Compound heterozygous CACNA1H mutations associated with severe congenital amyotrophy

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ABSTRACT
Neuromuscular disorders encompass a wide range of conditions often associated with a genetic component. In the present study, we report a patient with severe infantile-onset amyotrophy in whom two compound heterozygous variants in the gene CACNA1H encoding for Ca,v3.2 T-type calcium channels were identified. Functional analysis of Ca,v3.2 variants revealed several alterations of the gating properties of the channel that were in general consistent with a loss-of-channel function. Taken together, these findings suggest that severe congenital amyoplasia may be related to CACNA1H and would represent a new phenotype associated with mutations in this gene.

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Introduction
Neuromuscular disorders (NMD) encompass a wide range of conditions including amyotrophic lateral sclerosis (ALS), congenital muscular dystrophies and myopathies, Duchenne muscular dystrophy, and spinal muscular atrophy. They are characterized by progressive muscle degeneration and weakness that primarily or secondarily impair skeletal muscles and their innervation. The onset of NMD ranges from in utero to old age, but primarily affect infants, children, and teenagers [1]. Although some NMD are acquired, the vast majority are genetic, and may display autosomal recessive, autosomal dominant, or X-linked inheritance [2]. It is also apparent that NMD phenotypes may vary widely even in individuals carrying the same disease-causing mutation, suggesting the existence of additional factors such as modifier genes that contribute to the onset, severity, and duration of the disease [3].

Recently, whole exome sequence analysis has identified two compound heterozygous recessive missense mutations in the gene CACNA1H in a patient with ALS [4,5]. CACNA1H encodes for Ca,v3.2 channel, a member of the voltage-gated calcium channel family [6]. Ca,v3.2 channels are widely expressed throughout the body including the central and peripheral nervous system, heart, kidney, smooth muscle, as well as in several neuroendocrine organs [7]. Through their ability to support low-threshold calcium influx (T-type current), they serve essential physiological processes including neuronal firing, hormone secretion, smooth muscle contraction, and myoblast fusion [8]. Their physiological implication is further exemplified by the existence of polymorphisms in CACNA1H associated with a number of human disorders including several forms of epilepsy [9], autism spectrum disorders [10,11], congenital pain [12], primary aldosteronism [13,14], and ALS [4,5].

In the present study, we report a patient with severe congenital amyotrophy in whom two compound heterozygous variants in CACNA1H were identified. Functional analysis of Ca,v3.2 variants revealed altered channel gating, strengthening the genetic association of CACNA1H with NMD.

Materials and methods
Whole exome sequencing
Whole exome sequencing was performed at a commercial laboratory (GeneDx). Using genomic DNA from the proband and both parents, exonic regions and flanking splice junctions were selected, sequenced, and analyzed as per established proprietary protocols using an Illumina
sequencing system with 100 bp or greater paired end reads. Reads were aligned to human genome build GRCh37/UCSC hg19 and analyzed for sequence variants using a custom-developed analysis tool (Xome Analyzer). Mean depth of coverage was 100x.

**Plasmid cDNA constructs**

The human wild-type Ca\textsubscript{3.2} in pcDNA3.1 [15] was used as template to introduce separately the V681L and D1233H mutations by site-directed mutagenesis using the Q5\textregistered Site-Directed Mutagenesis Kit (NEB) and the following pairs of primers: p.V681L: 5'-GGGCTCAGTttgCCCTGGCC-3' (forward) and 5'-GACAGATGGCCAGGGCC-3' (reverse); p.D1233H: 5'-CCTGCGATCcacAGCCACCG-3' (forward) and 5'-AAGAAGTCGCTGGGCAGG-3' (reverse). Final constructs were verified by sequencing.

**Cell culture and heterologous expression**

Human embryonic kidney tsA-201 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all media purchased from Invitrogen) and maintained under standard conditions at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}. Heterologous expression of Ca\textsubscript{3.2} channels was performed by transfecting cells with plasmid cDNAs encoding for Ca\textsubscript{3.2} channel variants using the calcium/phosphate method as previously described [16].

