Rare *CACNA1A* mutations leading to congenital ataxia

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Abstract

Human mutations in the *CACNA1A* gene that encodes the pore-forming α_{1A} subunit of the voltage-gated Cav2.1 (P/Q-type) Ca^{2+} channel cause multiple neurological disorders including sporadic and familial hemiplegic migraine, as well as cerebellar pathologies such as episodic ataxia, progressive ataxia, and early-onset cerebellar syndrome consistent with the definition of congenital ataxia (CA), with presentation before the age of two years. Such a pathological role is in accordance with the physiological relevance of Cav2.1 in neuronal tissue, especially in the cerebellum. This review deals with the report of the main clinical features defining CA, along with the presentation of an increasing number of *CACNA1A* genetic variants linked to this severe cerebellar disorder in the context of Ca^{2+} homeostasis alteration. Moreover, the review describes each pathological mutation according to structural location and known molecular and cellular functional effects in both heterologous expression systems and animal models. In view of this information in correlation with the clinical phenotype, we take into consideration different pathomechanisms underlying the observed motor dysfunction in CA patients carrying *CACNA1A* mutations. Present therapeutic management in CA and options for the development of future personalized treatment based on Cav2.1 dysfunction are also discussed.

Keywords

Congenital ataxia; Cerebellar atrophy; Permanent ataxia; Early-onset cerebellar signs; *CACNA1A* gene; Cav2.1 (P/Q-type) voltage-dependent calcium channel

Declarations

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Introduction

The term congenital ataxia (CA) is applied to children with early evidence and symptoms of ataxia caused by cerebellar disease. However, the infant affected by a cerebellar disease does not show the typical signs of cerebellar syndrome, which can be detected later in life, due to the immaturity of the central nervous system and the chronology of motor development. This may delay clinical suspicion as the predominant signs at that moment are unspecific: muscular hypotonia, weak deep tendon reflexes, and delayed motor milestones. Since clear signs of a cerebellar syndrome, including ataxia, dysmetria, and dysarthria (speech delay), are usually not seen before the second or third year [7], the determination of CA is normally made retrospectively.

Another difficulty for the early identification of congenital cerebellar ataxia is the lack of clinical, radiological, or laboratory markers; like many genetic conditions, cerebellar atrophy may not be present in the first magnetic resonances. Cerebellar atrophy, detected with magnetic resonance imaging (MRI) as a widening of the interfolia spaces in the cerebellum, usually progresses during childhood [105], even when there is a lack of clinical correlation and the patients improve in milestone acquisitions. Some genetic disorders, particularly inborn errors of metabolism, may have blood biomarkers that should be studied to rule out treatable disorders [67]. However, this is not the case with channelopathies.

Pathogenic variants in the *CACNA1A* gene (encoding the pore-forming α_{1A} subunit of the Cav2.1 (P/Q-type) Ca^{2+} channel) have been related to several clinical phenotypes. Of these, some may be present early in life, and with some patients it is truly difficult to establish a unique phenotype, as the symptoms and the phenotypes may overlap.

Probably the earliest detectable syndrome related to pathogenic variants in *CACNA1A* is early infantile epileptic encephalopathy 42 (EIEE42, MIM#617106). It is associated with early onset epilepsy which interferes with normal neurodevelopment and impedes the possibility of detecting the above-mentioned unspecific signs of cerebellar involvement early in life [36]. However, patients develop cerebellar atrophy, showing a widening of the interfolia spaces on MRI. But epileptic encephalopathy predominates in the clinical symptoms [37]. The literature includes those pathogenic variants more as a cause of epileptic encephalopathy than a cause of cerebellar syndrome. Nonetheless, they probably overlap, as it has been recently reported in other calcium channelopathies (related to the *CACNA1G* gene, encoding the pore-forming α_{1G} subunit of the Cav3.1 (T-type) Ca^{2+} channel).

Concerning congenital or early onset ataxia related to pathogenic variants in *CACNA1A*, two phenotypes have been described, both with an underlying variable but chronic cerebellar syndrome with acute symptoms added episodically: episodic ataxia type 2 (EA2), MIM#108500 and familial hemiplegic migraine (FHM) with progressive cerebellar ataxia, MIM#141500. The youngest reported patients with specific cerebellar symptoms were 2 and 5 years of age, respectively, but there are reports of earlier symptoms with delayed developmental delay from the first months of life [88].

The complexity of both situations (difficulties in the early identification of CA and the diversity and frequent overlapping of the phenotypes related to *CACNA1A*) leave clinicians in a difficult position in terms of making an early diagnosis and determining the prognosis. Below we will review, within the guidelines of evidence following Scottish Intercollegiate Guidelines Network (SIGN) grading system (<http://www.sign.ac.uk>), the scientific knowledge concerning the pathogenic variants related to *CACNA1A* and CA, their impact on Cav2.1 channel gating and activity, and the correlation with phenotype, with a particular focus on management and treatment.

Structural and physiological features of the Cav2.1 channel

The Cav2.1 (P/Q-type) Ca²⁺ channel is a high-voltage-activated channel consisting of a principal pore-forming subunit (α_{1A} or CACNA1A), associated with auxiliary subunits (at least, the $\alpha_2\delta$ dimer and one or more β subunits (Cav β_{1-4})). As with the other nine members of the Cav family, the α_{1A} subunit consists of four repeated homologous domains (DI–DIV) each with six transmembrane regions (S1–S6) that make up two functional modules: a voltage sensor (S1–S4) and a Ca²⁺-selective pore (S5–P loop–S6) (Figs. 1A and 2) [21]. The N- and C-termini along with the intracellular loops between α_{1A} homologous domains serve as a platform for channel gating regulation through multiple intracellular signaling pathways, including presynaptic proteins [8, 21, 29, 64, 102, 103, 146] and Ca²⁺ influx through the channel in a calmodulin-dependent manner [19, 21, 24, 73, 104].

As with other voltage-gated ion channels, Cav2.1 opening induced by membrane depolarization is due to the movement of 5–6 positively charged residues located at the S4 α -helices (Arg⁺ (R) or Lys⁺ (K) periodically aligned, every 3rd–4th amino acid (R0–R5 positions), with intervening hydrophobic residues). They generate the conformational changes required for pore opening [1]. These S4 “gating charges” translocate intracellularly through a transmembrane electric field formed by the S1–S3 helices. In particular, conserved negative, polar, and hydrophobic residues on the S2 helices and an invariant aspartate residue

on the S3 segments are necessary contributors to the energy pathway for S4 gating charge movement [16, 20, 51, 93, 113]. This displacement in turn allows voltage sensor coupling to pore opening, most probably by an elbow connecting S4 to the S4-S5 linker, supporting a “sliding helix” mechanism of gating as suggested for voltage-gated Na⁺ channels [138].

Ca_v2.1 channel expression is mostly restricted to neuroendocrine cells and neurons throughout the nervous system. There, Ca_v2.1 channels are located at presynaptic terminals [137] in close coupling to the release of neurotransmitters [21]. They are also present in somatodendritic membranes, participating in the control of neuronal firing [86], gene expression [111], intracellular signaling [35], and neuronal viability [39].

Human Ca_v2.1 pathophysiology

Ca_v2.1 is expressed in all brain areas involved in the pathogenesis of migraine (including cerebral cortex, the trigeminal ganglia, and brainstem nuclei involved in nociception) [95, 98]. Increased or decreased Ca_v2.1-mediated Ca²⁺ influx has been linked to different clinical features. Most cases of sporadic and familial hemiplegic migraine (S/FHM) relate to gain-of-function mutations on the *CACNA1A* gene that specifically enhance excitatory synaptic transmission at cortical synapses [95, 126], mainly due to a reduction in the voltage threshold of channel activation [4, 17, 50, 52, 71, 72, 76, 87, 95, 103, 118–120] (as illustrated in Fig. 3). Such cortical hyperexcitability may be further enhanced by the lower direct G protein-mediated inhibition of Ca_v2.1 channels reported for some FHM mutations [46, 47, 81, 103, 136]. Taken together, these factors encourage initiation and propagation of cortical spreading depression (CSD), a key process in migraine pathophysiology [34, 76, 77, 95].

