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Gating defects of disease-causing \textit{de novo} mutations in \textit{Ca}_v1.3 \textit{Ca}^{2+} channels

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ABSTRACT
Recently, we and others identified somatic and germline \textit{de novo} gain-of-function mutations in CACNA1D, the gene encoding the a1-subunit of voltage-gated \textit{Ca}_v1.3 \textit{Ca}^{2+}-channels. While somatic mutations identified in aldosterone producing adenomas (APAs) underlie treatment-resistant hypertension, germline CACNA1D mutations are associated with a neurodevelopmental disorder characterized by a wide symptomatic spectrum, including autism spectrum disorder. The number of newly identified CACNA1D missense mutations is constantly growing, but their pathogenic potential is difficult to predict \textit{in silico}, making functional studies indispensable to assess their contribution to disease risk.

Here we report the functional characterization of previously identified CACNA1D APA mutations F747L and M1354I using whole-cell patch-clamp electrophysiology upon recombinant expression in tsA-201 cells. We also investigated if alternative splicing of \textit{Ca}_v1.3 affects the aberrant gating of the previously characterized APA mutation R990H and two mutations associated with autism spectrum disorder (A479G and G407R). Splice-variant dependent gating changes are of particular interest for germline mutations, since the relative expression of \textit{Ca}_v1.3 splice variants differs across different tissues and within brain regions and might therefore result in tissue-specific phenotypes. Our data revealed a complex gain-of-function phenotype for APA mutation F747L confirming its pathogenic role. Furthermore, we found splice-variant dependent gating changes in R990H, A479G and G407R. M1354I did not change channel function of \textit{Ca}_v1.3 splice variants and should therefore be considered a rare non-pathogenic variant until further proof for its pathogenicity is obtained. Our new findings together with previously published data allow classification of pathogenic CACNA1D mutations into four categories based on prototypical functional changes.

Introduction

Different classes of voltage-gated \textit{Ca}^{2+} channels serve a central role for key physiological processes by generating inward \textit{Ca}^{2+} currents in response to changes in membrane potentials in electrically excitable cells [1–3]. \textit{Ca}^{2+} influx through these channels drives not only membrane depolarization but, through \textit{Ca}^{2+} ions as universal second messenger, also regulates a large variety of intracellular \textit{Ca}^{2+} dependent processes. These include muscle contraction, gene transcription, hormone secretion, neurotransmitter release, synaptic excitability and plasticity [1–3]. Their subcellular targeting as well as the dynamics of \textit{Ca}^{2+} entry need to be tightly controlled by accessory subunits, post-translational modification, lipid- and protein interactions, as well as alternative splicing [1,2]. The occurrence of human channelopathies resulting from even minor changes in \textit{Ca}^{2+} channel activity [4–8] further emphasizes the importance of strictly regulated channel gating and targeting for normal physiological function.

We [9–12] and others [13–16] have recently described \textit{de novo} missense mutations in CACNA1D encoding the pore-forming a1-subunit of \textit{Ca}_v1.3 L-type channels. These included somatic mutations identified in aldosterone-producing adenomas [APAs, 11,12,15], as well as germline mutations from patients with neurodevelopmental disorders characterized by autism spectrum disorder (ASD) and/or intellectual disability with or without seizures [9,10,13–15]. Three of these patients further displayed primary aldosteronism or hyperinsulinism...
All so far functionally characterized mutations – both from germline and somatic mutations – induced pronounced gating changes, evident either as robust shifts in activation and inactivation voltage to more negative potentials and/or strong reductions of $I_{Ca}$ inactivation, thus permitting enhanced $Ca^{2+}$ entry through the channel. This suggests that $Ca_{v}1.3$ gain-of-function cannot only drive excessive aldosterone production in APAs ($Ca^{2+}$ is the critical steroidogenic second messenger in zona glomerulosa cells, 17) but might also confer high risk for neuropsychiatric and neurological disorders [18].

Due to the known physiological role of $Ca_{v}1.3$ for brain development and function [2,18] and the recent findings of recurrent de novo germline CACNA1D mutations in patients with neuropsychiatric and neurological symptoms this gene has been incorporated in custom genetic panels for clinical diagnosis, including autism/intellectual disability (Autism/ID Xpanded Panel).

