De novo CACNA1D Ca\textsuperscript{2+} channelopathies: clinical phenotypes and molecular mechanism

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Abstract
The identification of rare disease-causing variants in humans by large-scale next-generation sequencing (NGS) studies has also provided us with new insights into the pathophysiological role of de novo missense variants in the CACNA1D gene that encodes the pore-forming α1-subunit of voltage-gated Cav1.3 L-type Ca\textsuperscript{2+} channels. These CACNA1D variants have been identified somatically in aldosterone-producing adenomas as well as germline in patients with neurodevelopmental and in some cases endocrine symptoms. In vitro studies in heterologous expression systems have revealed typical gating changes that indicate enhanced Ca\textsuperscript{2+} influx through Cav1.3 channels as the underlying disease-causing mechanism. Here we summarize the clinical findings of 12 well-characterized individuals with a total of 9 high-risk pathogenic CACNA1D variants. Moreover, we propose how information from somatic mutations in aldosterone-producing adenomas could be used to predict the potential pathogenicity of novel germline variants. Since these pathogenic de novo variants can cause a channel-gain-of-function, we also discuss the use of L-type Ca\textsuperscript{2+} channel blockers as a potential therapeutic option.

Keywords Voltage-gated Ca\textsuperscript{2+} channels · CACNA1D · Neurodevelopmental disorders · Autism spectrum disorders · Calcium channel blockers · Variants

In the past few years the identification of rare disease-causing variants in humans by large-scale next-generation sequencing (NGS) studies has provided us with unprecedented new insights into the physiological and pathophysiological role of ion channels, including voltage-gated Ca\textsuperscript{2+} channels (Cavs, Table 1). The first disease-causing genetic Cav variants were inherited conditions in CACNA1A causing familial hemiplegic migraine type 1 and episodic ataxia type 2 [61, 63] or CACNA1F causing eye disorders such as congenital stationary night blindness type 2 [84, 86, 96]. The first de novo variants in L-type Cavs were found in CACNA1C leading to Timothy syndrome, a multisystem disorder [39, 80, 81]. More recently, high-throughput sequencing of family trios and quads in well-defined disease cohorts in combination with advanced bioinformatic pipelines and the availability of large genetic databases led to the discovery of disease-causing de novo missense variants in the pore-forming α1-subunits of several Cav subtypes (Table 1). This includes variants in CACNA1E (Cav2.3 α1) causing developmental epileptic encephalopathies with contractures, macrocephaly, and dyskinesia [22], in CACNA1G (Cav3.1 α1) causing childhood-onset cerebellar atrophy [13] and in CACNA1H (Cav3.2 α1) causing early-onset hypertension with primary aldosteronism [74]. In addition, there is accumulating evidence that CACNA1D (Cav1.3 α1) variants cause an often severe neurodevelopmental syndrome, which is reviewed in this article.
Interestingly, de novo pathogenic CACNA1D variants and similarly those in CACNA1C, CACNA1E, CACNA1G, and CACNA1H are not gene-disrupting resulting in a loss of channel function. Instead, they cause typical changes of channel gating, which can enhance channel activity (gain of channel function) during electrical activity patterns in neurons, endocrine, and other electrically excitable cells. These very characteristic gating changes allow the classification of these variants as pathogenic variants in electrophysiological recordings after heterologous expression in mammalian cells (Fig. 1, Table 2). Most variants are located within regions of the pore-forming α1-subunits, which are critical for the function of the activation gate and its control by the channels’ voltage-sensors (see chapter below). Recently published cryo-electron microscopy and crystal structures of two Cav α1-subunits (Cav1.1, Cav3.1; [93, 99]) and bacterial sodium channels (BacNavs) in different states [37, 92] now enable the construction of homology models, which help us to predict how these variants interfere with basic channel functions on the molecular level. Therefore, these variants provide important insight not only into disease but also into the structure-function relationship of Cav α1-subunits.

Gene disrupting de novo variants (frameshift, premature stop codon, splice-donor defect) causing loss of channel function can be reliably predicted using bioinformatic pipelines in most cases. In contrast, it is much more difficult to distinguish high-risk, disease-causing de novo missense variants, for which the functional consequences are difficult to predict in silico, from rare missense variants, which contribute only weakly to disease risk or are even benign. This has important clinical implications, because the symptomatic spectrum of a Ca\(^{2+}\) channelopathy should primarily be inferred from rare variants proven to confer high risk in functional studies.

In this review we summarize the spectrum of symptoms associated with a syndrome caused by de novo CACNA1D missense variants leading to aberrant gating properties of Cav1.3 Ca\(^{2+}\) channels, which support enhanced channel activity especially in cells firing from negative membrane potentials. We update the clinical phenotype of all well-documented pathogenic CACNA1D variants affecting a total of 12 individuals. This should help to guide clinical diagnosis and help to outline a rational strategy for potential personalized therapy with Ca\(^{2+}\) channel blockers.