Hemiplegic Migraine (HM)-linked *CACNA1A* gain-of-function mutations often produce cerebellar symptoms as well [4, 10, 17, 33, 38, 45, 68, 69, 88, 116, 131]. This is not surprising given the particularly high Ca_v2.1 expression in the cerebellum [94, 98]. Hence, different forms of ataxia (including some with cerebellar atrophy) are also associated with alteration of a narrow window of Ca²⁺ homeostasis in cerebellar neurons produced by either *CACNA1A* loss- or gain-of-function mutations [4, 25, 26, 44, 45, 49, 58, 59, 94, 97, 99, 101, 102, 108, 110, 116, 121, 129, 131, 133–135, 147]. Indeed, clinicians are aware of a widely recognized phenotypic overlap between HM and diverse forms of cerebellar dysfunction, including EA2, spinocerebellar ataxia type 6 (SCA6), and cerebellar syndrome [61, 79, 94]. This feature is extensive to CA as well, where *CACNA1A* is lately emerging as one of the important disease-causative genes. At least 17 different *CACNA1A* mutations have been reported in association with CA or with permanent ataxia or early-

onset cerebellar signs, consistent with CA [4, 10, 15, 33, 42, 45, 48, 68, 75, 88, 91, 112, 116, 121, 124, 130, 131] (Figs. 1A and 2, Table 1). All of them affect Cav2.1 residues highly conserved through evolution (see Fig. 4 for comparison of orthologous Cav2.1 channels) and with an important level of conservation among the 10 members of the human Cav channel family (Cav1.1-Cav1.4, Cav2.1-Cav2.3 and Cav3.1- Cav3.3) (Fig. 5).

Molecular distribution, functional consequences, and clinical features of CA-linked Cav2.1 mutations

Although structural data regarding the whole Cav2.1 channel complex are not yet available, the cryo-electron microscopy (cryo-EM) structure of the rabbit Cav1.1 complex, containing the pore-forming α_{1S} and several regulatory subunits [139], is of great help for the proper localization of CA-linked Cav2.1 mutations, given their high level of homology (Fig. 1B). In view of that, they distribute around the pore regions of Cav2.1 domains II (T666M, I712V, D715E), III (Y1385C, V1396M, Δ F1502), and IV (V1696F, S1799L, I1811L), the voltage sensors of Cav2.1 domains III (I1338T, I1342T, R1350Q/R1349Q, R1352Q, R1359W), and IV (R1664Q, R1673P), and the S4-S5 linker of Cav2.1 domain II (S218L) (Figs. 1A and 2, Table 1).

Mutations located at Ca²⁺-selective pore modules of Cav2.1

Regarding the mutations affecting the pore regions, there are functional studies for five of them. T666M (located at the selectivity filter in domain II) and I1811L (at the S6 segment of α_{1A} domain IV and contributing to the inner pore vestibule of the channel) induce a gain-of-function effect due to a shift in the Cav2.1 channel activation to lower voltages by $\sim$ 6-7 mV [71, 119]. T666M is one of the most frequently reported pathogenic variants and has been linked to a very wide spectrum of symptoms (Table 1) with a level of evidence: 2- (based on single patients or small cohorts of patients), and grade of recommendation: C (based on non-analytical studies) (SIGN grading system E:2-, R:C). Age and form of presentation may vary from 9 months of age, with a unspecific delayed psychomotor development [88], to an oligosymptomatic form of migraine without aura in adulthood [131]. Considering the chronic symptoms related to this variant, the reported features are from normal neurological exam to mild or moderate ataxia. About intellectual symptoms, normal and mild intellectual disabilities have been reported. Concerning the acute phenotype, episodic coma with focal deficits and S/FHM episodes in a sort of monomorphic presentation in every patient, but not intrafamilial, has been reported. In some reported cases, acetazolamide (more frequently used) and verapamil have shown positive responses in preventing the recurrence of the

acute phenotype (migraine, S/FHM or acute coma); however there are no clinical trials evidencing this benefit. MRI study results have ranged from normal posterior fossa structures to cerebellar atrophy restricted to the vermis or generalized. Although there are no correlational studies, it seems that in the literature there might be a good correlation between the severity of the clinical chronic symptomatology and the abnormalities in neuroimaging. Interestingly, the wide differences reported both in clinical pictures and neuroimaging can occur even in the same pedigree [41, 131].

I1811L has been not so frequently reported and the described phenotype includes a variable cerebellar syndrome with normal to mild intellectual disability and a predominance of FHM in the clinical picture (SIGN grading system E:2-; R:C) (Table 1) [116].

D715E and Δ F1502 (located at the S6 segment of α_{1A} domains II and III, respectively, contributing to the inner pore vestibule of the channel) dramatically decreases the voltage threshold for channel activation by ~ 17 -21 mV [4, 72]. In the case of mutation Δ F1502, this large enhancement of channel gating by voltage is accompanied by other gain-of-function effects including the acceleration of Cav2.1 activation kinetics and the slowing of channel deactivation and inactivation kinetics. Thus, despite a reduction in current density and the enhanced inactivation by voltage also found in the mutant channel (loss-of-function effects), the final consequence of mutation Δ F1502 is a huge increase in Ca^{2+} influx after stimulation of neuronal physiological relevance, including single action potentials and trains of action potentials [4]. Both pathogenic variants have been related to chronic cerebellar syndrome appearing in infancy/childhood with acute HM events associating focal deficits and epileptic seizures. Acetazolamide in some cases has been proven to be effective in limiting the frequency of the acute events (SIGN grading system E:2-; R:C) (Table 1) [4, 32, 33, 45, 72].

V1396M, due to its location close to the beginning of the pore-loop between S5 and S6 helices of α_{1A} domain III, could produce altered Ca^{2+} flux through the channel. The affected residue is in a region of invariant amino acids conserved in the rabbit Cav1.1 (Fig. 1B), in Cav2.1 of different species (Fig. 4) and in all human Cav channels (Fig. 5). Furthermore, this initial pore-loop region contributes to both a potential main entrance of Ca^{2+} to the selectivity filter vestibule and to the docking site for the $\alpha_2\delta$ subunit [139]. Consequently, valine to methionine substitution might alter the hydrophobic interactions with neighboring residues, modifying the three-dimensional arrangement of the helices in the pore domain and affecting not

just the flux of Ca^{2+} but also interaction with the above-mentioned regulatory subunit, which is essential for the proper functional expression of the pore-forming α_{1A} subunit [30]. In support of this hypothesis, the murine Cav2.1 channel carrying the equivalent V1396M mutation (V1347M) shows higher current density than the wild-type (WT) channel in a heterologous expression system [62]. Besides, as reported for the other CA-linked mutations described above, the introduction of methionine also hyperpolarizes (by ~ 5 mV) the voltage dependence activation of Cav2.1 [62]. V1396M has been described in patients presenting with CA and early onset epileptic encephalopathy including different kinds of refractory generalized seizures (myoclonic, tonic-clonic, atonic...) and sometimes fulfilling Lennox Gastout syndrome criteria [62, 75, 121]. As a consequence, neurodevelopment is severely affected, and severe intellectual disability has been described in the most severely affected patients. Additionally, patients harboring the V1396M variant may show acute episodes of coma and HM (SIGN grading system E:2-; R:C) (Table 1).

Although no functional data are available for CA-linked mutations I712V, Y1385C, V1696F, and S1799L, they might well induce gain-of-function effects on channel gating similar to those produced by the other pore mutations. Indeed, they share identical or close structural locations: the S6 segments lining the inner pore of the channel (I712V and D715E at α_{1A} domain II, Δ F1502 at α_{1A} domain III, S1799L and I1811L at α_{1A} domain IV) and the S5 segments that also make up the conduction pathway near the inner mouth of the pore and participate in the conformational rearrangements occurring during channel gating (Y1385C and V1396M at α_{1A} domain III, and V1696F at α_{1A} domain IV) [139]. Accordingly, another mutation affecting the V1696 residue that was found in association with FHM (V1696I) [32] reduces (by ~ 4 mV) the threshold for Cav2.1 voltage-dependent activation, slows channel inactivation, and lessens the extent of G protein-mediated inhibition by impairing interaction of the $\text{G}\beta\gamma$ dimer with Cav2.1 [47, 87].

Recurrence and severity of the FHM episodes may lead to supratentorial abnormalities such as progressive bilateral mesiotemporal sclerosis, reported in I712V and Y1385C, as well as cerebellar atrophy, altogether bringing about a neurological regression in the affected patients. In some cases acetazolamide has been effective in reducing the incidence of the episodes and stabilizing some cerebellar symptoms (SIGN grading system E:2-; R:C) (Table 1) [48, 124]. The phenotype related to V1696F is also severe, combining a severe form of cerebellar syndrome, tetraparesis with extrapyramidal movement disorder, epilepsy, and deleterious episodes of FHM that may lead to neurological regression. Some of these episodes overlap with

both alternating hemiplegia and FHM, and were treated with flunarizine in one patient to doubtful effect (SIGN grading system E:2-; R:C) (Table 1) [130].

In general, functional characterization of the different CA-linked Cav2.1 pore mutations unveils a bidirectional relationship within the two functional modules of the channel, voltage sensors, and pore domains. Not only do conformational changes in the voltage sensors during channel stimulation trigger pore opening, but also structural modifications of the pore segments, such as those induced by CA-linked mutations, influence voltage sensor operation, promoting their translocation in response to membrane depolarization [4, 62].

