome compatible with life. In the case of $Ca_v1.2$ (*CACNA1C* gene), such a scenario leads to Timothy syndrome (see article in this issue). Homozygous $Ca_v1.2$ knockout mice die during development before day 14.5 post-coitum which may be due to their prominent role in the cardiovascular system [65]. Like for $Ca_v1.2$, heterozygous $Ca_v1.3$ knockout mice were not distinguishable from wild type, suggesting that heterozygous loss-of-function mutations would also be clinically silent in humans. However, based on data from homozygous $Ca_v1.3$ knockout mice, it is very likely that complete loss of $Ca_v1.3$ function would not be lethal. Homozygous $Ca_v1.3$ knockouts are viable and have been successfully used to establish the role of this LTCC isoform for physiology (for review, see [77]). If $Ca_v1.3$ serves a similar role in humans, this mouse model predicts no clinical symptoms in heterozygous patients but congenital hearing impairment and sinoatrial node dysfunction in homozygous individuals. Sinoatrial node dysfunction is unlikely to be lethal because the bradycardia and sinoatrial node arrhythmia observed in $Ca_v1.3$ knockout mice are pronounced at rest and largely disappear during exercise. Such a syndrome may therefore be rare and present mainly in consanguineous deafness families.

$Ca_v1.4$ channelopathies (*CACNA1F* gene)

Incomplete congenital stationary night blindness type 2

Incomplete congenital stationary night blindness type 2 (CSNB2) is an X-linked form of congenital stationary night blindness which is caused by mutations in the voltage-gated calcium-channel gene *CACNA1F* encoding $Ca_v1.4$ LTCCs

(OMIM: 300110). CSNB2 is characterized by variable and usually mild clinical symptoms. The term is, however, misleading because night blindness may not be the major complaint, unlike in the complete form of stationary night blindness (CSNB1) which is caused by different genetic defects either in the nyctalopin (OMIM: 300278) or the metabotropic glutamate receptor-6 (OMIM: 604096). Typical symptoms in CSNB2 are moderately low visual acuity, myopia, nystagmus, and variable levels of night blindness, but one or more of these symptoms may be absent [6]. The eye fundus is normal but electroretinograms (ERGs) are abnormal [83]. CSNB2 patients show a very abnormal dim scotopic ERG and a typical negative bright-flash ERG which has large a-waves, but severely reduced b-waves. Oscillatory potentials are also missing [83]. The ERG data are compatible with a defect in neurotransmission within the retina between photoreceptors and second-order neurons [83]. LTCCs are the predominant channels controlling neurotransmitter secretion at the ribbon synapses of retinal photoreceptors (see references in [44]) and of cochlear inner hair cells [62]. These cell types show "tonic" neurotransmitter release in response to graded changes in the membrane potential, unlike in most other fast, chemical synapses in which non-LTCCs (such as $Ca_v2.1$ and $Ca_v2.2$) trigger neurotransmitter release during bursts of short action potentials ("phasic release") [14]. In the dark, photoreceptors depolarize to a resting membrane potential of -36 to -40 mV [17], enhancing tonic release. Light absorption in the photoreceptor outer segments and closure of cyclic guanosine monophosphate (cGMP)-gated cation channels hyperpolarizes the cells to below -55 mV [86]. Release occurs at so-called ribbon-type synapses where Ca^{2+} channels appear clustered. To support tonic release, retinal Ca^{2+} channels must activate rapidly at relatively negative voltages (below -40 mV) and inactivate slowly [63]. Identification of the genetic defect responsible for CSNB2 led to the discovery of a novel Ca^{2+} channel $\alpha 1$ subunit, $Ca_v1.4$ (see references in [44]), which carries the disease-related mutations, and is preferentially expressed in retinal synapses [5, 16]. It took several years until cloned $Ca_v1.4$ channel complexes could be functionally expressed in mammalian cells [44] to investigate their functional and pharmacological properties [4, 19, 20, 44, 53, 58, 59]. Similar to photoreceptor Ca^{2+} currents, recombinant $Ca_v1.4$ currents in cultured mammalian cells activate rapidly and inactivate very slowly during depolarizing pulses. Interestingly, this was due to a very slow voltage-dependent inactivation accompanied by complete absence of so-called calcium-dependent inactivation (CDI) [44]. CDI is considered an important negative feedback mechanism that protects cells from excess Ca^{2+} influx [1]. Similar to $Ca_v1.3$, $Ca_v1.4$ channels open at more negative membrane potentials than $Ca_v1.2$ [44], allowing the channel to conduct Ca^{2+} at potentials negative to -40 mV. Together, inactivation and activation characteristics of $Ca_v1.4$ channels reveal a substantial window current, which permits ion influx under constant depolarized conditions. Peloquin and colleagues observed that at near physiological temperatures, inactivation kinetics is accelerated but the window current is still preserved [58]. These biophysical properties make them ideally suited for tonic glutamate release from photoreceptor terminals. $Ca_v1.4$ $\alpha 1$ subunits are expressed at release sites of mammalian photoreceptors in the outer plexiform layer [3, 16] and channel loss-of-function would therefore be expected to decrease photoreceptor neurotransmitter release capacity, impair signaling to second-order retinal neurons, and thus explain the ERG abnormalities in CSNB2. $Ca_v1.4$ may also contribute to the LTCC currents measured in bipolar cell terminals, explaining punctate $Ca_v1.4$ $\alpha 1$ immunostaining in the mouse inner plexiform layer [5].