**Physiological role of Cav1.3 L-type Ca\(^{2+}\) channels**

The fact that aberrant gating of Cav1.3 Ca\(^{2+}\) channels can cause neurodevelopmental and endocrine symptoms can be
explained by the multiple functions of these channels in the mammalian organism. Of the L-type family (Cav1, Table 1), the Cav1.2 and Cav1.3 isoforms show a wide and often overlapping tissue distribution and can be found in most electrically excitable cells (for review see [96]). Cav1.3 channels, although classified as high-voltage activated (HVA, Table 1), can operate at much more negative membrane potentials compared with other HVA Cavs [40, 42], which enables them to support special functions within the auditory, cardiac, endocrine, and nervous system as outlined below. Insight into the physiological roles of Cav1.3 channels came from Cav1.3-knockout mice [54, 67] and humans harboring a mutation in the CACNA1D gene resulting in non-functional Cav1.3 channels [4]. In both loss of Cav1.3 function resulted in congenital deafness, bradycardia and sinoatrial node (SAN) arrhythmia (human SAN dysfunction and deafness syndrome, SANDD, OMIM # 614896). The hearing loss can be explained by the important role of presynaptically clustered Cav1.3 channels in cochlear hair cells where they provide Ca2+ influx to trigger neurotransmitter release at synaptic ribbons [10, 67, 83]. In contrast, neuronal Cav1.3 channels are predominantly expressed postsynaptically where they shape electrical activity patterns, contribute to dendritic Ca2+ signaling, and fine-tune Ca2+-dependent gene expression (for review see [96]). In the heart Cav1.3 channels predominate in the SAN and atrioventricular node where Cav1.3 Ca2+ influx at negative potentials drives the diastolic depolarization required for normal cardiac pacemaking [45, 47, 98], which explains the observed cardiac phenotype in Cav1.3-deficient humans and mice. Cav1.3 also controls endocrine functions in the pancreas and in the adrenal gland. In mice, Cav1.3 does not contribute much to the overall Ca2+ current and insulin release from pancreatic β cells [78]. However, in one Cav1.3-knockout mouse line genetic ablation of Cav1.3 induced hypoinsulinemia and impaired glucose tolerance, associated with a deficit in postnatal β cell generation/proliferation ([54], but see [67]). In human pancreatic β cells, Cav1.3 transcripts predominate and seem to be involved in exocytosis [68]. In cultured catecholamine-releasing chromaffin cells of the murine adrenal medulla, Cav1.3 Ca2+ channels mediate ~25% of the total Ca2+ current, support autonomous pacemaker activity [46], and shape secretion-associated firing patterns of these neuroendocrine cells [89]. Cav1.3 is also expressed in aldosterone-secreting zona glomerulosa cells of the adrenal cortex [16, 72]. In these cells, aldosterone synthesis is driven by a periodic intracellular Ca2+ signal that mainly depends on low-voltage activated Cav3.2 T-type channels; however, Cav1.3 also contributes to this Ca2+ signal (for review see [5]). These special functions of Cav1.3 channels in pancreatic β cells and aldosterone-producing cells nicely explain that individuals harboring an activity-promoting de novo CACNA1D variant (germline or somatically in aldosterone-
producing adrenomas, APAs) can present with primary aldosteronism and/or hyperinsulinemic hypoglycemia (see below, Table 3).

While the patients with de novo CACNA1D missense variants that are described in this review exhibit neurodevelopmental symptoms (Table 3), no brain pathologies have been reported so far for Cav1.3-deficient humans. However, using genetic and pharmacological approaches in mouse models helped to reveal several important functions of Cav1.3 in the central nervous system. Cav1.3 channels account for ~10% of total L-type Ca\(^{2+}\) channels in the brain and are expressed within multiple regions [6, 78, 79] where they can shape neuronal excitability and induce gene transcription, important for synaptic plasticity, memory formation, and neuronal development. Cav1.3-deficient mice show subtle deficits in proper brain development, evident from a lower number of dopamine-producing neurons in the substantia nigra [62] and a decreased volume and neuron number in the auditory brain stem [24, 25] and the dentate gyrus [48]. The latter was associated with reduced hippocampal neurogenesis and impairments in hippocampus-dependent cognitive functions [48]. Cav1.3 channels are also involved in mood and emotional behaviors. Loss of Cav1.3 resulted in an antidepressant-like phenotype in mice [11], while the opposite was observed upon selective Cav1.3 activation [78]. Also, stimulation of Cav1.3 within the ventral tegmental area (VTA; dopamine midbrain system) was sufficient to elicit the depressive-like behavior together with a social deficit and enhanced cocaine-associated behaviors [49]. This was in line with previous reports linking Cav1.3 activity to the development of psychostimulant-induced sensitized behaviors [20, 33, 71]. An anxiety-like phenotype upon global Cav1.3 knockout was most likely the consequence of the knockout-induced deafness [11], whereas fore-brain specific knockdown of Cav1.3 had no effect on anxiety-related parameters in mice [36]. However, the consolidation of conditioned contextual fear memory is facilitated by Cav1.3 activity, as it was impaired in Cav1.3-deficient mice and associated with significantly reduced long-term potentiation (LTP) in the basolateral amygdala [50, 51]. There is also evidence linking the oscillatory Cav1.3 Ca\(^{2+}\) influx in dendrites of autonomously spiking substantia nigra neurons to their selective cell death in Parkinson’s disease ([21] but see [62]).

Given the multiple functions of Cav1.3 Ca\(^{2+}\) channels throughout the body, it is plausible that interfering with the way these channels conduct Ca\(^{2+}\) ions can result in the dysregulation of several body functions and thus to human diseases.