Based on findings in $Ca_{v}1.3$-deficient mice [19] and humans [20] heterozygous de novo loss-of-function mutations are expected to be clinically silent and only missense mutations with gating changes permitting enhanced $Ca^{2+}$ signaling are likely to be disease relevant. However, the functional consequences of such mutations are difficult to predict in silico. Therefore, newly identified rare missense variants have to be functionally characterized in heterologous expression systems to confirm their disease causing potential. So far this has been accomplished for seven patients with germline CACNA1D mutations [six different mutations, 9,10,13–15] and six somatic mutations occurring in APAs [11,12,15,21]. Interestingly, several of the functionally verified germline mutations causing a neurodevelopmental disorder are also found in APAs [I750M, G403D, V401L, 18]. Therefore, if a germline mutation in patients with a neurodevelopmental syndrome is also reported in APAs this would be strong support for its pathogenic role and guide genetic and clinical diagnosis. The number of CACNA1D mutations in APAs in precursor lesions (aldosterone producing cell clusters, APCCs,) is constantly growing [>40, 18,22,23]. All these mutations are highly likely to allow enhanced $Ca^{2+}$ signaling though $Ca_{v}1.3$ channels because they are selected by their ability to drive $Ca^{2+}$-dependent aldosterone production. However, despite this selection pressure not all predicted high confidence CACNA1D APA and APCC mutations may strongly contribute to disease risk because other, yet unknown mechanisms could be responsible. Notably, a substantial fraction of excessive aldosterone production in APAs is due to mechanisms not explained by somatic variants in known genes [24]. This emphasizes the need for further functional characterization of CACNA1D APA mutations to verify their causative roles.

Here we report the analysis of three APA mutations (F747L, R990H, M1354I) which we have previously reported together with other CACNA1D mutations in a cohort of 152 subjects [11]. We also investigated if alternative splicing of $Ca_{v}1.3$ $\alpha1$ can affect mutational effects on channel function in R990H, M1354I and in two previously reported ASD mutations A749G and G407R. Indeed, we found splice-variant dependent gating changes in R990H, A749G and G407R. Our data confirm a gain-of-function phenotype also for F747L but not for M1354I. Finally, our experiments support our previous hypothesis that an $\omega$-current through mutant R990H rather than altered gating explains its pathogenic potential.

**Experimental procedures**

**Complementary DNA constructs**

Human wild-type $Ca_{v}1.3\alpha_{1}$-subunits (reference sequence EU363339) comprising alternative exons 8a and 42 (long C-terminal splice variant) or 43s [short C-terminal splice variant, 25] were previously cloned into the pGFPminus vector (no GFP tag, CMV promoter) [25]. Mutations were introduced into $Ca_{v}1.3$ splice variants using standard polymerase chain reaction approaches and verified by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). When co-expressed with auxiliary $\beta_{3}$ and $\alpha_{2}\delta$-1 subunits in tsA-201 cells, all mutant $\alpha_{1}$-subunits were detected with the expected molecular mass in Western blots and at densities comparable to wild-type (n ≥ 3 transfections for each mutant).

**Cell culture and transfection**

tsA-201 cells were maintained in culture and transiently transfected with wild-type (WT) or mutant
Ca_{1.3} \alpha_{1-} and auxiliary \beta_{3-} (NM_012828) and \alpha_{2} \delta_{1-} (NM_001082276) subunits as previously described \cite{9,26}. GFP was co-transfected to visualize transfected cells.

**Electrophysiological recordings in tsA-201 cells**

For whole-cell patch-clamp recordings borosilicate glass electrodes (203-776-0664 Warner Instruments and 64-0792, Harvard Apparatus, USA) with a final resistance of 1.5–3.0 MΩ were pulled using a micropipette puller (Sutter instruments, P-97) and fire polished afterwards (Microforge, Narishinge MF-830). All recordings were performed at room temperature (21–23 °C) in whole-cell configuration using the Axopatch 200B amplifier (Axon instruments), digitized at 50 kHz (Digitizer 1322A, Axon instruments), low-pass filtered at 5 kHz and compensated for 60–90 % of the series resistance. The recording solutions contained in mM: *bath*: 15 CaCl_{2}, 10 HEPES, 150 choline-Cl and 1 MgCl_{2}, adjusted to pH 7.4 with CsOH; *intracellular*: 135 CsCl, 10 HEPES, 10 Cs-EGTA, 1 MgCl_{2}, 4 Na_{2}ATP adjusted to pH 7.4 with CsOH. The holding potential (HP) was set to −80 mV. To determine the current-voltage (I-V) relationship, a 30 ms (short splice variants) or 50 ms (long splice variants) square pulse protocol to different voltages was applied. Resulting I-V curves were fitted from −80 mV to + 40 mV to the following equation:

\[ I = G_{\text{max}}(V - V_{\text{rev}})/(1 + \exp(-(V - V_{0.5}))/k) \]

where \( V_{\text{rev}} \) is the extrapolated reversal potential, \( V \) the test potential, \( I \) the peak current, \( G_{\text{max}} \) the maximum conductance, \( V_{0.5} \) the half maximal activation voltage and \( k \) the slope factor. The voltage-dependence of Ca^{2+} conductance was fitted according to a Boltzmann distribution:

\[ G = G_{\text{max}}/(1 + \exp(-(V - V_{0.5}))/k) \]

Estimates for changes in channel open probability and/or single channel conductance were obtained as described \cite{25} by normalizing the ionic tail current to the amplitude of the integrated ON-gating current (\( Q_{\text{ON}} \)) of the same pulse at the \( V_{\text{rev}} \). The \( Q_{\text{ON}} \) at the \( V_{\text{rev}} \) was also used to determine the surface expression of the channel complexes.

For analysis of current densities, surface expression and estimation of the open probability only recordings from the same transfections were compared.

Steady-state inactivation was measured by applying a control test pulse (20 ms to the voltage of maximal inward current, \( V_{\text{max}} \)) followed by 5-s conditioning steps to various potentials and a subsequent 20-ms test pulse to \( V_{\text{max}} \) (30-s recovery between protocols). Inactivation was calculated as the ratio between the current amplitudes of the test versus control pulse. Steady-state inactivation parameters were obtained by fitting the data to a modified Boltzmann equation:

\[ G = (1 - G_{\text{max}})/(1 + \exp((V - V_{0.5}))/k)) + G_{\text{max}} \]

To investigate inactivation kinetics, cells were depolarized for 5 s to the \( V_{\text{max}} \) normalized (\( I/I_{\text{max}} \)) and the remaining current (\( R \)) at different time points was determined. Recovery from inactivation was determined by a 10 ms test pulses to \( V_{\text{max}} \) at the indicated time after a 1-s conditioning pulse to \( V_{\text{max}} \). Differences in the inactivation after repeated stimuli were determined after applying 100-ms square pulses to \( V_{\text{max}} \) at a frequency of 3 Hz. Holding potential between the steps was −60 mV.

Leak subtraction was performed either offline (steady-state inactivation, 5-s inactivation, recovery from inactivation and pharmacological experiments) or online using P/4 protocol. Recordings were junction potential corrected by −9.3 mV for Ca^{2+} and by −8.6 for Ba^{2+} as charge carrier, as previously described \cite{27}.

**Statistics**

Data were analyzed using Clampfit 10.2 (Axon Instruments), Microsoft Excel, SigmaPlot 8 (Systat Software, Inc) and GraphPad Prism 5 software (GraphPad software, Inc). All values are presented as mean ± S.E.M. for the indicated number of experiments (\( n \)) unless stated otherwise. Data were analyzed by unpaired Student’s t-test, Mann-Whitney test, and one-way ANOVA followed by Bonferroni post-hoc test. Statistical significance was set at \( p < 0.05 \).
Results

Splice-variant dependent effects of ASD mutations G407R and A749G

We have recently shown that two de novo missense mutations, G407R and A749G (Figure 1), found in two patients with ASD cause severe gating changes that are expected to result in aberrant \( \text{Ca}_{1.3} \)-mediated \( \text{Ca}^{2+} \) signaling in the brain. Together with three other mutations (V401L, G407D, I750M) that cause an even more severe neurodevelopmental syndrome \[9,15]\, this strongly suggests that these mutations confer a high risk for these diseases \[18\]. \( \text{Ca}_{1.3} \) also undergoes extensive alternative splicing, giving rise to a major C-terminal long splice variant (\( \text{Ca}_{1.3L} \)) and a number of C-terminal short splice variants, with \( \text{Ca}_{1.3s} \) (\( \text{Ca}_{1.3S} \)) being the most abundant one in the brain \[25,28–30\]. C-terminal splicing removes a modulatory domain allowing channels to undergo major changes in voltage-and \( \text{Ca}^{2+} \)-dependent gating resulting in activation at lower voltages, enhanced \( \text{Ca}^{2+} \)-dependent inactivation and increased channel open probability \[25,27,28,31\]. We have previously reported gating effects of the germline mutations A749G and G407R (situated at the intracellular end of IS6 and IIS6, respectively) only in the human C-terminally long (\( \text{Ca}_{1.3L} \)) isoform \[10\]. To test for potential splice-variant dependent effects we now introduced both mutations into human \( \text{Ca}_{1.3S} \) to compare their functional consequences with those in \( \text{Ca}_{1.3L} \) under identical experimental conditions.