**Recording of T-type currents**

Patch clamp recording of T-type currents in tsA-201 cells expressing Ca\textsubscript{3.2} channels was performed 72 h after transfection in the whole-cell configuration at room temperature (22–24°C). The bath solution contained (in millimolar): 5 BaCl\textsubscript{2}, 5 KCl, 1 MgCl\textsubscript{2}, 128 NaCl, 10 TEA-Cl, 10 D-glucose, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2 with NaOH). Patch pipettes were filled with a solution containing (in millimolar): 110 CsCl, 3 Mg-ATP, 0.5 Na-GTP, 2.5 gCl\textsubscript{2}, 5 D-glucose, 10 EGTA, and 10 HEPES (pH 7.4 with CsOH), and had a resistance of 2–4 MΩ. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments) and acquisition and analysis were performed using pClamp 10 and Clampfit 10 software, respectively (Axon Instruments). The linear leak component of the current was corrected online, and current traces were digitized at 10 kHz and filtered at 2 kHz. The voltage dependence of activation of Ca\textsubscript{3.2} channels was determined by measuring the peak T-type current amplitude in response to 150 ms depolarizing steps to various potentials applied every 10 s from a holding membrane potential of −100 mV. The current-voltage relationship (I/V) curve was fitted with the following modified Boltzmann Equation (1):

$$I(V) = G_{\text{max}} \frac{(V - V_{\text{rev}})}{1 + \exp \left( \frac{(V_{0.5} - V)}{k} \right)}$$

with $I(V)$ being the peak current amplitude at the command potential $V$, $G_{\text{max}}$ the maximum conductance, $V_{\text{rev}}$ the reversal potential, $V_{0.5}$ the half-activation potential, and $k$ the slope factor. The voltage dependence of the whole-cell Ba\textsuperscript{2+} conductance was calculated using the following modified Boltzmann Equation (2):

$$G(V) = \frac{G_{\text{max}}}{1 + \exp \left( \frac{(V_{0.5} - V)}{k} \right)}$$

with $G(V)$ being the Ba\textsuperscript{2+} conductance at the command potential $V$.

The voltage dependence of the steady-state inactivation of Ca\textsubscript{3.2} channels was determined by measuring the peak T-type current amplitude in response to a 150 ms depolarizing step to −20 mV applied after a 5 s-long conditioning prepulse ranging from −120 mV to −30 mV. The current amplitude obtained during each test pulse was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. The voltage dependence of the steady-state inactivation was fitted with the following two-state Boltzmann function (3):

$$I(V) = \frac{I_{\text{max}}}{1 + \exp \left( \frac{(V - V_{0.5})}{k} \right)}$$

with $I_{\text{max}}$ corresponding to the maximal peak current amplitude and $V_{0.5}$ to the half-inactivation voltage.

The recovery from inactivation was assessed using a double-pulse protocol from a holding
potential of −100 mV. The cell membrane was depolarized for 2 s at 0 mV (inactivating prepulse) to ensure complete inactivation of the channel, and then to −20 mV for 150 ms (test pulse) after an increasing time period (interpulse) ranging between 0.1 ms and 2 s at −100 mV. The peak current from the test pulse was plotted as a ratio of the maximum prepulse current versus interpulse interval. The data were fitted with the following single-exponential function (4):

\[
\frac{I}{I_{max}} = A \times \left(1 - \exp\left(-\frac{t}{\tau}\right)\right)
\]

where \(\tau\) the time constant of channel recovery from inactivation.

**Statistical analysis**

Values are presented as mean ± S.E.M. for \(n\) experiments. Statistical significance was determined using a one-way ANOVA test followed by Dunnett multiple comparison test. * \(p<0.05\), ** \(p<0.005\), *** \(p<0.001\).