### Characteristic gating changes of Cav1.3 channels associated with high-risk pathogenic variants

The typical gating changes repeatedly observed in well-characterized patients with a pathogenic CACNA1D de novo variant have been described in previous publications (Fig. 1; for references see Table 2). It is important to note that some of these germline variants have also been reported as somatic variants in APAs ([3, 72], Table 2), which likely evolve from

### Table 2 Classification of CACNA1D missense variants by characteristic functional changes

| Type  | Mutation | Occurrence               | Functional changes                                      | ISR sensitivity | References |
|-------|----------|--------------------------|--------------------------------------------------------|-----------------|------------|
| 1     | G403D    | Germline                 | Inactivation almost abolished (voltage-dependence of inactivation not measurable) |                | [72]       |
|       | G403R    | Somatic                  | Voltage-dependence of activation shifted to hyperpolarized voltages or unchanged |                | [3, 72]   |
|       | G407R    | Germline                 | Inactivation shifted to hyperpolarized voltages         | HP -80 mV: enhanced (1.5-fold)                  | [65]       |
| 2     | V259D    | Somatic                  | Voltage-dependence of activation strongly shifted to hyperpolarized voltages | HP -50 mV: unchanged | [26]       |
|       | V401L    | Germline + somatic       | Voltage-dependence of inactivation strongly shifted to hyperpolarized voltages or unchanged | HP -80 mV: enhanced (3-fold) | [66]       |
|       | S652L    | Germline                 | Slower and less complete inactivation                   |                | [41, 64, 66] |
|       | F747L    | Somatic                  | Voltage-dependence of inactivation strongly shifted to hyperpolarized voltages or unchanged |                | [3, 72]   |
|       | A749G    | Somatic                  | No change in voltage-dependence of gating              |                | [87]       |
|       | I750M    | Germline + somatic       | Mutation-induced (depolarizing)\(\omega\)-currents      |                | [52, 66]   |

Table taken and modified from [66]. Functional changes of Cav1.3 α1 variants were determined upon heterologous expression in mammalian cells (HEK293, tsA201) together with auxiliary β3 (or β1b and β2α in [41]) and α2C-1 subunits. Isradipine sensitivity was measured using depolarizing standard square pulses to the \(V_{\text{max}}\) (voltage of maximal activation) elicited from a holding potential (HP) of −50 mV or −80 mV as indicated. *Q547H: this homozygous variant is not a de novo variant and therefore not further discussed in this review.
Table 3  High-risk disease-causing de novo germline CACNA1D variants

| Case no. | Variant      | Age first symptoms (sex) | ASD | Seizures | Limb spasticity | Hypotonia | Primary aldosteronism | Hypoglycemic hyperinsulinism | Intellectual impairment/disability |
|----------|--------------|--------------------------|-----|----------|-----------------|-----------|-----------------------|-------------------------------|----------------------------------|
| 1        | G403D (ex 8B)| 1 month (f)              |     |          | +               | +         | +                    |                               | +                                |
| 2        | G403D (ex 8B)| Birth (f)                |     | (+)      | +               | +         | +                    |                               | +                                |
| 3        | 1750M        | Birth (f)                |     | +        | +               | +         | +                    |                               | +                                |
| 4        | V259A        | 1.5 month (m)            |     |          | Normal EEG      |           | +                    |                               | +                                |
| 5        | A749G        | 8 years (f)              |     | +        | Normal EEG      |           | +                    |                               | +                                |
| 6        | G407R (ex 8A)| 15 years (m)            |     |          | +               | +         | +                    |                               | +                                |
| 7        | V401L        | 4 months (m)             |     | +        | +               | +         | +                    |                               | +                                |
| 8        | S652L        | Homozygotic twins (m)    |     | +        | (no recurrence)|           | -                    |                               | +                                |
| 9        | S652L        | Homozygotic twins (current age 13) | + |        | Normal EEG      |           | +                    | + (no recurrence)          | +                                |
| 10       | A749T        | 1 year (f)               |     |          | +               | +         | +                    |                               | +                                |
| 11       | A749T        | Details not reported     |     |          | +               | +         | +                    |                               | +                                |
| 12       | L271H        | Birth (f)                |     |          | +               | +         | +                    |                               | +                                |

For classifying the pathogenicity (Patho) of the CACNA1D variants, we used the criteria proposed in the ACMG classification system [69]. For each of the variants, the combination of the criteria for PS (strong evidence for pathogenicity) and PM (defining moderate evidence for pathogenicity) is given. All variants can be considered “pathogenic” based on ACMG criteria, with the exception of L271H, which is considered “likely pathogenic.” However, as argued in the text, it should be considered a high-risk disease-causing de novo variant. ASD, autism spectrum disorder; CS, cesarean section; ex, exon; f, female; m, male; +, symptom reported; −, symptom reported to be absent.
The functional characterization of a larger set of both germline and somatic variants using whole-cell patch-clamp studies by us and others [3, 26, 41, 52, 64–66, 72, 87] now allows to propose at least four characteristic types of functional alterations leading to a channel gain of function. These have been published recently [66] and are depicted in (Table 2).

Type 1 are the inactivation-deficient variants, such as G403D, G403R, and G407R, in which most of the Cav1.3 current fails to inactivate (Fig. 1B). They could also be classified as “Timothy syndrome like” variants because they induce similar gating changes as the Cav1.2 α1C-subunit (CACNA1C) variants G402S and G406R that affect the corresponding amino acid residue and cause Timothy syndrome, a multisystem disorder also associated with autism [7, 80, 81, 96]. An example is illustrated in Fig. 1A, B for variant G407R (introduced into the short splice-variant of Cav1.3 [66]). Type 2 variants still inactivate to variable extents (i.e., faster or slower than wild-type) but are characterized by pronounced negative shifts of the voltage-dependence of activation with or without a strong negative shift also of the inactivation voltage. An example is shown in Fig. 1C, D for the recently published S652L variant [26]. Type 3 is characterized only by slower and less complete inactivation after 3–5 s at the voltage of maximal activation (V_{max}), which should favor persistent current in cells during prolonged depolarizations (Table 2). Type 4 are variants in the voltage sensor (e.g., S4 helix positive charges), which enable depolarizing ω-currents as described in several other voltage-gated Ca^{2+} channels [12, 29, 32, 84]. For variants without a functional phenotype in vitro, we cannot exclude pathogenicity through molecular mechanisms which escape our in vitro functional analysis (such as tissue-specific protein-protein interactions disrupted by the variant). However, their role for the disease etiology remains uncertain until further functional or clinical data provide more conclusive insight (e.g., by demonstrating their presence in a larger number of patients and/or in APAs/APCCs).