Mutations located at voltage sensitive modules of Cav2.1

Functional characterization of three CA-linked mutations affecting the voltage sensors has also been described. R1350Q (also referred to as R1349Q) affects the R2 residue of the voltage sensor S4 segment of α_{1A} domain III and is homologous to the mouse R1252Q allele of the tottering, *Cacna1atg*, mutant series (Figs. 1, 2, 4 and 5). This murine variant is a gain-of-function mutation that shifts Cav2.1 channel activation to lower voltages by ~12 mV [82]. Interestingly, persistent tonic upgaze has been reported to be the first symptom leading to the diagnosis in patients bearing mutation R1350Q, before the whole clinical spectrum is developed months later, initially, with a global developmental delay (first manifestation of CA), and later, with FHM episodes. Moreover, extrapyramidal (involuntary movement of limbs, horizontal rhythmic head movements, and stereotypic movements) and pyramidal signs have been described (SIGN grading system E:2-; R:C) (Table 1) [9, 10, 15, 112].

The functional consequences of mutations R1664Q and R1673P (altering the gating charges at positions R2 and R5, respectively, of the voltage sensor S4 segment of α_{1A} domain IV, Figs. 1, 2, 4 and 5) have been studied *in vivo* using transgenic flies. When expressing the *Drosophila* equivalent of WT Cav2.1 (cacophony calcium-channel), Cav2.1 R1664Q, or Cav2.1 R1673P in a Cav2.1-deficient background, the R1673P mutant channel was able to rescue synaptic transmission in photoreceptors in 3-day-old larvae to a greater extent than the WT channel, whereas Cav2.1 R1664Q failed to rescue the synaptic transmission defect of Cav2.1-deficient flies. These results suggest that R1673P and R1664Q are gain- and loss-of-function mutations, respectively [75]. Furthermore, at 30 days the rescue of synaptic function observed for Cav2.1 R1673P was not present; substantial photoreceptor degeneration was observed only in those flies

expressing the R1673P channel but never in those with the WT or R1664Q channels nor in Cav2.1-deficient flies. Therefore, it can be argued that the early gain-of-function favoring Ca²⁺ influx may end later in neurodegeneration due to Ca²⁺ toxicity [75]. In contrast, more recently analysis of the Cav2.1 R1673P rat ortholog (R1624P) (Fig. 4) in a heterologous expression system provides evidences to consider this genetic variant as a loss-of-function mutation due to a reduction in Ca²⁺ current density, an increase in the threshold for channel activation by ~ 25 mV, and accelerated kinetic of inactivation [123]. Clarifying the final effect of mutations R1664Q and R1673P in human Cav2.1 activity at neuronal level will require further research. From the phenotypical point of view, R1664Q seems to be related to two kinds of phenotypes among the reported patients: those presenting a congenital cerebellar syndrome and those presenting episodic ataxia that may respond to acetazolamide therapy to prevent the episodes (SIGN grading system E:2-; R:C) [28, 75, 117]. To date, in relation to R1664Q, migraine without focal deficits has been reported, but not coma episodes or FHM. Similarly, R1673P associates early onset ataxia with psychomotor delay and cerebellar atrophy in neuroimaging, but again in the absence of FHM events (SIGN grading system E:2-; R:C) (Table 1) [123].

Other CA-linked voltage sensor mutations lack functional studies but also affect residues equivalent to the above indicated and might have similar consequences in Cav2.1 activity. This is the case of mutations R1352Q and R1359W, located at the R3 and R5 positions, respectively, of voltage sensor S4 segment of α_{1A} domain III (Figs. 1, 2, 4 and 5). R1352Q has been related to a CA with early oculomotor manifestations and later HM episodes (SIGN grading system E:2-; R:C) (Table 1). However, the reported clinical phenotype for R1359W is different, presenting with early onset epilepsy and development of pyramidal signs, in addition to chronic cerebellar syndrome with global developmental delay. No acute events have been reported (SIGN grading system E:2-; R:C) (Table 1). Nevertheless, more patients harboring this variant should be studied to learn whether the clinical picture is consistent, since the literature is scarce [91].

I1338T and I1342T locate instead at the extracellular linker between S3 and S4 segments of the voltage sensor in α_{1A} domain III. They change hydrophobic residues (highly conserved/semiconserved in all human Cav channels) to polar uncharged amino acids a few residues upstream from the first positively charged residue (R0) of the S4 segment, and therefore it would not be a surprise that these genetic variants alter Cav2.1 voltage sensitivity in a similar way to mutations directly affecting S4 gating charges. In support of

this idea, another mutation (found in association with HM) in this same region, K1336E, shifts Cav2.1 channel activation to lower voltages by ~ 4-10 mV, slows inactivation kinetics, and reduces the direct G-protein-mediated Cav2.1 inhibition by affecting the voltage-dependent dissociation/re-association rates of the Gβγ dimer from the channel complex [46, 87]. Nevertheless, the final demonstration of a similar gain-of-function effect for I1338T and I1342T would require functional characterization of the human mutant channel. Both pathogenic variants have been related to a congenital form of ataxia with global developmental delay, intellectual compromise, and cerebellar atrophy with acute events of SHM (SIGN grading system E:2-; R:C). Patients bearing I1342T mutation may suffer from epilepsy and develop pyramidal signs during their evolution (Table 1).

Finally, functional data are also available for mutation S218L, located in the S4-S5 linker at domain I (Figs. 1A and 2), initially reported in association with S/FHM, slowly progressive cerebellar ataxia and atrophy, epileptic seizures, hemiconvulsion-hemiplegia-epilepsy syndrome, coma or profound stupor, and fatal, cerebral edema [22, 27, 69, 109, 141, 144]. Not surprisingly the term “Early seizures and cerebral oedema after trivial head trauma” (ESCEATHHT) to design these acute events was proposed. Acetazolamide or flunarizine may be successfully used in some patients since, as a matter of fact, the severe and reiterative episodes may lead to permanent hemispheric brain atrophy (SIGN grading system E:2-; R:C) (Table 1) [22, 27, 109, 120, 141]. According to analysis performed in both heterologous expression system and in neurons from knock-in (KI) mice, S218L shows a strong gain-of-function effect due to a decrease in the voltage threshold for Cav2.1 activation by ~ 15 mV that allows Ca²⁺ influx at membrane voltages that are insufficient to open WT channels [50, 120]. This may be enhanced by other gain-of-function effects of the mutation, such as the slowing of inactivation kinetics, the increased rate of recovery from inactivation, and the reduction in direct G-protein Cav2.1 inhibition [120, 136]. Besides, homozygous KI mice carrying the S218L mutation are a good clinical model of the main phenotypic characteristics observed in sick humans, including mild permanent cerebellar ataxia [77]. Therefore, physiological studies from this animal model may help us to understand the link between the molecular mechanisms altering Cav2.1 activity and the clinical phenotype.

Role of Cav2.1 gain- and loss-of-function mutations in congenital ataxia

Focusing on cerebellar function, how does the gain-of-function mutation S218L affect cerebellar performance in KI mice? Purkinje cells (PCs) constitute the only output from the cerebellar cortex allowing movement coordination, maintenance of balance, and motor learning. The cerebellum performs these tasks after integration of multiple motor and sensory synaptic inputs, which arrive at PCs via two main excitatory afferent pathways: the climbing fiber coming from neurons at the inferior olivary nucleus and the parallel fibers provided by cerebellar granule cells. Due to gain-of-function effects, higher Ca^{2+} influx through the mutant $\text{Cav}2.1$ S218L channel at the soma and dendrites of mouse Purkinje Cells (PCs) promotes the generation of somatic action potentials and dendritic Ca^{2+} spikes, and generates irregular activity patterns [44]. Such PCs hyperexcitability is further enhanced by increased Ca^{2+} influx through the mutant channels at presynaptic terminals leading to higher neurotransmitter release from the excitatory afferent neurons mentioned above [44]. Furthermore, the S218L-induced hyperexcitability of PCs underlies the ataxic phenotype in S218L KI mice since both irregular PCs spiking and ataxic motor behaviour are neutralized by activators of the hyperpolarizing small-conductance Ca^{2+} -activated K^+ (SK) channel [44]. These effects on the activity of the cerebellar circuitry can be extrapolated to the rest of CA-linked $\text{Cav}2.1$ gain-of-function mutations reported in humans (which are the majority among those for which functional studies are available; see Table 1). Such abnormal cerebellar activity may explain why CA patients display neurological signs soon after birth and before neuroimaging can disclose the cerebellar atrophy, whose subsequent progression may be related to PCs degeneration due to Ca^{2+} excitotoxicity, as proposed for spinocerebellar ataxias [65].