Mutation A749G\(_S\) resulted in a similarly strong negative shift of the voltage dependence of activation and steady-state inactivation as the long splice variant (Figure 2(a,b), Table 1; historical data for long splice variants measured under identical conditions are given for comparison). Since wild-type \( \text{Ca}_{1.3S} \) activates and inactivates at more negative voltages than \( \text{Ca}_{1.3L} \) (Figure 2(a,b), Table 1, 26), a substantial fraction of A749G\(_L\) inactivated already at negative voltages unable to activate channels (closed-state inactivation), as evident from the steady-state activation and inactivation curves (Figure 2(b)). At \(-50\) mV, where channels were not activated, only \(1.13 \pm 0.01\%\) and \(2.51 \pm 0.01\%\) of WT\(_L\) and WT\(_S\) channels are inactivated, whereas for A749G\(_L\) 19.2 \pm 0.03 \% and for A749G\(_S\) even 41.8 \pm 0.07 \% were already unavailable (\(p < 0.0001\) for A749G\(_L\) vs. WT\(_L\), A749G\(_S\) vs. WT\(_S\) and A749G\(_L\) vs. A749G\(_S\), one-way ANOVA followed by Bonferroni post hoc test).

**Figure 1.** Transmembrane topology of the \( \text{Ca}_{1.3} \) \( \alpha_1 \)-subunit. The pore-forming subunit of voltage gated \( \text{Ca}^{2+} \) channels consist of four transmembrane repeats each comprising six membrane spanning helices connected by intra- and extracellular linkers. Segments (S) 1–4 of each repeat comprise the voltage-sensing domain, whereas segments 5–6 line the pore. The positions of missense mutations identified in ASD (G407R, A749G) and APAs (F747L, R990H, M1354I) are indicated. Mutations G407R (I56), F747L (I56) and A749G (I56) reside within the so-called activation gate at the distal part of the S6 segment. Mutation R990H affects the third of the five gating charges within the III54 helix of the voltage sensing domain and M1354I is localized at the distal part of segment IV55 facing the extracellular side. For references see text.
Since our measurements require recordings with 15 mM Ca\(^{2+}\) as a charge carrier, voltage-dependent activation and inactivation curves would be shifted by about \(-18\) mV at physiological Ca\(^{2+}\) concentrations [2 mM Ca\(^{2+}\); 25]. Therefore, about half of the A749G\(_S\) channels are predicted to

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**Figure 2.** Biophysical properties of Ca\(_v\)1.3 mutant A749G\(_S\). (a) Current-voltage relationships (\(I_{Ca}\), mean ± S.E.M.; 50-ms depolarizations to indicated voltages) of WT\(_S\) and A749G\(_S\). Only WT data recorded in parallel on the same days (> 3 different transfections) were included. Inset (upper): Box plots of WT\(_S\) and A749G\(_S\) peak current densities. In contrast to the long splice variant, no significant difference was observed in the current amplitudes (median, 5/95 percentile, [pA/pF]): WT\(_S\): \(-8.74 (−22.4/-5.54)\) n = 10; A749G\(_S\): \(-6.18 (−26.4/-4.94)\), n = 12; p = 0.339, Mann Whitney test). Inset (lower): Representative \(I_{Ca}\) traces from WT\(_S\) and A749G\(_S\) during depolarizations to \(V_{\text{max}}\). (b) Steady-state activation (circles, solid lines) and inactivation (squares, dashed lines) of WT\(_S\) (black symbols) and A749G\(_S\) (orange symbols). For statistics and n-numbers see Table 1. Lines without symbols represent the corresponding voltage-dependence of activation and inactivation for full length WT\(_L\) and A749G\(_L\) recorded under identical conditions (taken from reference 10). (c) Inactivation time course of WT\(_S\) (n = 35) and A749G\(_S\) (n = 9) during depolarization to \(V_{\text{max}}\) for 5 s. Normalized traces are shown as mean ± S.E.M. Inactivation was best described by a bi-exponential decay. The faster inactivation of the mutant resulted from a slightly larger contribution of the fast inactivating component (WT\(_S\): 79.1 ± 1.11 %, n = 26, A749G\(_S\): 84.3 ± 2.21 %, p = 0.036, n = 8) and faster inactivation of the slow component (WT\(_S\): \(\tau_{\text{slow}}\) = 602 ± 21.8 ms, A749G\(_S\): 420 ± 59.6 ms, p = 0.0012), despite a moderately slower fast inactivation (WT\(_S\): \(\tau_{\text{fast}}\) = 36.5 ± 1.8 ms, A749G\(_S\): 57.9 ± 7.99 ms, p = 0.0004). Additionally, inactivation was more complete in the mutant (remaining current: WT\(_S\): 14.6 ± 2.5 %, A749G\(_S\): 5.65 ± 1.19 %, p = 0.061, for statistics see Table 2). Statistical significance has been determined by unpaired Student's t-test.