Clinical characteristics of germline CACNA1D variants

Here we summarize the clinical presentation of 12 individuals with a confirmed or predicted high-risk, pathogenic CACNA1D variant for which a well-documented clinical history is available and the “diagnostic” gating changes of Cav1.3 have either been confirmed in functional studies or are strongly supported (variant A749T) by molecular modeling (see below).

The first reported variant (A749G) has been identified in an individual with autism spectrum disorder (ASD) and intellectual impairment but without evidence for additional neurological symptoms ([58, 64]; case no. 5 in Table 3). A subsequent report [72] described pathogenic CACNA1D variants in two patients with congenital primary aldosteronism, seizures, and neurological abnormalities (PASNA, OMIM # 615474; cases no. 1, no. 3 in Table 3). However, for both patients it could not be excluded that birth complications contributed to their neurological symptoms. Despite the report of another subject (G407R; case no. 6 in Table 3) affected by ASD without intellectual impairment and neurological symptoms [28, 64], the majority of variants we currently oversee leads to a severe developmental disorder also associated with developmental delay, intellectual impairment, neurological symptoms (including seizures in some of them), and, in several cases, endocrine symptoms, evident as primary aldosteronism, congenital hyperinsulinemic hypoglycemia, or both (Table 3).

Recently, two cases affected by the de novo CACNA1D variant A749T were diagnosed (cases no. 10, no. 11 in Table 3). It is located in the same position as the A749G variant described above within the “LAIA’’ motif discussed in more detail below. Based on its location and the nature of the amino acid exchange, we also predict pathogenicity for this variant, although this needs confirmation in functional studies as for other germline de novo variants (Table 3). Because clinical characteristics have not yet been reported and are also not deposited in online databases, the clinical presentation of subject no. 10 is reported here.

The female patient was referred to a local autism center at 3 years of age. Her medical history revealed diagnoses of ASD, global developmental delay, muscle hypotonia, delayed vision maturation, and difficulty walking. A chromosomal microarray was performed at 20 months which was unremarkable, but a whole exome sequence was completed 2 years later revealing the CACNA1D variant. No other genetic abnormalities were found that could explain her symptoms. At the time of presentation to the autism clinic, the patient had been under specialty care from gastroenterology (due to early childhood gastroesophageal reflux and a milk protein allergy), neurology, developmental neurology, developmental pediatrics (medication management of self-harming behaviors), ophthalmology (visual impairment), and orthopedics (for leg/foot...
misalignment). Despite patient being good-natured and inquisitive, the family was seeking medication management for impulsive and unpredictable self-harming events which occurred multiple times a day including biting herself, scratching to the point of bleeding, throwing herself to the ground, hitting her head against hard surfaces, punching a car so hard it “bloodied her knuckles,” and other significant self-injurious events. Over 2 years of being in the autism clinic, many medications were trialed to provide relief including amantadine, risperidone, memantine, buspirone, quetiapine, gabapentin, and medications targeting underlying anxiety and sleep disorders. Despite periodic improvements with medication management in addition to robust therapeutic services and weekly Applied Behavior Analysis, there was no consistent regimen that controlled her self-harming behaviors and treatment was frequently adjusted. Additionally, a short trial with immediate release isradipine was initiated in an effort to address the underlying calcium channel mutation but was stopped because of concern for worsened symptoms, inefficacy, and complication by a common cold which developed during therapy. During this time, no evidence for cardiovascular abnormalities was found and blood pressure was normal.

From the clinical reports of this and 11 other subjects (Table 3), the following clinically relevant conclusions can be drawn:

1. All affected individuals, except for those with congenital primary aldosteronism (and severe PASNA symptoms, cases no. 1, no. 3, no. 4, and no. 12 in Table 3), have been diagnosed with ASD.
2. Only 2 of the 12 cases (no. 5, no. 6 in Table 3) have been reported with no other neurological or endocrine symptoms in addition to ASD (with or without intellectual impairment) at the time the medical history was published.
3. Endocrine symptoms, driving early genetic diagnosis and immediate therapeutic intervention in the PASNA patients and the individuals affected by congenital hyperinsulinemic hypoglycemia (G403D) or both (L271H, [14]), are observed only in a minority of cases. Their absence does not rule out a CACNA1D channelopathy, and their presence cannot be predicted from the variant gating changes: (i) even variants (such as A749G or S652L) causing almost identical biophysical changes in APAs, it is likely that the altered gating changes also increase Cav1.3 channel activity in heterologous expression systems and promoted Ca2+ signaling in APAs, it is likely that the altered gating changes also increase Cav1.3 channel activity in some populations of neurons. Thus, inhibition of Cav1.3 channel activity with drugs appears as a potential treatment option in affected individuals. Although neurodevelopmental defects are unlikely to be completely reversible, treatment of some otherwise difficult to control symptoms (such as seizures, self-harming behaviors, or muscle hypotonia) may considerably improve the quality of life of patients and their caregivers.

As described above, Cav1.3 L-type channels are also present in other tissues. Although homozygous loss of Cav1.3 function results in congenital deafness and sinoatrial node dysfunction, it is at present unclear if the heterozygous gain-of-function gating changes of the reported variants result in clinically relevant symptoms on hearing or directly cause the cardiac abnormalities observed in some cases (Table 3).