$\text{Cav}2.1$ loss-of-function has been considered the main pathological mechanism employed by *CACNA1A* mutations linked to some particular ataxic disorders. This applies to SCA6-associated mutations [94, 135, 147] and most EA2-linked genetic variants [25, 58, 59, 94, 97, 101, 102, 108, 110, 129, 133, 134]. Functional analysis in SCA6 KI mice shows that although $\text{Cav}2.1$ biophysical properties in PCs remain unaltered by *CACNA1A* mutations, the presence of expansions of a polyglutamine (polyQ) sequence at the $\text{Cav}2.1$ C-terminal promotes the toxic aggregation of the expanded polyQ channel in an age-dependent manner [135]. This most probably produces the selective PCs degeneration leading to the ataxic phenotype [135]. Studies performed in EA2 mouse models carrying loss-of-function missense or nonsense mutations suggest that the deficit in motor performance results from impaired $\text{Cav}2.1$ activity/expression, producing dysfunction of multiple cerebellar cell types concomitantly [31, 99]. Loss-of-function *CACNA1A* mutations

linked to CA might have similar consequences for the proper development and performance of the cerebellar circuit.

Congenital ataxia is related to mutations affecting cerebellar Ca^{2+} homeostasis

Abundant evidence points to the need for proper Ca^{2+} homeostasis in the cerebellum to avoid pathogenicity. Disruption of Ca^{2+} fluxes in cerebellar neurons produced by mutations in the genes *CA8* (encoding carbonic anhydrase 8 (CA8)), *ITPR1* (encoding inositol 1,4,5-trisphosphate receptor, type 1 (IP₃R1)), *PMCA2* and *PMCA3* (encoding plasma membrane Ca^{2+} ATPases 2 and 3 (PMCA2 and 3), respectively), and *CACNA2D2* (encoding $\alpha_2\delta_2$ auxiliary subunit of high voltage-gated Ca^{2+} channels) also leads to functional and/or developmental defects in the cerebellum, and to CA forms in humans and similar ataxic signs in animal models.

CA8 is mainly expressed in cerebellar PCs, where it binds to the inositol 1,4,5-trisphosphate receptor type 1 (IP₃R1). Such interaction impairs IP₃ binding to IP₃R1 by reducing receptor affinity for IP₃, thereby lessening the ability of IP₃R1 to rapidly release Ca^{2+} from the endoplasmatic reticulum (ER) [54, 83]. CA-linked *CA8* missense mutations exert a deleterious effect on function and expression of *CA8* [66, 122]. Lower CA8 levels enhance ER-mediated intracellular Ca^{2+} signaling that may affect synaptic inputs received by PCs and cause the ataxic phenotype [11, 107]. Reinforcing this hypothesis, *CA8* null mutations or knockdown in animal models results in abnormal cerebellar synaptic morphology and function, increased neuronal cell death in the cerebellum, and defects in motor and coordination behaviors [3, 53, 63].

As indicated above, IP₃R1 is a Ca^{2+} channel placed at intracellular Ca^{2+} stores including the ER. It is highly expressed in cerebellar PCs and opens to allow Ca^{2+} efflux from the ER following the binding of IP₃, a second messenger produced upon the activation of G-protein-coupled receptors at the plasma membrane [6, 83]. Multiple missense mutations in IP₃R1 have been reported in association with CA [5, 18, 40, 55, 57, 89, 91, 100, 106, 143]. In agreement with the increased intracellular Ca^{2+} signaling produced by CA-linked *CA8* genetic variants, some of the IP₃R1 mutations produce channel gain-of-function, either by preventing the inhibitory binding of CA8 or by improving IP₃ binding affinity [2, 18]. Nevertheless, most of the CA-linked IP₃R1 mutations abolish channel activity due to reduced IP₃ binding or impaired channel gating [2]. This again shows that, as seen in *CACNA1A* mutations, an aberrant Ca^{2+} homeostasis in cerebellar neurons due to both upward and downward modifications underlies the pathogenesis of CA.

PMCA2 and 3 are abundantly expressed in the cerebellum, in particular at PCs and at the excitatory presynaptic terminals of granule cells that synapse onto PCs, respectively. Several *PMCA2* and 3 missense mutations are linked to a cerebellar phenotype compatible with CA. Mutated pumps show reduced capacity to extrude Ca^{2+} from the neurons, which may lead to higher Ca^{2+} baseline levels and/or altered local Ca^{2+} signaling, affecting synaptic efficiency and fostering PCs hyperexcitability that ends in the ataxic phenotype [13, 14, 127, 128, 145].

The Ca_v regulatory subunit $\alpha_2\delta_2$ is highly expressed in the cerebellar cortex, where it is essential for the proper assembly and membrane trafficking of the $\text{Ca}_v2.1$ channel complex to increase channel density. Furthermore, $\alpha_2\delta_2$ also modulates $\text{Ca}_v2.1$ voltage-dependent gating and kinetics properties for its appropriate activity [30]. At least one homozygous genetic variant in $\alpha_2\delta_2$ is associated with CA [125]. It affects splicing and produces a truncated form of the regulatory channel subunit [125], which is expected to negatively affect $\text{Ca}_v2.1$ function to explain its pathogenic effect.

Personalized treatment based on $\text{Ca}_v2.1$ dysfunction

Overall, the findings discussed above reveal that there is no general pathomechanism by which alteration of cerebellar Ca^{2+} homeostasis causes CA. Therefore, specific and thoughtful functional characterization of each CA-linked mutation is required to distinguish between gain- and loss-of-function effects in order to formulate a rational, personalized therapeutic approach. Focusing on CA-linked *CACNA1A* mutations, such tailored disease treatment should target either the identified $\text{Ca}_v2.1$ dysfunction or its consequence in the cerebellar neuronal network.

Targeting $\text{Ca}_v2.1$ channel activity

Pharmacological evidence suggests that $\text{Ca}_v2.1$ inhibitors can become novel therapeutic tools to treat *CACNA1A*-linked disorders of the nervous system involving gain of channel function. For example, 2,50-di(tertbutyl)-1,4-benzohydroquinone (BHQ), by inhibiting $\text{Ca}_v2.1$ partially reverts the gain-of-function effects produced on both $\text{Ca}_v2.1$ gating and subsequent synaptic transmission by mutation S218L [60], associated with both CA [121] and a severe form of FHM that includes slowly progressive cerebellar ataxia and atrophy [22, 27, 69, 109, 141, 144]. However, BHQ is in fact an inhibitor of sarcoendoplasmic Ca^{2+} ATPases (SERCAs) that also has pro-oxidant properties [43, 85]. Thus, its lack of selectivity for $\text{Ca}_v2.1$ prevents it from effective therapeutic application due to undesirable side effects.

Furthermore, it has been suggested that Cav2.1 inhibition is, at least in part, the mechanism of action through which medicinal plants (Feverfew, or Butterbur) show efficacy in the relief of migraine in general, as a number of natural inhibitors of the Cav2.1 channel have been isolated from the plant [56].

Furthermore, the employment of the nonspecific Cav channel blocker verapamil has been shown to be successful for the reduction of neuronal hyperexcitability related to increased Cav2.1 activity produced by *CACNA1A* mutations [68].

In view of all these clues, scientists have already highlighted the requirement for selective channel inhibitors to test the potential of Cav2.1 channel negative modulation as a safe and effective treatment for nervous system disorders related to channel gain-of-function [90, 98].

Mirroring this therapeutic strategy, the use of Cav2.1 openers would be expected to restore the channel activity altered by pathological loss-of-function mutations. In this respect, derivatives of roscovitine, a cyclin-dependent kinase (Cdk) inhibitor [80] also displaying agonist action on Cav channels [12, 23, 142], appear to be effective activators of Cav2 channels (including Cav2.1) for further therapeutic development. Roscovitine-derivative compounds have lower Cdk inhibition potency and higher Cav2 agonist efficacy [74, 115]. They slow Cav2 deactivation, which leads to a large increase in presynaptic Ca²⁺ entry and higher neurotransmitter release during neuronal activity. Accordingly, these agonists can partially restore deficient channel activity and synaptic transmission due to impaired Cav2.1 function [115, 123, 140].

Another candidate offering new opportunities for the development of novel and selective Cav2.1 openers is a cyclooctapeptide analogue of ω -agatoxin IVB named EP14. This cyclooctapeptide, which mimics the most preeminent interacting domain of the toxin with Cav2.1, enhances Ca²⁺ flux through the channel and potentiates spontaneous excitatory synaptic transmission in neuronal networks, without affecting cell survival [96].