**Table 1.** Activation and inactivation parameters for ASD mutations A749G and G407R.

|            | activation | inactivation |
|------------|------------|--------------|
|            | \(V_{\text{rev}}\) [mV] | Slope [mV] | \(V_{0.5}\) [mV] | \(n\) | \(V_{0.5}\) [mV] | Slope [mV] | Remaining current [%] | \(n\) |
| WT\(_S\)   | 64.9 ± 1.04 | 7.71 ± 0.11 | −9.49 ± 0.42 | 66 | −31.7 ± 0.88 | 4.31 ± 0.12 | 11.5 ± 2.18 | 25 |
| AG\(_G\)   | 54.7 ± 1.46*** | 6.61 ± 0.11*** | −18.3 ± 1.34*** | 13 | −48.6 ± 1.58*** | 4.27 ± 0.16 | 6.01 ± 1.50 | 9 |
| GR\(_G\)   | 61.6 ± 1.77 | 7.68 ± 0.28 | −15.3 ± 2.20*** | 14 | nd | | | |
| WT\(_L\)   | 67.7 ± 1.14 | 8.92 ± 0.20 | −2.55 ± 1.05 | 29 | 25.7 ± 2.08 | 5.56 ± 0.23 | 14.4 ± 3.12 | 18 |
| AG\(_L\)   | 60.3 ± 0.83*** | 7.14 ± 0.26*** | −12.3 ± 0.87*** | 27 | −41.1 ± 1.07*** | 5.82 ± 0.18 | 9.43 ± 1.49 | 14 |
| GR\(_L\)   | 54.7 ± 2.32*** | 7.90 ± 0.30 | −6.58 ± 1.41 | 13 | nd | | | |

All values are presented as mean ± S.E.M. Number of independent transfections >3. Parameters were obtained after fitting normalized (\(I/I_{\text{max}}\)) I-V relationships or normalized (\(I/I_{\text{max}}\)) steady-state inactivation curves, as described in Methods. Statistical analysis was performed using one-way ANOVA and Bonferroni post-hoc test (activation) or unpaired Student’s t-test (steady-state inactivation). Significance ** * p < 0.01, *** p < 0.001 in comparison to WT\(_L\) (*) or WT\(_S\) (†) (statistical analysis has been performed separately for long and short splice variants). n, number of experiments; nd, not determined; \(V_{0.5}\), half maximal activation/inactivation voltage; \(V_{\text{rev}}\), reversal potential; WT, wild-type. Note small differences among WT recordings within different sets of experiments (Tables 1, 3 and 5) because WT control experiments were always carried out in parallel with mutated channels. Reference data, which were already published previously, are indicated in italics (taken from reference 10).
be already inactivated at membrane potentials close to resting potentials. In the heterozygous individuals this would decrease the availability of A749Gs in comparison to wild-type channels in neurons with more positive membrane potentials, such as for example substantia nigra and ventral tegmental area dopamine neurons. This reveals both, gain- and loss of function properties of these mutations depending not only on the activity patterns of different neurons but also strongly affected by the relative abundance of long and short splice variants.