**Repurposing of Ca2+ channel blockers (“Ca2+ antagonists”) for symptom control**

As outlined above, the heterozygous de novo CACNA1D variants are dominant in nature and the disease cannot be explained by heterozygous loss of channel function, which is apparently asymptomatic in mice and humans. This is further supported by at least 10 heterozygous protein loss of function variants (stop gained or frameshift located N-terminal to the beginning of the C-terminus) reported in gnomAD (gnomad.broadinstitute.org) samples so far. Based on the enhanced channel activity in heterologous expression systems and promoted Ca2+ signaling in APAs, it is likely that the altered gating changes also increase Cav1.3 channel activity in some populations of neurons. Thus, inhibition of Cav1.3 channel activity with drugs appears as a potential treatment option in affected individuals. Although neurodevelopmental defects are unlikely to be completely reversible, treatment of some otherwise difficult to control symptoms (such as seizures, self-harming behaviors, or muscle hypotonia) may considerably improve the quality of life of patients and their caregivers.
Treatment with Ca^{2+} channel blockers has already been described in some of the patients, but no conclusive results regarding improvement of neurological or neuropsychiatric symptoms have yet been obtained. The clinical course of the patient described in case no. 1 (G403D, PASNA) was notable for uncontrolled hypertension with hypokalemia (due to primary aldosteronism). Treatment with the dihydropyridine (DHP) Ca^{2+} channel blocker amlodipine normalized blood pressure and resolved biventricular hypertrophy. Effects of this treatment on other underlying symptoms were not reported [72]. The patient of case no. 2 (same variant) with congenital hyperinsulinemic hypoglycemia received diazoxide (only required until age of 5 years), which successfully controlled blood glucose. Although considered a therapeutic option, treatment with a Ca^{2+} channel blocker was not reported [18]. As mentioned in the detailed case report above, short treatment of subject no. 10 with the DHP isradipine was also inconclusive. Interestingly, the young subject no. 12 was treated with nifedipine oral solution every 8 h to control hypertension. This not only controlled hypertension but also improved muscle hypotonia. The extent of this improvement was not quantified, and the long-term outcome as well as the tolerability of this treatment was not reported [14]. Nevertheless, it supports the hypothesis that some symptoms may improve upon treatment with DHPs.

What is known about the pharmacology of Cav1.3 that could guide off-label treatment trials in subjects with confirmed pathogenic CACNA1D variants? Both pharmacokinetic and pharmacodynamics need to be considered.

The first question is if Ca^{2+} channel blockers can sufficiently engage Cav1.3 Ca^{2+} channels in the brain at plasma levels achieved for antihypertensive therapy. Pharmacokinetic studies in rodents clearly show that some DHPs, such as felodipine and isradipine, used since decades for the treatment of high blood pressure, can quickly and efficiently cross the blood-brain barrier ([76], for review see [43]). An exception seems to be amlodipine. For this widely used DHP, brain exposure after a single dose seems to be lower [88]. Since this compound’s long half-life requires many days of dosing to reach steady state, it is unclear if brain exposure can further increase after multiple dosing.

The second question is if Cav1.3 channels in the brain are efficiently blocked at therapeutic doses of DHPs. The therapeutic target of DHPs for cardiovascular indications is Cav1.2 L-type channels in arterial smooth muscle cells [78, 85]. It is known that under identical experimental conditions Cav1.2 channels are about 5 times more sensitive to inhibition by DHPs than Cav1.3 [62, 94]. This means that higher doses may be required to inhibit Cav1.3 channels in the brain compared with Cav1.2 in the periphery [62]. However, we have recently discovered that some variants can enhance the sensitivity of Cav1.3 channels for inhibition by the DHP isradipine, as shown for S652L [26] and, to a smaller extent, for V401L [65]. This has meanwhile been confirmed also for other variants (NJO, unpublished data). However, we also found the opposite, variants which significantly reduce sensitivity to isradipine (unpublished). This strongly suggests that therapeutic trials should be first started in subjects with DHP-sensitizing variants. If therapy fails in these patients it is
unlikely that subjects with other variants would benefit from DHP therapy.

In addition, other factors could be therapy limiting: DHPs are very well tolerated by most patients but may cause hypotension and dizziness at higher doses. Immediate release preparations should be avoided because fast blood pressure lowering may induce sympathetic activation, reflex tachycardia, and flushing, which may cause unwanted behavioral reactions in some patients. Therefore, suitable DHPs require extended release formulations to prevent fast onset, which can be a problem in patients with feeding problems or younger patients, in which oral solutions are more appropriate for administration and correct dosing. In addition, DHPs are cytochrome-P450-3A4 substrates and drug-drug interactions with concomitant therapies (e.g., antiepileptic or psychiatric drugs) have to be considered.

Nevertheless, despite many open questions and potentially therapy-limiting considerations, treatment with Ca\(^{2+}\) \(\alpha\)-channel blockers remains a therapeutic option that should be explored carefully in these patients. These experimental therapies will require not only a skilled therapist but also the patience and support of cooperative parents and their qualified assessment of predefined treatment outcomes.