So far, more than 40 structural aberrations were identified in the $Ca_v1.4$ $\alpha 1$ subunit gene of CSNB2 patients (Fig. 3). Most of them are predicted to cause severe structural changes, such as truncated $\alpha 1$ subunits, unlikely to support significant channel activity. Moreover, premature stop codons in regions followed by splice sites at a distance of 50–55 nucleotides downstream-yield mRNAs should be eliminated by nonsense-mediated mRNA decay [48] and thus might not even lead to expression of the truncated $Ca_v1.4$ $\alpha 1$ subunit protein. Due to the X-linked condition, CSNB2 results in a complete loss of $Ca_v1.4$ channel function only in affected males. However, some missense mutations are unlikely to lead to a complete loss-of-channel function (Fig. 3). Hoda et al. [32] characterized a mutation G369D in the pore-lining region of segment IS6 that caused pronounced changes of the channel's inactivation gating and also shifted the $V_{0.5,act}$ to more negative voltages compatible with an overall $Ca_v1.4$ channel gain-of-function. Furthermore, ion selectivity was affected, suggesting that the negative charge introduced by the G369D mutation at the cytoplasmic side of IS6 not only affects conformational changes associated with channel activation but also interferes with cation permeation through the pore. Interestingly, G369 corresponds to G402 in $Ca_v1.2$ $\alpha 1$, which is mutated to serine in some patients with Timothy syndrome [71] and strongly inhibits voltage-dependent inactivation (VDI). In $Ca_v1.2$, VDI is also inhibited by mutation of nearby residues such as a serine residue important for slow inactivation in IS6 and G406 in Timothy syndrome (G406R) [72]. Obviously, channelopathies in different LTCC $\alpha 1$ subunits have identified a region forming a critical "hotspot" for channel gating.

Another gain-of-function mutation was discovered in a New Zealand family showing a similar but more severe clinical phenotype than in CSNB2. The missense mutation I745T in the pore helix IIS6 produced a remarkable -30-mV