**Results**

**Clinical characteristics and genetic analysis**

The patient is a 13-month-old girl who was born at term following a pregnancy complicated by severe polyhydramnios treated with amnioreduction at 31 weeks. Her birth weight was 2.9 kg (20% ile). Her Apgar score (Appearance, Pulse, Grimace, Activity, and Respiration) used to summarize the health of newborn children was critically low (2 at 1-min and 5-min) and was intubated for poor respiratory effort, absent gag and suck. She had arthrogryposis, areflexia, and no antigravity movements. Nerve conduction studies showed intact sensory but absent motor responses. Magnetic resonance imaging (MRI) of her brain was unremarkable. However, the MRI of her muscles noted severe and diffuse amyoplasia with largest muscles being right biceps femoris (8 x 5 mm at widest diameter) and medial gastrocnemius (8 x 3 mm) (Figure 1(a,b)). A biopsy of the vastus lateralis revealed no viable muscle and repeat biopsies of the gastrocnemius noted a strong predominance of type 1 fibers and a marked variability of fiber size. She remains fully ventilated via tracheostomy with no antigravity movement at 13 months old. Whole exome sequencing identified two compound heterozygous variants (c.2041G>T and c.3697G>C) in CACNA1H, causing the substitution of the valine (V) 681 by a leucine (L) (p.V681L) and of the aspartic acid (D) 1233 by a histidine (H) (p.D1233H) in the Ca\(_{\text{3.2}}\) T-type calcium channel, respectively. These mutations were inherited from the father and mother, respectively, who have no symptoms of muscle disease (Figure 1(c)). The p. V681L and p.D1233H mutations are located within the intracellular loops connecting domains I-II and domains II-III of Ca\(_{\text{3.2}}\) channel, respectively (Figure 1(d)).

**Expression of Ca\(_{\text{3.2}}\) channel variants**

To assess the functional consequence of CACNA1H variants, the two mutations were introduced separately into the human Ca\(_{\text{3.2}}\) channel, and functional expression was assessed by patch clamp recording of T-type currents in tsA-201 cells expressing Ca\(_{\text{3.2}}\) channel variants. Cells expressing WT, V681L and D1233H Ca\(_{\text{3.2}}\) channel variants, or co-expressing the two-channel variants (V681L + D1233H) to resemble the heterozygous status of the patient for the two mutations, displayed characteristic low-threshold voltage-activated T-type currents (Figure 2(a,b)). The maximal T-type conductance in cells expressing Ca\(_{\text{3.2}}\) variants was similar (V681L: 756 ± 49 pS/pF, \(n = 45\); D1233H: 640 ± 60 pS/pF, \(n = 48\)) and was not significantly different from cells expressing the WT channel (688 ± 59, \(n = 45\)) (Figure 2(c,d)) and Table (1). These results indicate that the V681L and D1233H mutations do not alter the expression level of Ca\(_{\text{3.2}}\) channels.

**Gating properties of Ca\(_{\text{3.2}}\) channel variants**

To assess the functional properties of Ca\(_{\text{3.2}}\) variants, we analyzed the voltage-dependence of T-type current activation in cells expressing WT, V681L, D1233H, or co-expressing the V681L and D1233H channel variants (co-expression of the two separate cDNA constructs each carrying one mutation). The mean half-activation potential in cells expressing the
V681L variant was shifted by 3.5 mV ($p = 0.0039$) toward hyperpolarized potentials ($-43.4 \pm 0.9$ mV, $n = 45$) compared to cells expressing the WT channel ($-39.9 \pm 0.6$ mV, $n = 45$) (Figure 3(a,b) and Table 1). Although the mean half-activation potential of T-type currents measured in cells expressing the D1233H variant was not significantly altered, we observed a significant decrease ($p = 0.0429$) of the inactivation slope factor (5.0 $\pm$ 0.2, $n = 48$) compared to WT channels (5.6 $\pm$ 0.2, $n = 45$) (Figure 3(c) and Table 1)). To further investigate the gating properties of Ca$_{v}$3.2 variants, we assessed the voltage-dependence of the steady-state inactivation of the T-type current. We observed a hyperpolarized shift of the mean half-inactivation potential by 5.5 mV ($p < 0.0001$) in cells expressing the V681L variant alone ($-75.3 \pm 1.2$ mV, $n = 30$) and by 4.2 mV ($p = 0.0027$) in cells co-expressing the V681L and D1233H variants ($-74.0 \pm 1.0$ mV, $n = 31$) compared to cells expressing the WT channel ($-69.8 \pm 0.6$ mV, $n = 32$) (Figure 3(d–f) and Table 1). In contrast, the voltage-dependence of steady-state inactivation of the T-type current in cells expressing the D1233H remained unaltered (Figure 3(d–f) and Table 1). In addition,
recovery from inactivation (Figure 3(g,h) and Table (1)) as well as inactivation and inactivation kinetics remain similar between the channel variants, and only a mild acceleration of the inactivation kinetic of the V681L variant (18.4 ± 0.9, n = 30) was observed compared to the WT channel (21.9 ± 1.0, n = 45) (Figure 3(i) and Table (1)).