**Somatic CACNA1D APA/APCC variants can help to classify germline variants as high risk**

As outlined above, somatic CACNA1D variants in APAs and APCCs cause excess aldosterone production and primary aldosteronism although they seem not to directly contribute to abnormal cell proliferation and adenoma formation [97]. This finding strongly suggests that the gating changes induced by these variants indeed permit enhanced Ca\(^{2+}\) signaling through Cav1.3 channels in these human tumor cells. Accordingly, several variants found in APAs were also reported as pathogenic germline variants (Fig. 2) in a subject with (I750M) and others without endocrine symptoms (S652L, V401L). Therefore, APA/APCC variants could guide the assessment of the potential pathogenicity of new germline variants for which no functional data are available and thus could aid clinical diagnosis. However, a small percentage of APAs and APCCs cannot yet be explained by known somatic de novo mutations [9]. Therefore, it cannot be ruled out completely that a CACNA1D variant is in fact benign and other yet unknown genetic factors account for excess aldosterone production in a given APA/APCC. We therefore propose criteria which classify these somatic variants as pathogenic, likely pathogenic, likely benign or of (yet) uncertain pathogenicity (Table 4). The classified somatic APA/APCC variants as well as their approximate position within the Cav1.3 \(\alpha\1\)-subunit are given in Table 5 and Supplementary Figure 1, respectively.

**Molecular mechanism of altered channel gating in high-risk variants**

As mentioned above all germline variants and the vast majority of APA/APCC variants occur in the voltage-sensing or pore-forming domain of the channel (Fig. 2).
| Position | Variant | Reference | Gating change | Times reported | Reported in gnomAD (#) | Pathogenicity |
|----------|---------|-----------|---------------|----------------|-------------------------|---------------|
| E124     | E124K   | [60]      | -             | 1              | E124Q (1×)              | Likely pathogenic |
|          |         |           |               |                | E124D (3×)              |               |
| L248     | L248F   | [60]      | -             | 1              | -                       | Likely pathogenic |
| V259     | V259A   | [75]      | -             | 1× (germline)  | -                       |               |
|          | V259D   | [3, 17]   | Type-2 [3]    | 3              | -                       | Pathogenic |
| V259G    | [60]    |           | 1             | -              | -                       | Pathogenic |
| L272     | L272R   | [60]      | 1             | -              | Likely pathogenic        |               |
| V309     | V309A   | [56]      | 1             | -              | Likely pathogenic        |               |
|          |         |           |               |                | V309I (84×; HOM: 1×)     |               |
| G323     | G323R   | [60]      | -             | 1              | -                       | Likely pathogenic |
| V401     | V401L   | [65]      | Type-2 [65]   | 1× (germline)  | -                       |               |
|          |         |           |               | 4              | Pathogenic               |               |
| G403     | G403D   | [18, 72]  | Type-2 [72]   | 2× (germline)  | -                       |               |
|          | G403R   | [1, 3, 17, 34, 56, 57, 59, 60, 72, 73, 95, 100] | Type-1 [3,72] | 54 | Pathogenic |
|          |         |           | (exon 8a)     |                |                         | (exon 8a: 32; 8b: 12; ns: 10) |
| S410     | S410L   | [59, 60]  | -             | 2              | -                       | Pathogenic |
| E412     | E412D   | [95]      | -             | 1              | -                       | Likely pathogenic |
| G457     | G457R   | [59]      | -             | 1              | 1×                      | Uncertain |
| R510 (R530) | R510X | [59] | - | 1 | - | Likely benign |
|          |         |           |               |                | G457del (2×)            |               |
| P548 (P568) | P548L | [59] | - | 1 | - | Likely pathogenic |
| L613 (L633) | L613Q | [57] | - | 1 | - | Likely pathogenic |
| R619 (R639) | R619P | [56] | - | 1 | - | Likely pathogenic |
| R619W    | [57]    |           | 1             | 1×              | -                       | Uncertain |
| S652 (S672) | S652L | [15] | - | 2× (germline)* | - | |
|          | S652L   | [17, 56, 60, 95] | Type-2 [26] | 5       | -                       | Pathogenic |
| S652 (S672) | S652W | - | - | S652W (3×)---no gating change [26] | - | |
| L653 (L673) | L653P | [60] | - | 1 | - | Likely pathogenic |
| L655 (L675) | L655P | [17] | - | 1 | - | Likely pathogenic |
| Position  | Variant   | Reference | Gating change | Times reported | Reported in gnomAD (#) | Pathogenicity |
|----------|-----------|-----------|---------------|----------------|------------------------|---------------|
| S724 (S744) | S724L | [60] | - | 1 | - | Likely pathogenic |
| V728 (V748) | V728I | [90] | - | 1 | - | Likely benign |
| Y741 (Y761) | Y741C | [17] | - | 1 | - | Likely pathogenic |
| F747 (F767) | F747C | [55, 56, 59] | - | 3 | - | Pathogenic |
| | F747L | [1, 3, 17, 56, 57, 59, 60, 87, 95] | Type-2 [66] | 21 | - | Pathogenic |
| | F747V | [17, 55, 57, 59, 60, 73, 95] | - | 18 | - | Pathogenic |
| L748 (L768) | L748S | [60] | - | 1 | - | Likely pathogenic |
| I750 (I770) | I750M | [72] | Type-2 [72] | 1× (germline) | - | Pathogenic |
| | I750F | [17, 56] | - | 2 | - | Pathogenic |
| V752 (V772) | V752G | [95] | - | 1 | - | Likely pathogenic |
| 755-757del | Deletion of “LAD” | [60] | - | 1 | - | Likely pathogenic |
| S969 (S989) | S969L | [60] | - | 1 | - | Likely pathogenic |
| V979 (V999) | V979D | [17] | - | 1 | - | Likely pathogenic |
| Other gnomAD entries at this position: | A756T | 1× | | | | |
| Other gnomAD entries at this position: | V979I | 1× | | | | |
| K981 (K1001) | K981N | [17] | - | 1 | - | Likely pathogenic |
| R990 (R1010) | R990G | [56] | - | 1 | - | Pathogenic |
| | R990H | [3, 59, 60, 87, 95] | Type-4 [52] | 9 | - | Pathogenic |
| R993 (R1013) | R993T | [56] | - | 3 | - | Pathogenic |
| | R993S | [95] | - | 1 | - | Pathogenic |
| A998 (A1018) | A998I | [17] | - | 3 | - | Pathogenic |
| | A998V | [17, 56, 60, 95] | - | 9 | - | Pathogenic |
| C1007 (C1027) | C1007R | [56] | - | 1 | - | Likely pathogenic |
| A1011 (A1031) | A1011T | [60] | - | 1 | - | Likely pathogenic |
| I1015 (I1035) | I1015S | [56] | - | 1 | - | Pathogenic |
| | I1015V | [60, 95] | - | 2 | - | Pathogenic |
| F1147 (F1167) | F1147C | [59] | - | 1 | - | Pathogenic |
| | F1147L | [59, 60] | - | 2 | - | Pathogenic |
| V1151 (V1171) | V1151F | [17, 56] | - | 3 | - | Pathogenic |
| I1152 (I1172) | I1152N | [17] | - | 1 | - | Likely pathogenic |
| V1153 (V1173) | V1153G | [87] | Type-2 [87] | 1 | - | Pathogenic |
| Other gnomAD entries at this position: | V1153I | 3× | | | | |
| Other gnomAD entries at this position: | R1183C | 2× | | | | |
| R1183 (R1203) | R1183H | [60] | - | 1 | - | Likely pathogenic |
We have used the SWISS-MODEL webserver [91] to generate a homology model of CACNA1D based on the apo structure of the inactivated rabbit Cav1.1 α1-subunit in complex with α2δ-, β-, and γ-subunits (PDB entry 5gjv, [93]). Mapping of the mutated residues included in Table 3 onto this structural model revealed spatial clustering around the activation gate of repeats I and II (Fig. 3A). In detail, V401, G403, G407, A749, and I750 are part of the pore-forming S6 segments, whereas V259 and S652 are located in close proximity on neighboring S4-S5 linkers (which connect the voltage-sensing domain (VSD) to the activation gate). These sections of the protein are critical determinants of multiple channel properties: V401 for example is part of a set of hydrophobic residues (V401, F747, F1147, and F1444) lining the pore. These residues form hydrophobic interactions when the pore is closed, thereby prohibiting Ca2+ conductance. Interestingly, variants of two other amino acids of this hydrophobic cluster—F747 and F1147—have been identified in APAs (Table 5). This suggests that disruption of these hydrophobic interactions generally alters channel function, independently of the exact residue affected, and it will be interesting to see whether a variant of the fourth residue in this cluster—F1444—will be observed in patients in the future.