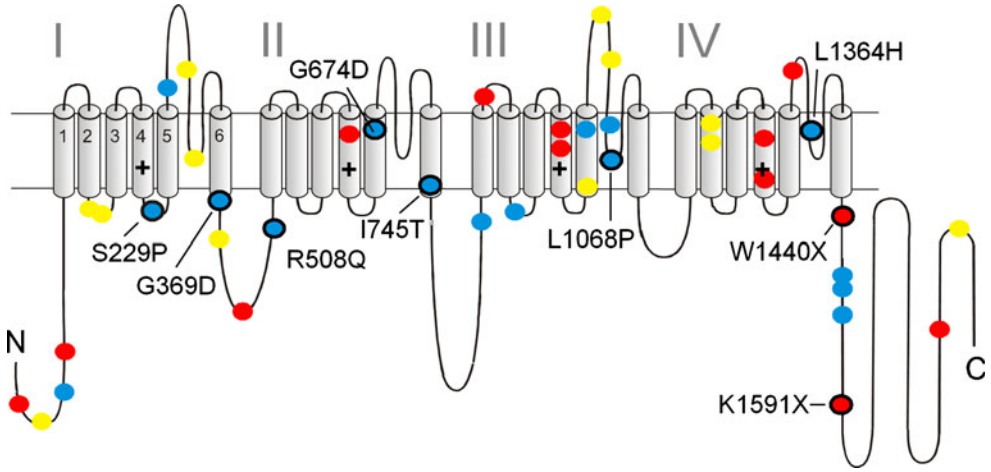

Fig. 3 Mutations in Ca^{2+} channel $Ca_v1.4$ α1 subunits identified in patients with CSNB2: a folding model of α1 subunits based on hydrophobicity analysis is shown. *Plus sign* indicates several positive charges within the transmembrane S4 helices within the hydrophobic repeats I–IV. Position of CSNB2 mutations is indicated. *Colors* indicate the predicted structural changes: *blue*, single missense mutations; *yellow*, in-frame amino acid deletions or insertions; *red*, truncated protein due to single mutations that introduce stop codons. *Black circles* refer to mutations that are functionally characterized [31–33, 53, 59, 67]

shift in the voltage dependence of $Ca_v1.4$ channel activation as well as significantly slower inactivation kinetics when expressed in tsA-201 cells [31]. This observation triggered a detailed analysis of the role of the equivalent residue in $Ca_v1.2$ for channel gating [34], indicating that substitution of this residue destabilizes the closed and favor the open conformation of the pore. Molecular dynamics simulations suggest that this may also involve mutation-induced conformational alterations of other interacting transmembrane segments [75, 76].

In contrast, no channel activity could be measured for mutants S229P and W1440X after expression in *Xenopus* oocytes, and mutant L1068P yielded currents only in the presence of the channel activator BayK8644 [32]. Mutations S229P, G369D, and L1068P α1 subunits were expressed at levels indistinguishable from wild-type channels, but no protein was detected for the truncation mutation W1440X after expression in tsA-201 cells [32]. Two other missense mutations, R508Q and L1364H, reduced protein expression in transfected tsA-201 cells, which may, although not yet proven, also decrease retinal Ca^{2+} current density [33]. However, McRory et al. found that two missense mutations, G674D and A928D, and the W1459X truncation mutation in the C-terminus exerted no detectable changes in the activation, inactivation, or conductance properties of expressed $Ca_v1.4$ channels. For the mutation G369D, they only found a slight, but statistically significant increase in the slope factor of the activation curve and a less pronounced shift of the half-activation potential with Ca^{2+} as compared to Ba^{2+} as charge carrier. This discrepant finding might be explained by the fact that their $Ca_v1.4$ α1 subunit [44] differed in four amino acid positions from the human $Ca_v1.4$ α1 subunits used by Hoda et al. [53]. This also includes neutralization of a negative charge in the IS6 helix which may by required to "sense" the additional negative charge introduced by the G369D mutation. The possibility that the mutations affect $Ca_v1.4$ α1 protein expression has not been tested in their study.

Clinical CSNB2 symptoms might therefore result not only from complete loss of function and/or decreased expression of mutant channels with unchanged gating behavior but also from gating changes including a channel gain-of-function. The gain-of-function mutations should promote Ca^{2+} entry through the channel raising the important question about how increased channel function could impair light-induced signaling between photoreceptors and second-order neurons. One possible interpretation is as follows. Because the half-maximal voltage of activation for retinal LTCCs (and $Ca_v1.4$) [44, 53] is clearly above -40 mV [17, 85], the retinal operating range of membrane potential changes is at the "foot" of the LTCC activation curve and thus Ca^{2+}-influx becomes very small or not measurable [86] at hyperpolarized voltages (e.g., -55 mV, Fig. 4) during illumination. From the activation curve, an about 50-fold increase of $Ca_v1.4$ inward current can be predicted upon depolarization to -35 mV. A pronounced negative shift of the activation curve by a CSNB2 mutation would result in a significant increase of Ca^{2+} influx during illumination at negative voltages, but at the same time, would reduce the increase upon depolarization, leading to a reduced dynamic range (Fig. 4). The corresponding change in the dynamic range of tonic glutamate release could then explain how the synaptic gain between first- and second-order neurons is reduced in CSNB2 retinas.