Targeting Cav2.1 cellular functions

Indirect therapies acting downstream of Cav2.1 channels can be also considered for *CACNA1A*-linked diseases. In particular, therapeutic agents that modulate synaptic efficacy have already been employed. Acetazolamide, a carbonic anhydrase inhibitor, has shown effectiveness in the treatment of diseases associated with both gain- and loss-of-function *CACNA1A* mutations, such as FHM [92] and EA2 [70], respectively. More recently, it has been shown that hypoglycosylation of Cav2.1 channel subunits (α_{1A} and $\alpha_{2\delta}$) induced gain-of-function effects on channel gating that echoed those reported for pathogenic *CACNA1A* mutations linked to FHM and ataxia. Hypoglycosylation is caused by a wide group of diseases

known as congenital disorders of glycosylation (CDG). This finding, accompanied by the great similarity in the clinical, neuroimaging, and neurophysiological characteristics between PMM2-CDG patients and patients with mutations in *CACNA1A*, suggests that increased Cav2.1 activity due to deficient N-glycosylation may contribute to the development of cerebellar syndrome in PMM2-CDG [61]. All these discoveries led to the first clinical trial for acetazolamide repurposing (compassionate use in PMM2-CDG), which produced substantial improvements in cerebellar syndrome in PMM2-CDG patients [79]. The therapeutic mode of action of acetazolamide is not fully understood. For the prevention of neuronal hyperexcitability related to Cav2.1 gain-of-function, it has been suggested that inhibition of carbonic anhydrase in the central nervous system causes accumulation of carbonic acid and the consequent increase in the concentration of H^+ intracellularly. This made K^+ ions shift to the extracellular compartment to buffer the altered acid-base status, producing the hyperpolarization of neurons and increasing their threshold for firing to minimize abnormal and excessive neuronal discharge [84]. The mechanism used by acetazolamide in the treatment of disorders due to Cav2.1 loss-of-function may be related to the recovery of regular pacemaking in PCs [132].

K^+ channel blockers have been approved to treat pathological conditions related to decreased Cav2.1 activity. By increasing the duration of action potentials, and in so doing promoting Ca^{2+} entry through deficient Cav2.1 channels, the administration of either 3,4-diaminopyridine alone or 4-aminopyridine in combination with roscovitine derivatives has proven capable of correcting the Cav2.1-related defective synaptic transmission in this kind of neurological disorders [78, 114].

In conclusion, there is an important range of pharmacological options that may be of use for both the development of new therapeutic tools and for the repurposing of existing drugs for the individualized treatment of CA and other pathologies that involve altered Cav2.1 activity. Choosing the best drugs for this development and repurposing requires adequate prior knowledge of the genetic and functional causes underlying the disease.

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A

B

A

B

Ca _v 2.1		S4-S5 loop		Ca _v 2.1		P-loop	
	DI				DII		
		218				666	
<i>Homo sapiens</i>	210	PSLQVVLK S IMKAMIPLL	227	<i>Homo sapiens</i>	655	PAAIMTVFQIL T GEDWNEVMYDG	677
<i>Mus musculus</i>	212	PSLQVVLK S IMKAMIPLL	229	<i>Mus musculus</i>	657	PAAIMTVFQIL T GEDWNEVMYDG	679
<i>Rattus norvegicus</i>	212	PSLQVVLK S IMKAMIPLL	229	<i>Rattus norvegicus</i>	657	PAAIMTVFQIL T GEDWNEVMYDE	679
<i>Oryctolagus cuniculus</i>	210	PSLQVVLK S IMKAMIPLL	227	<i>Oryctolagus cuniculus</i>	655	PAAIMTVFQIL T GEDWNEVMYDG	677
<i>Pan troglodytes</i>	210	PSLQVVLK S IMKAMIPLL	227	<i>Pan troglodytes</i>	654	PAAIMTVFQIL T GEDWNEVMYDG	676
<i>Bos taurus</i>	210	PSLQVVLK S IMKAMIPLL	227	<i>Bos taurus</i>	656	PAAIMTVFQIL T GEDWNEVMYDG	678
<i>Danio rerio</i>	116	PSLQVVLK S IMKAMIPLL	133	<i>Danio rerio</i>	552	PAAIMTVFQIL T GEDWN M VMYDG	574
<i>Drosophila melanogaster</i>	153	PSLQVVLK S IIKAMAPLL	170	<i>Drosophila melanogaster</i>	608	PIALLTVFQIL T GEDWNEVMYQ Q	630
		*****:***	***			* *:*****	***:
Ca _v 2.1		S4		Ca _v 2.1		S4	
	DI	R0	R1	R2	R3	R4	R5
		1338	1342	1350/1349	1352	1359	
<i>Homo sapiens</i>	1338	DINTIKSLRVLRLRPLK T IKRLP	1361				
<i>Mus musculus</i>	1289	DINTIKSLRVLRLRPLK T IKRLP	1312				
<i>Rattus norvegicus</i>	1289	DINTIKSLRVLRLRPLK T IKRLP	1312				
<i>Oryctolagus cuniculus</i>	1347	DINTIKSLRVLRLRPLK T IKRLP	1370				
<i>Pan troglodytes</i>	1337	DINTIKSLRVLRLRPLK T IKRLP	1360				
<i>Bos taurus</i>	1340	DINTIKSLRVLRLRPLK T IKRLP	1363				
<i>Danio rerio</i>	1148	DISTIKSLRVLRLRPLK T IKRLP	1171				
<i>Drosophila melanogaster</i>	865	NLSTIKSLRVLRLRPLK T IKRVP	888				
		: : .*****:	*				
Ca _v 2.1		S5		Ca _v 2.1		S5	
	DI	1385	1396		DIV	1696	
<i>Homo sapiens</i>	1378	FNILIV Y MLFMFIFAVVAVQLFK G	1401	<i>Homo sapiens</i>	1692	LPY V CLLIAMLFFIYAIIGMQVFG	1715
<i>Mus musculus</i>	1329	FNILIV Y MLFMFIFAVVAVQLFK G	1352	<i>Mus musculus</i>	1643	LPYVCLLIAMLFFIYAIIGMQVFG	1666
<i>Rattus norvegicus</i>	1329	FNILIV Y MLFMFIFAVVAVQLFK G	1352	<i>Rattus norvegicus</i>	1643	LPYVCLLIAMLFFIYAIIGMQVFG	1666
<i>Oryctolagus cuniculus</i>	1387	FNILIV Y MLFMFIFAVVAVQLFK G	1410	<i>Oryctolagus cuniculus</i>	1701	LPYVCLLIAMLFFIYAIIGMQVFG	1724
<i>Pan troglodytes</i>	1377	FNILIV Y MLFMFIFAVVAVQLFK G	1400	<i>Pan troglodytes</i>	1691	LPYVCLLIAMLFFIYAIIGMQVFG	1714
<i>Bos taurus</i>	1380	FNILIV Y MLFMFIFAVVAVQLFK G	1403	<i>Bos taurus</i>	1694	LPYVCLLIAMLFFIYAIIGMQVFG	1717
<i>Danio rerio</i>	1188	LNILIV Y MLFMFIFAVVAVQLFK G	1211	<i>Danio rerio</i>	1503	LPYVCLLIAMLFFIYAIIGMQ L FG	1526
<i>Drosophila melanogaster</i>	905	VNILIV Y ILFQFIFSVIGVQLF N G	928	<i>Drosophila melanogaster</i>	1224	LPYVCLLIAMLFFIYAIIGMQVFG	1247
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Ca _v 2.1		S6		Ca _v 2.1		S6	
	DII	712	715		DIII	1502	
<i>Homo sapiens</i>	691	SIYFIVLTLFGNYTLLNVFLAI A VDN	716	1488	SIFYVVYFVVFPFF F VNIFVALIIIT	1513	1788
<i>Mus musculus</i>	693	SIYFIVLTLFGNYTLLNVFLAI A VDN	718	1439	SIFYVVYFVVFPFF F VNIFVALIIIT	1464	1739
<i>Rattus norvegicus</i>	693	SIYFIVLTLFGNYTLLNVFLAI A VDN	718	1439	SIFYVVYFVVFPFF F VNIFVALIIIT	1464	1739
<i>Oryctolagus cuniculus</i>	691	SIYFIVLTLFGNYTLLNVFLAI A VDN	716	1497	SIFYVVYFVVFPFF F VNIFVALIIIT	1522	1797
<i>Pan troglodytes</i>	690	SIYFIVLTLFGNYTLLNVFLAI A VDN	715	1487	SIFYVVYFVVFPFF F VNIFVALIIIT	1512	1787
<i>Bos taurus</i>	692	SIYFIVLTLFGNYTLLNVFLAI A VDN	717	1490	SIFYVVYFVVFPFF F VNIFVALIIIT	1515	1790
<i>Danio rerio</i>	588	SVFFIVLTLFGNYTLLNVFLAI A VDN	613	1299	SIFYVVYFVVFPFF F VNIFVALIIIT	1324	1593
<i>Drosophila melanogaster</i>	644	SIYFIVLVLFNGYTLLNVFLAI A VDN	669	1017	SIFYIVYFIVFPFF F VNIFVALIIIT	1042	1314
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	DIV	1799	1811		DIV	1799	1811
<i>Homo sapiens</i>	1813	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Homo sapiens</i>	1813	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Mus musculus</i>	1764	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Mus musculus</i>	1764	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Rattus norvegicus</i>	1764	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Rattus norvegicus</i>	1764	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Oryctolagus cuniculus</i>	1822	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Oryctolagus cuniculus</i>	1822	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Pan troglodytes</i>	1812	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Pan troglodytes</i>	1812	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Bos taurus</i>	1815	YFYFVSFIFLCSFLMLNLFVAV I MDN		<i>Bos taurus</i>	1815	YFYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Danio rerio</i>	1618	YLYFVSFIFLCSFLMLNLFVAV I MDN		<i>Danio rerio</i>	1618	YLYFVSFIFLCSFLMLNLFVAV I MDN	
<i>Drosophila melanogaster</i>	1339	YAYFVSFIFFCSFLMLNLFVAV I MDN		<i>Drosophila melanogaster</i>	1339	YAYFVSFIFFCSFLMLNLFVAV I MDN	
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S4-S5 loop