G403 and A749 are part of the S6 G/A/G/A ring found at the intersection of the S4-S5 linker, S5, and S6 helices. They are involved in linker-S6 interactions and have been implicated in coupling of the VSD and the pore [23]. In a homology model of the resting state (based on the resting state of a BacNav channel, PDB entry 6p6w, [92]), these residues fit tightly in the intersection point (Fig. 3B). Therefore, any variant either increasing flexibility and diminishing hydrophobic contacts (i.e., alanine to glycine) or introducing larger and/or polar residues (i.e., alanine to threonine) is expected to interfere with, and thus destabilize, the modeled resting closed state conformation and promote open channel states.

In addition, A749, together with I750, is part of the "LAIA" motif that is highly conserved among L-type Ca2+ channels and mutation of any residue of the motif to proline resulted in altered activation properties in the related Cav1.2 channel [27]. Similar to the G/A/G/A ring, S652 and V259 are within another critical interaction site, connecting the S4-S5 linker of repeats I and II. Previous modeling studies suggested that loss of hydrogen bonds in the S652L variant could be responsible for the effect on channel function [26].

Finally, comparing the resting and activated (based on the active state structure of a BacNav channel, PDB entry 5vb8, [37]) state models of CACNA1D shows that G407 in the S6 segment is located within the pore region, where large movements of the backbone facilitate channel opening and closure (Fig. 3C). Please note that G407 corresponds to the last S6 residue that is still resolved in the template of the BacNav crystal structure, suggesting that downstream residues are subject to conformational flexibility.

Table 5

| Position | Variant | Reference | Gating change | Times reported | Pathogenicity |
|----------|---------|-----------|---------------|----------------|---------------|
| F1248 (F1268) | F1248L | [59, 60] | Pathogenic | 4 | Pathogenic |
| D1273 (D1293) | D1273N | [60] | Pathogenic | 1 | Pathogenic |
| P1336 (P1371) | P1336R | [3, 17, 59] | Pathogenic | 4 | Pathogenic |
| V1338 (V1373) | V1338M | [1, 17, 57, 59, 60, 73] | Pathogenic | 13 | Pathogenic |
| I1352 (I1387) | I1352T | [59] | Pathogenic | 1 | Pathogenic |
| M1354 (M1389) | M1354I | [3, 17] | No gating change [66] | 2 | Pathogenic/uncertain |
| P1499 (P1534) | P1499L | [59] | Pathogenic | 1 | Pathogenic |
| T1835 (T1879) | T1835I | [60] | Pathogenic | 1 | Pathogenic |
| W1836 (W1880) | W1836X | [59] | Pathogenic | 1 | Pathogenic |

Criteria for the classification of the pathogenicity are given in Table 4. The reference CACNA1D sequence EU_363339 contains exons 8a but not exons 8b or 8c. The respective NM_000720 reference sequence contains exons 8b and 8c but not exon 8a. Therefore, not all residues are measured (gating change) or not reported in gnomAD. "OM" indicates not measured gating change. "HOM" indicates not measured homology model. "X" indicates a STOP. "O" indicates a transition. "S" indicates a single amino acid change. "C" indicates a conservative amino acid change. Pathogenic: variant lacks the distal C-terminal regulatory domain (DCRD), which disrupts an autoregulatory interaction with the proximal C-terminus (PCRD), thereby interfering with channel gating (enhanced Ca2+-dependent inactivation and voltage sensitivity). The premature stop in the R510X variant likely results in a truncated, non-functional channel (loss of function).