Collectively, these data indicate that the V681L and D1233H mutations produce mild but significant alterations of the gating of Ca<sub>v</sub>3.2 by altering the voltage dependence of activation and inactivation of the channel.

**Window current of Ca<sub>v</sub>3.2 channel variants**

Considering the effect of the V681L and D1233H mutations on the voltage dependence of activation and inactivation of Ca<sub>v</sub>3.2 channels, we analyzed the window current of Ca<sub>v</sub>3.2 variants as the area under the overlap between the activation and inactivation curves (Figure 4(a)). We observed a decrease of the window current generated by Ca<sub>v</sub>3.2 variants. For instance, the mean area of the window current was decreased by 22% (p = 0.0055) and 19% (p = 0.0188) in cells expressing the V681L and D1233H variants, respectively (Figure 4(b,c)). Consistent with this observation, the window current in cells co-expressing the V681L and D1233H channel variants were decreased by 22% (p = 0.0079) compared to cells expressing the WT channel (Figure 4(b,c)). In addition, we observed a hyperpolarized shift of the peak-voltage of the window current by 4.6 mV (p < 0.0001) in cells expressing the V681L variant (Figure 4(d)).

Altogether, these data indicate that alteration of the voltage-dependence of activation and inactivation caused by the V681L and D1233H mutations ultimately translates into a decrease of the window current supported by Ca<sub>v</sub>3.2 variants.

**Discussion**

Here we describe two compounds heterozygous CACNA1H mutations identified in a case-unaffected-parents trio with infantile-onset NMD. The two mutations, V681L and D1233H, are located within the intracellular loops connecting domains I and II (I-II loop) and II and III (II-III loop) of the Ca<sub>v</sub>3.2 T-type calcium channel. Previous structure-function studies have documented the role of the I-II loop of Ca<sub>v</sub>3.2 in the control of the gating and surface expression of the channel [17,18]. Additionally, several human mutations associated with idiopathic generalized
epilepsy and located within the I-II loop and II-III-loop of Ca\textsubscript{v}3.2 alter channel gating [19–22]. Consistent with these observations, our functional analysis of Ca\textsubscript{v}3.2 missense variants revealed several alterations of the gating properties of the channel. For instance, the V681L mutation produced a hyperpolarized shift of the voltage dependence of activation, as well as a concomitant hyperpolarized shift of the voltage dependence of inactivation. From a mechanistic viewpoint, this apparent mix gain- and loss-of-channel function can be explained by a model in which the V681L mutation may destabilize the closed state of the channel. This notion has recently been proposed for Ca\textsubscript{v}3.3 channels [23] and is consistent with the observation that T-type channels inactivate from both open and closed states [24,25]. Of particular importance is the observation that as a result of the effect on the voltage-dependence of activation and inactivation, the V681L mutation caused a marked decrease of the window current supported by Ca\textsubscript{v}3.2 channel. Likewise, decrease of the window current was also observed with the D1233H as a result of the increased slope of the activation curve without alteration of the mid-voltage.