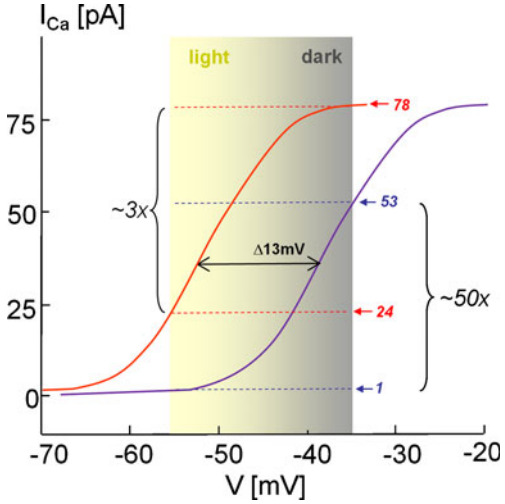

Fig. 4 Functional CSNB2 mutations in $Ca_v1.4$ α1 cause a decreased dynamic range of photoreceptor signaling: the operation range of photoreceptors (between -35 mV (*dark*) and approximately -55 mV (*light*) is near the foot of the I_{Ca} activation curve at physiological Ca^{2+} concentrations to ensure Ca^{2+} influx necessary for tonic glutamate release (see also text). A hyperpolarizing shift of the current–voltage relationship (*I–V*) is predicted to result in higher glutamate release at a given illumination level, causing a decreased dynamic range of photoreceptor signaling (here shown for mutation K1591X). According to the L-type current *I–V* relationship measured in photoreceptors (*black curve* [80]), a 13-mV hyperpolarizing shift of the I_{Ca} *I–V* relationship as observed for K1591X [67] would predict a smaller increase of I_{Ca} and exocytosis (predicted: normal ~50-fold, K1591X ~3-fold) when moving from the light (-55 mV) to the dark membrane potential (-35 mV)

In addition to CACANA1F, mutations in other genes can also cause incomplete forms of CSNB. Ca^{2+}-binding protein 4 (CaBP4) belongs to a protein family structurally similar to calmodulin (CaM). It is specifically found in photoreceptor synaptic terminals [29], modulates $Ca_v1.4$ Ca^{2+} channels by binding to the C-terminus [29], and the phenotype of $CaBP^{-/-}$ mice shares similarities with that of CSNB2 patients [29]. It therefore appeared as a disease candidate in CSNB2 patients without CACNA1F mutations. Zeitz and colleagues indeed found mutations in CaBP4 that account for an autosomal recessive form of CSNB2.

A homozygous nonsense mutation in the human gene for the accessory Ca^{2+} channel α2-δ4-subunit (CACNA2D4) was also found in patients with an electronegative electroretinogram and an initial diagnosis of night blindness [88]. Detailed clinical examination finally revealed a mild form of cone dystrophy. In mice, a protein-truncating frameshift of this subunit leads to abnormal electroretinograms, a reduction in the photoreceptor synaptic layer and a profound loss of synaptic ribbons between rods and rod bipolar cells [64, 87]. This emphasizes a key role of this accessory subunit for normal retinal function in humans and mice.

A truncating CSNB2 mutation reveals an intrinsic gating modulator in $Ca_v1.4$

Upon functional characterization of the CSNB2 C-terminal truncation mutant K1591X, Singh et al. [67] recently discovered that the absence of CDI in $Ca_v1.4$ channels is due to its active suppression by a C-terminal inhibitory domain. Like other VGCCs (such as $Ca_v1.2$ and $Ca_v1.3$) $Ca_v1.4$ channels are capable of undergoing robust CDI in a CaM-dependent manner [67] when this inhibitory domain is removed. In wild-type $Ca_v1.4$, this intrinsic gating modulator resides within the C-terminal tail downstream of an IQ domain which is required for CaM binding (Figs. 5 and 6). K1591X channels lack this modulator and therefore exhibit fast CaM-dependent CDI and a more negative activation voltage range than the wild type. These findings [67, 27, 84] revealed inhibition of CDI as a novel modulatory concept that contributes to the fine-tuning of $Ca_v1.4$ gating to prevent inactivation and thus support tonic neurotransmitter release in sensory cells and normal visual function in humans. The molecular basis of this modulatory mechanism itself is discussed controversially. Wahl-Schott and colleagues postulated binding of the distal C-terminus (termed ICDI, inhibitor of CDI, in their publication) to the EF hand motif in the proximal C-terminus, thereby, uncoupling the EF hand from the Ca^{2+} sensing apparatus. Based on their co-immunoprecipitation studies, loss of CaM-interaction with the C-terminus as underlying mechanism was excluded [84]. Instead, Singh and colleagues [67] postulated that the distal C-terminus (ICDI) binds to a segment comprising the EF hand, the pre-IQ and the IQ regions (Fig. 5a). In addition, their functional experiments also suggested a role for the post-IQ domain. Notably, they found that deletion of the C-terminal domain not only restored robust CDI but also induced a strong hyperpolarizing shift of the voltage dependence of $Ca_v1.4$ activation [67]. Therefore, they termed this domain "C-terminal modulator" (CTM) instead of ICDI, emphasizing this additional regulatory effect. Protein–protein interactions of C-terminal channel fragments and CaM expressed in HEK-293 cells measured using fluorescence resonance energy transfer (FRET), revealed that at resting calcium concentrations, apo-CaM binds to a C-terminal fragment containing the known CaM binding domains identified previously in other L-type Ca^{2+} channels (pre-IQ, IQ domains; [23, 60, 94]). Calcification of CaM at higher Ca^{2+} concentrations further stimulated CaM binding. In contrast, when the complete C-terminus was expressed (also containing the CTM) no apo-CaM binding occurred at resting Ca^{2+} concentrations (Fig. 5b) but was restored at higher Ca^{2+} concentrations, suggesting that the CTM modulates pre-association of CaM with the C-terminus. This could explain the lack of CDI in the wild-type $Ca_v1.4$