Table 5 (continued)

| Position | Variant | Reference | Gating change | Times reported | Pathogenicity |
|----------|---------|-----------|---------------|----------------|---------------|
| F1248 (F1268) | F1248L | [59, 60] | Pathogenic | 4 | Pathogenic |
| D1273 (D1293) | D1273N | [60] | Pathogenic | 1 | Pathogenic |
| P1336 (P1371) | P1336R | [3, 17, 59] | Pathogenic | 4 | Pathogenic |
| V1338 (V1373) | V1338M | [1, 17, 57, 59, 60, 73] | Pathogenic | 13 | Pathogenic |
| I1352 (I1387) | I1352T | [59] | Pathogenic | 1 | Pathogenic |
| M1354 (M1389) | M1354I | [3, 17] | No gating change [66] | 2 | Pathogenic/uncertain |
| P1499 (P1534) | P1499L | [59] | Pathogenic | 1 | Pathogenic |
| T1835 (T1879) | T1835I | [60] | Pathogenic | 1 | Pathogenic |
| W1836 (W1880) | W1836X | [59] | Pathogenic | 1 | Pathogenic |

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Given that structural components regulating both VSD-pore coupling and channel activation as well as pore opening/closure are concentrated in regions surrounding the activation gate, it should not come as a surprise that residues within this area are particularly susceptible to structural changes and give rise to variants that alter the biophysical properties of the channel. We therefore propose that new variants in this region are likely to have a functional impact and should be characterized in detail.

Molecular modeling can also provide evidence for variant R619P as a likely-pathogenic type 4 variant (Table 2). R619W and R619P were reported in APAs and APCCs (Table 5). However, R619W was also reported in a control subject in the gnomAD database (Table 5), casting doubt on its pathogenicity. R619 is the first positive charge in the VSD of repeat II (Fig. 4A). Both the wild-type R619 and the R619W variant provide a steric barrier that seals the VSD and prevents ion leakage through the surface of the VSD (Fig. 4B, D, E). Modeling of the R619P variant suggests that the smaller side chain fails to provide this seal and thus creates a tunnel (Fig. 4C, F). This allows for ion leakage through the VSD in the resting state and thus generates a so-called \( \omega \)-current, which favors membrane depolarization. Please note that mutation of the uppermost arginine in the VSD has been described as a general mechanism to create gating pores in voltage-gated ion channels [53] and that the Cav1.3 R619P mutant directly corresponds to the Nav1.5 R808P mutant associated with Brugada syndrome (R809P in the rat; [30]). Altogether, this suggests that the Cav1.3 R619P variant also has a high probability of being pathogenic.

Estimated prevalence of high-risk CACNA1D de novo variants in neurodevelopmental disorders

Two recently published germline de novo CACNA1D variants identified in small patient cohorts of ASD without (T1376M, T1376M, T1376M,
[31]) or with epilepsy (V1447L, [44]) could suggest that the prevalence of such de novo CACNA1D variants associated with neurodevelopmental disorders is higher as currently deduced from NGS of large patient cohorts. Both variants are de novo and absent in healthy controls (gnomAD database); however, due to a lack of detailed clinical or functional data, they were not included in Table 3. According to the ACMG classification system [69], we consider the V1447L variant as likely pathogenic due to its location within a well-established functional domain (S6, repeat IV). Importantly, residue V1447 is highly conserved among all Cavs, lies adjacent to the S6 G/A/G/A ring of repeat IV, and corresponds to I750 in repeat II (discussed above), further strengthening the assumption that mutation of this residue can indeed interfere with normal Cav1.3 channel function. This is less clear for the T1376M variant which is located at the beginning of the S5-S6 pore loop of repeat IV, and a functional impact on channel gating cannot be presumed in absence of functional data.

So far only 6 de novo CACNA1D variants have been identified in large-scale genetic studies in cohorts of ASD (A749G, G407R [70], 11,986 individuals; T1376M [31], 59 individuals), ASD with epilepsy (V1447L, [44], 103 individuals), and severe developmental disorders (2 x S652L [15], 1133 individuals). In these cohorts, the occurrence of CACNA1D variants is relatively rare (together 1:2214); thus, they are often not identified as high-risk genetic variants (as happened for S652L in [15] but see [26]). Additionally, patients with pathogenic CACNA1D variants can be affected by endocrine and/or neurological symptoms (Table 3) and might therefore meet the exclusion criteria of such studies, further contributing to their underrepresentation. Since the majority of the known de novo CACNA1D germline variants have been published as case reports or were even unpublished (we are aware of at least two additional unpublished patients affected by one of the pathogenic variants in Table 3), this supports the notion that such CACNA1D variants are likely underreported in the literature.

Summary and perspectives

As described in this review, we begin to understand the clinical disease spectrum associated with pathogenic de novo CACNA1D missense variants. Since the symptoms can only be explained by a channel gain-of-function, L-type Ca^{2+} channel blockers are a mechanism-based and still promising therapeutic option. However, current data predict that not all variants may be responsive to these drugs and it still remains unclear which of the many neurological and neuropsychiatric symptoms will respond to therapy. To learn more about potential drug effects, it is important to collect information about as many patients as possible which requires that clinicians and
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Code availability Not applicable

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Data availability Not applicable

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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