DI

218

Ca_v2.1 210 PSLQVVLK**S**IMKAMIPLL 227

Ca_v1.1 180 PSLQVVLNSIFKAMPLF 197

Ca_v1.2 252 PSLQVVLNSIIKAMVPLL 269

Ca_v1.3 255 PSLQVVLNSIIKAMVPLL 272

Ca_v1.4 221 PSLHIVLNSIMKALVPLL 238

Ca_v2.2 207 PSLQVVLK**S**IMKAMVPLL 224

Ca_v2.3 205 PSLQIVLK**S**IMKAMVPLL 222

Ca_v3.1 195 PSMRILV**T**LLLD**T**LPMLG 212

Ca_v3.2 214 PSMRILV**T**LLLD**T**LPMLG 231

Ca_v3.3 193 PSMRILV**N**LLLD**T**LPMLG 210

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S4

DI

R0 R1 R2 R3 R4 R5

1338 1342 1350/1349 1352 1359

Ca_v2.1 1338 D**I**N**T**I**K**S**L**R**V**L**R**V**L**R**P**L**K**T**I**K**R**L**P** 1361

Ca_v1.1 889 A**I**S**V**V**K**I**L**R**V**L**R**V**L**R**P**L**R**A**I**N**R**A**K** 912

Ca_v1.2 1010 A**I**N**V**V**K**I**L**R**V**L**R**V**L**R**P**L**R**A**I**N**R**A**K** 1033

Ca_v1.3 976 A**I**S**V**V**K**I**L**R**V**L**R**V**L**R**P**L**R**A**I**N**R**A**K** 999

Ca_v1.4 961 A**I**S**V**V**K**I**L**R**V**L**R**V**L**R**P**L**R**A**I**N**R**A**K** 984

Ca_v2.2 1243 D**I**N**T**I**K**S**L**R**V**L**R**V**L**R**P**L**K**T**I**K**R**L**P** 1266

Ca_v2.3 1250 D**I**K**T**I**K**S**L**R**V**L**R**V**L**R**P**L**K**T**I**K**R**L**P** 1273

Ca_v3.1 1373 I**L**G**M**L**R**V**L**R**L**L**R**T**L**R**P**L**R**V**I**S**R**A**Q** 1396

Ca_v3.2 1391 I**L**G**V**L**R**V**L**R**L**L**R**T**L**R**P**L**R**V**I**S**R**A**P** 1414

Ca_v3.3 1267 I**L**G**V**L**R**V**L**R**L**L**R**T**L**R**P**L**R**V**I**S**R**A**P** 1290

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S5

DI

1385 1396

Ca_v2.1 1378 F**N**I**L**I**V****Y**M**L**F**M**F**I**F**A**V**V**A**V**Q**L**F**K**G 1401

Ca_v1.1 929 G**N**I**V**L**V****T**T**L**L**Q**F**M**F**A**C**I**G**V**Q**L**F**K**G 952

Ca_v1.2 1380 G**N**I**V**I**V****T**T**L**L**Q**F**M**F**A**C**I**G**V**Q**L**F**K**G 1403

Ca_v1.3 1322 G**N**I**M**I**V****T**T**L**L**Q**F**M**F**A**C**I**G**V**Q**L**F**K**G 1345

Ca_v1.4 1299 G**N**I**M**I**V****T**T**L**L**Q**F**M**F**A**C**I**G**V**Q**L**F**K**G 1322

Ca_v2.2 1571 L**N**I**L**I**V****Y**M**L**F**M**F**I**F**A**V**I**A**V**Q**L**F**K**G 1594

Ca_v2.3 1579 F**N**I**L**I**V****Y**K**L**F**M**F**I**F**A**V**I**A**V**Q**L**F**K**G 1602

Ca_v3.1 1715 G**N**I**V**V**I**C**C**A**F**F**I**I**F**G**I**L**G**V**Q**L**F**K**G** 1738

Ca_v3.2 1721 G**N**I**V**L**I**C**C**A**F**F**I**I**F**G**I**L**G**V**Q**L**F**K**G** 1744

Ca_v3.3 1591 G**N**I**V**L**I**C**C**A**F**F**I**I**F**G**I**L**G**V**Q**L**F**K**G** 1614

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S6

DI

712 715

Ca_v2.1 691 S**I**Y**F**I**V**L**T**L**F**G**N**Y**T**L**L**N**V**F**L**A**I**A**V**D**N** 716

Ca_v1.1 638 C**I**Y**F**I**I**L**F**V**C**G**N**Y**I**L**L**N**V**F**L**A**I**A**V**D**N** 663

Ca_v1.2 730 C**I**Y**F**I**I**L**F**I**C**G**N**Y**I**L**L**N**V**F**L**A**I**A**V**D**N** 755

Ca_v1.3 729 C**I**Y**F**I**I**L**F**I**C**G**N**Y**I**L**L**N**V**F**L**A**I**A**V**D**N** 754

Ca_v1.4 735 C**I**Y**F**I**I**L**F**I**C**G**N**Y**I**L**L**N**V**F**L**A**I**A**V**D**N** 760

Ca_v2.2 686 S**F**Y**F**I**V**L**T**L**F**G**N**Y**T**L**L**N**V**F**L**A**I**A**V**D**N** 711

Ca_v2.3 680 A**I**Y**F**I**V**L**T**L**F**G**N**Y**T**L**L**N**V**F**L**A**I**A**V**D**N** 705

Ca_v3.1 941 A**L**Y**F**I**A**L**M**T**F**G**N**Y**V**L**F**N**L**L**V**A**I**L**V**E**G** 966

Ca_v3.2 992 A**L**Y**F**V**A**L**M**T**F**G**N**Y**V**L**F**N**L**L**V**A**I**L**V**E**G** 1017

Ca_v3.3 839 S**L**Y**F**V**A**L**M**T**F**G**N**Y**V**L**F**N**L**L**V**A**I**L**V**E**G** 864

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S6

DI

1799 1811

Ca_v2.1 1788 Y**F**Y**F**V**S**F**I**F**L**C**S**F**L**M**L**N**L**F**V**A**V**I**M**D**N** 1813

Ca_v1.1 1358 Y**Y**F**I**S**F**Y**M**L**C**A**F**L**I**N**L**F**V**A**V**I**M**D**N** 1383

Ca_v1.2 1501 V**F**Y**F**I**S**F**Y**M**L**C**A**F**L**I**I**N**L**F**V**A**V**I**M**D**N** 1526

Ca_v1.3 1441 I**V**Y**F**I**S**F**Y**M**L**C**A**F**L**I**I**N**L**F**V**A**V**I**M**D**N** 1466

Ca_v1.4 1418 I**A**Y**F**I**S**F**F**M**L**C**A**F**L**I**I**N**L**F**V**A**V**I**M**D**N** 1443

Ca_v2.2 1685 Y**F**Y**F**V**S**F**I**F**L**C**S**F**L**M**L**N**L**F**V**A**V**I**M**D**N** 1710

Ca_v2.3 1700 Y**V**Y**F**V**S**F**I**F**F**C**S**F**L**M**L**N**L**F**V**A**V**I**M**D**N** 1725

Ca_v3.1 1828 P**I**Y**F**V**S**F**V**L**T**A**Q**F**V**L**V**N**V**V**I**A**V**L**M**K**H** 1853

Ca_v3.2 1837 P**V**Y**F**V**T**F**V**L**V**A**Q**F**V**L**V**N**V**V**A**V**L**M**K**H 1862

Ca_v3.3 1707 P**L**Y**F**V**S**F**V**L**T**A**Q**F**V**L**I**N**V**V**A**V**L**M**K**H 1732

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P-loop

DII

666

Ca_v2.1 655 P**A**A**I**M**T**V**F**Q**I**L**T**G**E**D**W**N**E**V**M**Y**D**G 677

Ca_v1.1 601 P**Q**A**L**I**S**V**F**Q**V**L**T**G**E**D**W**T**S**M**M**Y**N**G 623

Ca_v1.2 693 P**Q**S**L**L**T**V**F**Q**I**L**T**G**E**D**W**N**S**V**M**Y**D**G 715

Ca_v1.3 692 P**Q**A**L**L**T**V**F**Q**I**L**T**G**E**D**W**N**A**V**M**Y**D**G 714

Ca_v1.4 698 P**Q**A**L**L**T**V**F**Q**I**L**T**G**E**D**W**N**V**V**M**Y**D**G 720

Ca_v2.2 650 P**A**A**I**L**T**V**F**Q**I**L**T**G**E**D**W**N**A**V**M**Y**H**G 672

Ca_v2.3 644 P**A**A**I**M**T**V**F**Q**I**L**T**G**E**D**W**N**E**V**M**Y**N**G 666

Ca_v3.1 910 L**W**A**I**V**T**V**F**Q**I**L**T**Q**E**D**W**N**K**V**L**Y**N**G 932

Ca_v3.2 961 L**W**A**I**V**T**V**F**Q**I**L**T**Q**E**D**W**N**V**V**L**Y**N**G 983