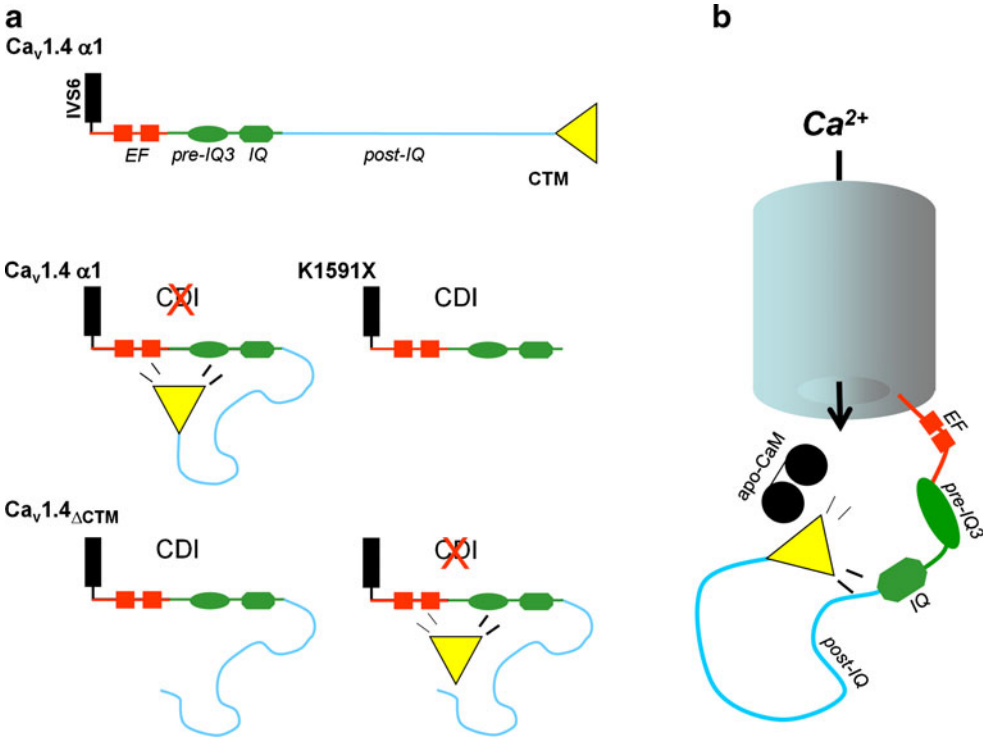

Fig. 5 Hypothetical model of $Ca_v1.4$ C-terminal modulation. **a** Motifs previously demonstrated to be important for CaM modulation of other Ca^{2+} isoforms (*red*: EF hand; *green*: pre-IQ regions, IQ domain) are illustrated. In wild-type $Ca_v1.4$ channels, the CTM predominantly interacts with a region comprising the EF hand, pre-IQ, and IQ domains and thereby inhibits CDI [67]. The CTM and the post-IQ motif (*light blue*) are missing in truncation mutant K1591X and therefore intrinsic CDI of $Ca_v1.4$ becomes apparent. CDI is present after deletion of the last 122 residues which comprises the CTM. When co-expressed with the truncated channel ($Ca_v1.4_{\Delta CTM}$), the CTM-peptide inhibits CDI and restores wild-type gating properties. This modulation requires the presence of the post-IQ region. In addition, Singh et al. imply a role of the post-IQ motif for voltage-dependent inactivation [67]. **b** As shown in FRET experiments [70], the $Ca_v1.4$ CTM interferes with CaM binding to one or more sites responsible for CaM pre-association (apo-CaM) in intact cells. Therefore, interference with CaM coordination is suggested, the likely mechanism explaining the inhibition of CDI