The question then arises as to how channel defects caused by the V681L and D1233H mutations may contribute to the disease phenotype. Alteration of the window current appears particularly relevant as an underlying mechanism of the atrophy observed in the proband for several reasons. First, Ca\textsubscript{v}3.2 channels are functionally expressed in embryonic skeletal muscle fibers where they present a developmental expression pattern consistent with a role in early-stage muscle differentiation. For instance, the T-type current density in muscle fibers increases transiently during prenatal myogenesis to reach a maximum expression at embryonic day E16 before to decrease dramatically until birth [26]. Second, extracellular calcium is required for the fusion of myoblasts [27] and a rise in intracellular calcium level precedes fusion [28,29]. Third, it was proposed that rise in intracellular calcium levels occurs via the window current supported by Ca\textsubscript{v}3.2 channels in response to the hyperpolarization of the plasma membrane that proceeds the fusion of the myoblasts [30]. Therefore, our observation

| Table 1. Electrophysiological properties of human Ca\textsubscript{v}3.2 channel variants expressed in tsA-201 cells. Activation and inactivation kinetic values are shown for a depolarization step to −40 mV from a holding potential of −100 mV. | 
|---|---|---|---|---|---|---|---|---|---|---|
| Ca\textsubscript{v}3.2 variant | V\textsubscript{0.5} (mV) | k (nS/µF) | τ (ms) (n) | G\textsubscript{max} (pS/pF) (n) | V\textsubscript{0.5} (mV) | k (nS/µF) | τ (ms) (n) | G\textsubscript{max} (pS/pF) (n) |
| WT | −39.9 ± 0.6 | 5.6 ± 0.2 | 45 | 377 ± 35 | 11 | −69.8 ± 0.6 | 4.4 ± 0.1 | 32 | 377 ± 35 | 11 |
| V681L | −43.4 ± 0.9** | 5.4 ± 0.2 | 45 | 377 ± 35 | 11 | −75.3 ± 1.2*** | 4.4 ± 0.1 | 32 | 377 ± 35 | 11 |
| D1233H | −41.1 ± 0.8 | 5.0 ± 0.2* | 48 | 377 ± 35 | 11 | −76.9 ± 1.2* | 4.4 ± 0.1 | 32 | 377 ± 35 | 11 |
| V681L+D1233H | −40.8 ± 0.6 | 5.6 ± 0.1 | 30 | 377 ± 35 | 11 | −74.0 ± 1.0** | 4.7 ± 0.2 | 31 | 377 ± 35 | 11 |
that the p.V681L and p.D1233H mutations caused a reduction of the window current could have an important consequence at early stages of muscle differentiation and may represent an underlying mechanism of infantile-onset amyotrophy (Figure 5(a)). Additionally, several studies have reported the expression of T-type channels in motor neurons [31,32]. Although the role of T-type channels in motor neurons has not been investigated yet, it is likely that they contribute to some forms of neuronal excitability. Therefore, alteration of T-type channel activity is likely to affect muscle afferent signaling which may participate to some extend to motor symptoms.

In summary, we identified new CACNA1H variants in an individual with infantile-onset NMD. Clinical, histopathological, and electrophysiological findings indicating a loss-of-Ca,3.2 channel function suggest that severe congenital amyoplasia may be related to CACNA1H, which add to the

Figure 3. Electrophysiological properties of Ca,3.2 variants. (a) Mean normalized voltage-dependence of T-type current activation for cells expressing WT (white circles), V681L (blue circles), D1233H (orange circles), and co-expressing V681L and D1233H channel variants (purple circles). (b) Corresponding mean half-activation potential values obtained from the fit of the activation curves with the modified Boltzmann Equation (2). (c) Corresponding mean half-activation slope factor. (d) Mean normalized voltage-dependence of steady-state inactivation. (e) Corresponding mean half-inactivation potential values obtained from the fit of the inactivation curves with the two-state Boltzmann function (3). (f) Corresponding mean inactivation slope factor. (g) Mean normalized recovery from inactivation kinetics. (h) Corresponding mean time constant τ values of recovery from inactivation obtained by fitting recovery curves with the single-exponential function (4). (i) Mean time constant τ values of activation and inactivation of T-type currents obtained by fitting the rising and decay phase of the current with a single exponential function, respectively.
growing list of CACNA1H channelopathies and would represent a new phenotype associated with mutations in this gene.

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**Disclosure statement**

The authors report no conflict of interest.

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