Ca_v3.3 808 L**W**A**I**V**T**V**F**Q**I**L**T**Q**E**D**W**N**V**V**L**Y**N**G 830

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S4

DIV

R0 R1 R2 R3 R4 R5

1664 1673

Ca_v2.1 1653 N**F**I**N**L**S**F**L**R**L**F**R**A**A**R**L**I**K**L**L**R**Q**G**Y** 1676

Ca_v1.1 1228 A**R**I**S**S**A**F**F**R**L**F**R**V**M**R**L**I**K**L**L**S**R**A**E** 1251

Ca_v1.2 1369 S**R**I**S**I**T**F**F**R**L**F**R**V**M**R**L**V**K**L**L**S**R**G**E** 1392

Ca_v1.3 1311 N**R**I**S**I**T**F**F**R**L**F**R**V**M**R**L**V**K**L**L**S**R**G**E** 1334

Ca_v1.4 1288 S**R**I**S**I**T**F**F**R**L**F**R**V**M**R**L**V**K**L**L**S**K**G**E** 1311

Ca_v2.2 1560 N**F**I**N**L**S**F**L**R**L**F**R**A**A**R**L**I**K**L**L**R**Q**G**Y** 1583

Ca_v2.3 1568 S**G**F**N**M**S**F**L**K**L**F**R**A**A**R**L**I**K**L**L**R**Q**G**Y** 1591

Ca_v3.1 1704 N**P**T**I**I**R**I**M**R**V**L**R**I**A**R**V**L**K**L**K**M**A**V 1727

Ca_v3.2 1710 N**P**T**I**I**R**I**M**R**V**L**R**I**A**R**V**L**K**L**K**M**A**T 1733

Ca_v3.3 1580 N**P**T**I**I**R**I**M**R**V**L**R**I**A**R**V**L**K**L**K**M**A**T 1603

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S5

DIV

1696

Ca_v2.1 1692 L**P**Y**V**C**L**L**I**A**M**L**F**F**I**Y**A**I**I**G**M**Q**V**F**G** 1715

Ca_v1.1 1267 L**P**Y**V**A**L**L**I**V**M**L**F**F**I**Y**A**V**I**G**M**Q**M**F**G** 1290

Ca_v1.2 1408 L**P**Y**V**A**L**L**I**V**M**L**F**F**I**Y**A**V**I**G**M**Q**V**F**G** 1431

Ca_v1.3 1350 L**P**Y**V**A**L**L**I**A**M**L**F**F**I**Y**A**V**I**G**M**Q**M**F**G** 1373

Ca_v1.4 1327 L**P**Y**V**A**L**L**I**A**M**I**F**F**I**Y**A**V**I**G**M**Q**M**F**G** 1350

Ca_v2.2 1599 L**P**Y**V**C**L**L**I**A**M**L**F**F**I**Y**A**I**I**G**M**Q**V**F**G** 1622

Ca_v2.3 1607 L**P**Y**V**C**L**L**I**A**M**L**F**F**I**Y**A**I**I**G**M**Q**V**F**G** 1630

Ca_v3.1 1743 V**G**N**L**G**L**L**F**M**L**L**F**F**I**F**A**A**L**G**V**E**L**F**G** 1766

Ca_v3.2 1749 V**G**N**L**G**L**L**F**M**L**L**F**F**I**Y**A**A**L**G**V**E**L**F**G** 1772

Ca_v3.3 1619 V**G**N**L**G**L**L**F**M**L**L**F**F**I**Y**A**A**L**G**V**E**L**F**G** 1642

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S6

DI

1502

Ca_v2.1 1488 S**I**F**Y**V**V**Y**F**V**V**F**P**F**F**F**V**N**I**F**V**A**L**I**I**I**T** 1513

Ca_v1.1 1042 A**I**F**F**I**I**Y**I**I**L**I**A**F**F**M**M**N**I**F**V**G**F**V**I**V**T** 1067

Ca_v1.2 1163 S**I**F**F**I**I**Y**I**I**I**I**A**F**F**M**M**N**I**F**V**G**F**V**I**V**T** 1188

Ca_v1.3 1129 S**I**F**F**I**I**Y**I**I**I**I**A**F**F**M**M**N**I**F**V**G**F**V**I**V**T** 1154

Ca_v1.4 1114 S**V**F**F**I**V**Y**I**I**I**I**A**F**F**M**M**N**I**F**V**G**F**V**I**I**T** 1139

Ca_v2.2 1393 S**I**F**Y**V**V**Y**F**V**V**F**P**F**F**F**V**N**I**F**V**A**L**I**I**I**T** 1418

Ca_v2.3 1400 S**I**F**Y**V**V**Y**F**V**V**F**P**F**F**F**V**N**I**F**V**A**L**I**I**I**T** 1425

Ca_v3.1 1514 L**L**Y**F**I**S**F**L**L**I**V**A**F**F**V**L**N**M**F**V**G**V**V**V**E**N** 1539

Ca_v3.2 1532 L**L**Y**F**I**S**F**L**L**I**V**S**F**F**V**L**N**M**F**V**G**V**V**V**E**N** 1557

Ca_v3.3 1408 L**L**Y**F**I**S**F**L**L**I**V**S**F**F**V**L**N**M**F**V**G**V**V**V**E**N** 1433

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Figure Legends

Figure 1. Location and conservation of CA-linked mutations between Cav2.1 and Cav1.1. (A)

Localization of the CA-linked mutations in the secondary structure of the Cav2.1 α_{1A} channel subunit according to the cryo-electron microscopy (cryo-EM) structure of the rabbit Cav1.1 complex, containing the pore-forming α_{1S} and several regulatory subunits [139] (see also Fig. 2). N- and C-termini and the intracellular loops are shown in gray, voltage-sensor modules (S1-S4) from the four domains are in green –with the S4 α -helixes colored in dark green– and Ca^{2+} -selective pore modules (S5-P loop-S6) are in orange. Purple and pink circles indicate CA-linked mutations associated with hemiplegic migraine episodes or not, respectively. Residues with functional data of the mutated channel are labeled in bold. (B) Sequence alignment between the regions of α_1 subunits for Cav2.1 and Cav1.1 channels affected by CA-linked mutations. The α_{1A} subunit region of each alignment is indicated on the top. CA-linked mutations are highlighted with yellow circles on the human Cav2.1 (hCav2.1) sequence. The selectivity filter residues (TGEDW) and the gating charged residues of S4 segments (labeled R0-R5) are shaded in red. Amino acids are colored depending on their physicochemical properties: small and hydrophobic are in red; acidic in blue; basic in magenta and G and amino acids containing hydroxyl, sulfhydryl or amine groups are colored in green. Below, a consensus code indicates fully conserved residues (*), conservation between residues with strongly similar properties (:) or with weakly similar properties (.). The Uniport IDs of the sequences aligned are hCav2.1: O00555-4 (corresponding to the isoform 4 of the protein) and hCav1.1: Q13698. The sequence alignments were performed using Multiple Sequence Alignment Clustal Omega. Although the isoform 4 of hCav2.1 is the one that matches better the nomenclature of the mutations found in the literature, there are some residue numbers that do not correspond: I1338 is I339 in our sequence, Y1385 is Y1384, V1696 is V1695 and I1811 is I1810 in sequence of isoform 4.