channel. By generation of different $Ca_v1.4$ truncation mutants, the critical residues comprising the CTM (and ICDI) were restricted to a stretch of about 25 amino acid residues within the distal C-terminus, which is highly conserved between $Ca_v1.4$, $Ca_v1.3$, and $Ca_v1.2$ (Fig. 6). Further FRET data were recently reported by the Biel group [27], which support the hypothesis that motifs further downstream of the EF hand are important for the intramolecular interaction in the $Ca_v1.4$ α1 C-terminus. More recently, David Yue's group confirmed the interference of the CTM with apoCaM binding. They provided evidence for a competitive mechanism in which CTM reduces the apparent affinity for apoCaM for the channel [46]. As the concentration of the CTM remains constant, the channel occupancy by apoCaM (and therefore CDI) becomes a function of the intracellular concentration of CaM.

CSNB2 mutations reveal an intrinsic gating modulator in $Ca_v1.3$

$Ca_v1.4$ α1 subunit mutations have provided valuable insight into the molecular mechanisms underlying the regulation not only of $Ca_v1.4$ but also $Ca_v1.3$ LTCCs. Given the high sequence homology in the C-terminus of LTCCs (Fig. 6), channel modulation by an intramolecular C-terminal protein–protein interaction may represent a general regulatory concept of LTCCs not limited to $Ca_v1.4$. Notably, alternative splicing in exon 42 in the C-terminus of $Ca_v1.3$ channels gives rise to naturally occurring channels with different lengths [35, 66]. Singh and colleagues [66] exploited the presence of a $Ca_v1.3$ CTM by functionally investigating the two human $Ca_v1.3$ α1 subunit splice variants. Similar to the $Ca_v1.4$ truncation mutant K1591X, the short splice form terminates shortly after the IQ motif, and therefore, also lacks the conserved region forming the CTM (Fig. 6). Indeed, the existence of a C-terminal modulation in human $Ca_v1.3$ is manifested by the pronounced gating differences between the long and short splice variant. This revealed an exciting novel mechanism by which $Ca_v1.3$ channel activity can be adjusted by splicing. Like $Ca_v1.4$ K1491X, the absence of the CTM in the short splice form led to $Ca_v1.3$ channels that activate and inactivate at lower voltages, resulting in a hyperpolarizing shift in the window current. Its stronger