Figure 2. Three-dimensional location of CA-linked mutations on a Cav channel model. The structure

of the α_{1A} subunit of the Cav1.1 channel (PDB 5GJV) [139] was used as a model considering their high level of homology (Fig. 1B). N- and C-termini and the intracellular loops are shown in light gray, voltage-sensor modules (S1-S4) are in gray and Ca^{2+} -selective pore modules (S5-P loop-S6) are in dark gray. Regions where CA-linked mutations have been described are colored corresponding to their domain: DII (in green, P-loop and S6), DIII (in orange, S4 and S5-P loop-S6) and DIV (in blue, S4 and S5-P loop-S6). The residues of Cav1.1 (α_{1S}) equivalent to those mutated in Cav2.1 (α_{1A}) and linked to congenital ataxia,

according to the sequence alignment on Figure 1B, have been highlighted in magenta and labeled as in Figure 1A. Note that the position of the mutation I1338T (dashed line arrow) is approximative, as it is located in a not resolved region of the channel structure.

Figure 3. Ca^{2+} currents through both wild-type and a gain-of-function mutant Cav2.1 channels. (A) Representative whole-cell current density from a wild-type Cav2.1 channel (upper traces) or a mutant channel carrying one of the Cav2.1 gain-of-function variants linked to congenital ataxia (ΔF1502 , lower traces) [4]. Ca^{2+} currents are induced by 20 ms depolarizing pulses from -80 mV to the indicated voltages. The wild-type or the mutant α_{1A} subunits, with the auxiliary subunits β_3 and $\alpha_2\delta$, were heterologously expressed in HEK293 cell line and Cav2.1 channel activity was recorded with whole-cell patch-clamp as previously described [4] (B) Corresponding I-V curves normalized to the maximal Ca^{2+} current density. Both panels illustrate the main effect observed in gain-of-function variants of Cav2.1, showing a decrease in the threshold for channel activation. This shift in the channel activation causes an increase of Ca^{2+} influx at lower voltages.

Figure 4. Sequence alignment between different species of the α_{1A} subunit regions affected by CA-linked mutations. The α_{1A} region of each alignment is indicated on the top. Cav2.1 mutations are indicated with yellow circles on the human sequence. The selectivity filter residues (TGEDW) and the gating charged amino acids of S4 segments (labeled R0-R5) and are shaded in red. Residues are colored given their physicochemical properties: small and hydrophobic are in red; acidic in blue; basic in magenta and G and residues containing hydroxyl, sulfhydryl or amine groups are colored in green. The consensus code below indicates fully conserved residues (*), conservative (:) or semi-conservative amino acid substitutions (.). The Uniport IDs of the aligned orthologous Cav2.1 sequences are: *Homo sapiens*: O00555-4; *Mus musculus*: P97445; *Rattus norvegicus*: P54282; *Oryctolagus cuniculus*: P27884; *Pan troglodytes*: A0A2I3T217; *Bos taurus*: F1N1E0; *Danio rerio*: E9QJF6; *Drosophila melanogaster*: P91645. The sequence alignments were performed using Multiple Sequence Alignment Clustal Omega.

Figure 5. Sequence alignment between all human Cav channel family considering the α_{1A} subunit regions affected by CA-linked mutations. The segment of the α_1 channel subunit at each alignment is indicated on the top. CA-linked mutations are highlighted with yellow circles on the hCav2.1 sequence.

The selectivity filter residues (TGEDW) and the gating charged residues of S4 segments (labeled R0-R5) are shaded in red. Amino acids are colored depending on their physicochemical properties, as mentioned in Figure 1 and 4. Below each alignment there is a consensus code indicating fully conserved residues (*), conservation between residues with strongly similar properties (:) or with weakly similar properties (.). The Uniport IDs of the human Cav channel family aligned are the following: hCa_v2.1: O00555-4; hCa_v1.1: Q13698; hCa_v1.2: Q13936; hCa_v1.3: Q01668; hCa_v1.4: O60840; hCa_v2.2: Q00975; hCa_v2.3: Q15878; hCa_v3.1: O43497; hCa_v3.2: O95180; hCa_v3.3: Q9P0X4. The sequence alignments were performed using Multiple Sequence Alignment Clustal Omega.

	Variant (p.)	Domain	Age of presentation	First symptoms	Cerebellar syndrome*	Intellectual disability	Epilepsy	Acute events: coma, S/FHM	MRI findings	Treatment	Other
Gain of Function (GoF)	T666M	Pore	Infancy to adulthood	Psychomotor delay or normal development	Normal to mild -moderate CS	Normal and mild ID	No	Acute coma, S/FHM	Normal / vermian or generalized cerebellar atrophy	Acetazolamide, verapamil	Hemispheric brain atrophy after severe acute episodes
	D715E	Pore	Childhood	S/FHM	Normal to mild -moderate CS	NR	NR	Acute coma, S/FHM	Normal / vermian or generalized cerebellar atrophy	NR	
	V1396M	Pore	Infancy	Psychomotor delay/ early ataxia/febrile seizures	Mild to severe CS	Mild to severe ID	EE: Lennox-Gastaut syndrome, refractory epilepsy	Acute coma, S/FHM	Cerebellar atrophy	NR	
	ΔF1502	Pore	Infancy	Psychomotor delay	CS	ID	Epilepsy related to acute events	SHM	Predominantly vermian cerebellar atrophy	Acetazolamide Worsening with flunarizine + CBZ.	
	I1811L	Pore	Infancy to adulthood	FHM/ataxia	CS	Normal and mild ID	No	FHM	Normal / generalized cerebellar atrophy	NR	
	R1350Q/ R1349Q	Voltage sensor	Early infancy	Persistent tonic upgaze, psychomotor delay	CS	ID	Febrile seizures	F/SHM	Normal / generalized cerebellar atrophy	Corticosteroids and hypertonic solution for events.	Dyskinesia, horizontal rhythmic head movements stereotypies. Pyramidal signs
	S218L	Linker	Infancy to childhood	Psychomotor delay or normal development	Normal to mild -moderate CS	Normal and mild ID	Febrile seizures and epilepsy related to acute events	Acute coma, S/FHM Early seizures and cerebral oedema after trivial head trauma(ESCEATHHT)	Normal / vermian or generalized cerebellar atrophy	Antiepileptic drugs for seizures. Acetazolamide and flunarizine in one patient	Hemispheric brain atrophy described after severe acute episodes
Loss of function (LoF)	R1664Q	Voltage sensor	Infancy to adulthood	Psychomotor delay or normal development	CS but also EA	ID or normal	No	Migraine without focal deficits	Normal / cerebellar atrophy	Acetazolamide for EA	Two different phenotypes appear in literature: EA and congenital ataxia
GoF and LoF	R1673P	Voltage sensor	Infancy	Psychomotor delay	CS	ID	No	No	Cerebellar atrophy	NR	Thin corpus callosum, delayed myelination
Not known	I712V	Pore	Infancy	Status epilepticus	CS	ID	Epilepsy related to acute events	Acute coma, S/FHM	Mild cerebellar atrophy	Acetazolamide	Developmental regression. Progressive bilateral mesiotemporal sclerosis
	Y1385C	Pore	Infancy	Psychomotor delay	CS	Moderate ID	Epilepsy related to acute events	S/FHM	Severe cerebellar atrophy		Hemispheric brain atrophy described after severe acute episodes
	V1696F	Pore	Infancy	Psychomotor delay	CS	ID	Epilepsy	FHM	No cerebellar atrophy	Flunarizine with doubtful effect	Tetraparesis, athetotic movements. Acute events fulfil criteria of AH
	S1799L	Pore	Infancy	Psychomotor delay	CS	ID	Yes	EA, coma, S/FHM	Cerebellar atrophy	NR	
	I1338T	Voltage sensor	Infancy	Psychomotor delay	CS	ID	No	SHM	Cerebellar atrophy	NR	
	I1342T	Voltage sensor	Infancy	Psychomotor delay	CS	ID	Complex partial seizures	SHM	Cerebellar atrophy	NR	Pyramidal signs
	R1352Q	Voltage sensor	Infancy	Nystagmus, strabismus	CS	Moderate ID	No	SHM	Cerebellar atrophy	NR	
	R1359W	Voltage sensor	Infancy	Epilepsy	CS	ID	Yes		Cerebellar atrophy	NR	Pyramidal signs

Table Legend

Table 1. Complete phenotype of *CACNA1A* variants related to Congenital Ataxia. AH: Alternating hemiplegia; CBZ: carbamazepine; CS cerebellar syndrome; EA: episodic ataxia; EE: Early epileptic encephalopathy; ID: Intellectual disability; NR: not reported; S/FHM: Sporadic/Familial hemiplegic migraine.

* Cerebellar syndrome includes impairment in several neurological functions causing different signs and symptoms. Cerebellar syndrome alters posture and gait (causing ataxia), limb coordination (causing dysmetria and tremor), speech (causing dysarthria and slurred speech) and external ocular movements (causing nystagmus, abnormal saccade, strabismus, eye dysmetria...)