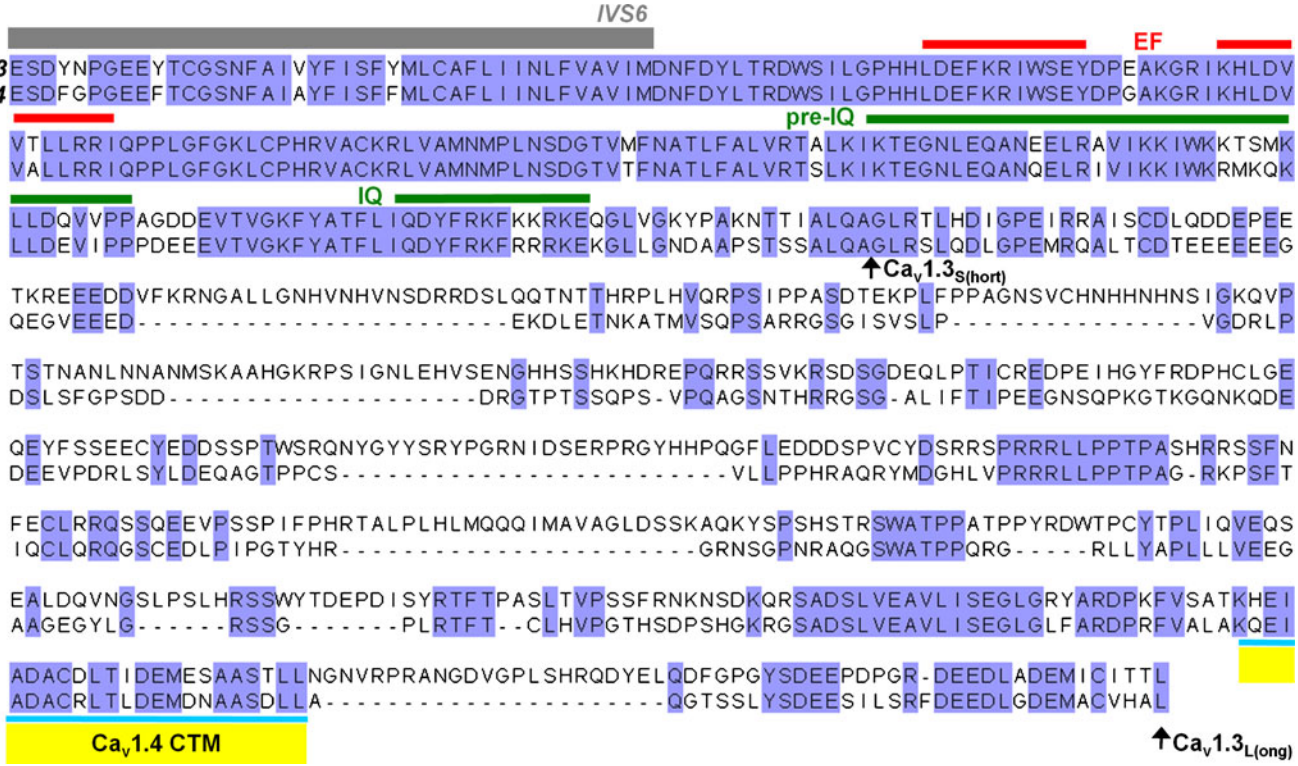

Fig. 6 Sequence alignment of C-terminal tails of human $Ca_v1.3$ and $Ca_v1.4$ L-type channels: a sequence alignment of human $Ca_v1.3$ (Genbank accession number EU363339) and $Ca_v1.4$ (Genbank accession number AJ224874) $\alpha 1$ subunits is shown. Sequence identity (*blue*) and gaps (-) are indicated. Regions previously shown to be important for channel modulation by CaM in other voltage-gated Ca^{2+} channel isoforms are depicted (EF hand, pre-IQ, and IQ domain). The position of long and short $Ca_v1.3$ channels is indicated by *black arrows* ($Ca_v1.3_L$ and $Ca_v1.3_S$, respectively). Position of the $Ca_v1.4$ CTM is given in *yellow*; - indicates residues absent in this sequence

CDI also caused more pronounced inactivation of I_{Ca} without affecting the voltage-dependent inactivation (VDI) time course. Interestingly, this regulation has not been reported for rat $Ca_v1.3$ analogs [89]. Many unique physiological functions of $Ca_v1.3$, including sensory and neuroendocrine cell signaling [49, 50, 62], pacemaking in neurons [57] and sinoatrial node cells [47], as well as its proposed role in the pathology of Parkinson's disease [15, 28] depend on the negative activation range and the amount of Ca^{2+} ions entering during plateau [57] or single action potentials [30]. Accordingly, the $Ca_v1.3$-CTM and factors that modify its activity (such as alternative splicing or interaction with other proteins [8, 43, 93]) appear as crucial determinants of electrical excitability. It can be predicted that the expression of short $Ca_v1.3$ channels would allow a cell to promote Ca^{2+} entry through $Ca_v1.3$ channels at sub-threshold voltages due to the more negative window current. Stronger activation at more negative voltages may also facilitate the onset of upstate potentials in neurons. Whereas negative activation of an even small $Ca_v1.3$ current could trigger pacemaking, faster CDI would limit Ca^{2+} entry during ensuing action potentials. This effect may be important in neurons which are susceptible to Ca^{2+} toxicity and neurodegeneration in Parkinson's disease [15]. In contrast, the CTM in the long $Ca_v1.3$ channels may be required for longer lasting Ca^{2+} signals triggered by stronger depolarization inducing cyclic adenosin mono-phosphate response element binding protein (CREB) phosphorylation and synaptic plasticity [92], or in sensory cells with tonic neurotransmitter release, such as cochlear inner hair cells or photoreceptors [62, 91] .

Non L-type Ca^{2+} channelopathies leading to altered LTCC function

Brain LTCCs are mainly located at somatodendritic locations. Rather than contributing to fast neurotransmitter release at nerve terminals, their somatodendritic Ca^{2+} signals play a major role in coupling synaptic activity to gene-transcription through different intracellular signaling pathways (for review, see [18]). These properties allow them to contribute to synaptic plasticity and control neuronal functions of pharmacotherapeutic relevance, including drug taking behavior, mood behavior, and fear memory (for reviews, see [18, 78]). Due to this special role, the question arises whether pathological changes in other (i.e., non-L-type) Ca^{2+} channel isoforms [14] can lead to *secondary* changes in LTCC expression and thereby allow them to contribute to disease-

related processes. This question has already been addressed in *tottering* mice, a natural mouse mutant. The *tottering* phenotype, an autosomal recessive mouse disease, is associated with mild ataxia, spontaneous behavioral arrest associated with synchronous, bilateral cortical polyspike discharges (resembling human absence epilepsy), and attacks of paroxysmal dystonia [10, 61]. A missense mutation (P601L, IIS5-S6 pore-loop) in the $Ca_v2.1$ $\alpha1$ subunit (forming P/Q-type Ca^{2+} channels, [14]) was found to underlie this phenotype (for review, see [61]). Interestingly, the paroxysmal dystonic symptoms, which can be reproducibly triggered, e.g., by immobilization stress, are prevented by subcutaneous or intracerebroventricular injection of different chemical classes of LTCC blockers, whereas ataxia is not ameliorated [10]. In accordance with these findings, dystonic episodes in *tottering* are also triggered by the LTCC activator Bay K8644 at doses not affecting wild-type mice [10]. Biochemical studies revealed significant upregulation of $Ca_v1.2$ $\alpha1$ subunits in *tottering* brains. Enhanced expression is mainly restricted to cerebellar Purkinje cells, suggesting that LTCCs in these cells can mediate episodic dystonia. This finding is surprising because LTCC expression in these neurons is very low, thus mediating only about 7% of the total Ca^{2+} channel current [21, 38]. L-type currents increased by 2.2-fold were recorded from *tottering* Purkinje cells already at early postnatal stages (P15), indicating developmental changes preceding the appearance of behavioral deficits [22]. Interestingly, $Ca_v2.1$-deficient mice, which also develop severe dystonia, show an increased contribution of L-type currents in Purkinje but not in cerebellar granule cells [38]. Somehow, altered $Ca_v2.1$ channel signaling appears to activate pathways that enhance $Ca_v1.2$ (but not $Ca_v1.3$; [38]) LTCC expression. The finding that enhanced LTCC expression and most likely activity in Purkinje cells contributes to the paroxysmal dystonia of the *tottering* phenotype is in good agreement with the observation that dystonic episodes lead to neuronal activation in the cerebellum and its relay nuclei in these mice [9], and that the dystonic phenotype is absent in *tottering* mice lacking Purkinje cells [11].

Further support for an isoform-specific role of $Ca_v1.2$ LTCCs in the induction of dystonic behavior comes from a mouse mutant in that a single targeted mutation within the dihydropyridine binding pocket eliminates BayK 8644 sensitivity but causes no detectable changes in $Ca_v1.2$ function and expression [68]. These mice are completely resistant to the typical BayK 8644-induced dystonic behavior observed in wild-type mice [37], indicating that this drug effect cannot be mediated by $Ca_v1.3$ activation alone but requires $Ca_v1.2$ [68].

Taken together, these findings are an important first step to address the general question about the role of LTCCs for the pathophysiology of paroxysmal dyskinesias. As demonstrated here, dysregulation of these channels, even in neurons where they only contribute marginally to total Ca^{2+} channel currents, can be relevant for disease.

Conclusions

So far, Ca^{2+} channelopathies have been described for $Ca_v1.1$, Cav1.2, and $Ca_v1.4$, but not yet for $Ca_v1.3$ LTCCs. $Ca_v1.1$ mutations associated with HPP-1 have provided valuable insight into the function of the voltage-sensing domains of voltage-gated Ca^{2+} channels and their dual role as voltage sensors and ion pores. Although the molecular details of how $\alpha1$ mutations sensitize excitation–contraction coupling between plasmalemmal $Ca_v1.1$ and SR RyR1 in skeletal muscle, and thereby cause susceptibility to MH, are not yet fully understood, they point to functionally critical regions in $\alpha1$ which were not detected in previous mutational studies investigating the orthograde coupling between these two ion channels. Finally, $Ca_v1.4$ mutations led to the discovery of a novel intramolecular protein interaction by which LTCCs modulate their gating behavior. This opened a new field of research also on $Ca_v1.3$ channels, which use this mechanism to adjust their activity by intracellular Ca^{2+} activity and alternative splicing. Given their delicate role in the pathophysiology of Parkinson's disease, this mechanism may also become a target for the development of novel therapies.

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