Mutation A749G slightly accelerated inactivation kinetics of both splice variants (Table 2). Bi-exponential inactivation over a 5-s time course of A749GS was faster (Figure 2), due to a significant decrease of τslow and an increase of the contribution of the fast inactivating component (Figure 2(c), see legend for statistics). τfast was slightly prolonged, which was also evident from a trend to more remaining current after 50 ms (Table 2). Although we made no attempts to separate the contribution of CDI and VDI for inactivation, the decrease of τslow must reflect a slowing of VDI by the mutation, whereas the increase in τfast suggests a slowing of CDI. This agrees with the observation of reduced CDI and enhanced VDI of this mutation in a rat Cav1.3 splice variant [16]. The authors predicted that the increase in VDI by the mutation may act in opposition to the reduced of CDI. Our data provide direct experimental support for their prediction.

As described previously [12,25] ON-gating currents (QON) were very small or absent for WT5 preventing measurements of the ratio of maximal tail current amplitude vs. integrated ON-gating current as an estimate for changes in open probability. Whereas A749GL significantly enhanced peak current amplitudes [10] this was not observed for A749GS. Instead, depolarization to voltages causing maximal activation (Vmax) of WT channels resulted in smaller current amplitudes in the mutant (Figure 2(a)). This indicated reduced A749GS current compared to WT, which was compensated by a higher driving force at peak current amplitudes due to its more negative activation voltage range. Since analysis of the QON in the long splice variants revealed reduced surface expression of A749GL [10], this suggests that the smaller current at WT Vmax observed with A749GS might also be due to a reduction in channel numbers at the cell surface.

Splicing-dependent differences were also found for ASD mutation G407R. G407R reduced maximal current amplitudes in both C-terminally long [Table 1, 10] and short CaV1.3 splice variants (Figure 3(a), Table 1). However, in contrast to G407RD, the voltage dependence of activation was significantly shifted in G407RS to more negative voltages (Figure 3(b)). Like for G407RD the inactivation time course during 5-s depolarizations was also dramatically slowed for G407RS (Figure 3, Table 2). G407RS, in contrast to G407RD [10] retained an initial fast inactivating component, most likely representing residual CDI (Figure 3(c,d)), which is more pronounced in short CaV1.3 splice variants [25,31,32]. Despite reduced maximal current amplitudes, the slow inactivation resulted in larger absolute current amplitudes during prolonged depolarization than in WT5 channels (Figure 3(c,d)). The fraction of ICa became larger after about 200 ms and was remarkably enhanced at the end of a 5-s pulse to Vmax for G407RS (59.9 ± 5.62 %, n = 8), compared to WT5 (13.5 ± 2.09 %, n = 35) (Figure 3(c), Table 2). The failure of G407R currents to inactivate prevented the measurement of steady-state inactivation parameters. Together with the more negative activation voltage range of G407RS, this predicts persistent inward current at even more negative (subthreshold) potentials than for G407RD (Figure 3).

### Table 2. Remaining current upon 5-s depolarization for ASD mutations A749G and G407R.

|       | R50 (%) | R500 (%) | R5000 (%) | n  |
|-------|---------|----------|-----------|----|
| WT5   | 41.1 ± 1.84 | 20.7 ± 2.05 | 13.5 ± 2.09 | 35 |
| AG5   | 46.2 ± 3.92 | 11.8 ± 1.87 | 8.16 ± 1.84 | 9  |
| GR5   | 73.6 ± 4.52 *** | 62.6 ± 5.39 *** | 59.9 ± 5.62 *** | 8  |
| WTL   | 69.5 ± 3.2  | 22.8 ± 2.42 | 6.12 ± 1.13 | 15 |
| AGL   | 60.0 ± 4.31 | 7.22 ± 1.76 *** | 3.12 ± 0.56 *** | 6  |
| GRL   | 99.2 ± 0.65 *** | 98.1 ± 1.33 *** | 80.2 ± 3.52 *** | 13 |

All values are presented as mean ± S.E.M. Number of independent transfections ≥2. ICa inactivation normalized to ICa max was obtained 50, 500 and 5000 ms after depolarization to Vmax. Statistical analysis was performed using one-way ANOVA and Bonferroni post-hoc test. Significance *** p < 0.001 in comparison to WT5 (*) or WT1 (†). n, number of experiments; R, remaining current; WT, wild-type. Reference data, which were already published previously, are indicated in italics (taken from reference 10).

APA mutations F747L and R990H – but not M1354I – alter channel gating

We next determined if APA mutations F747L, R990H and M1354I (Figure 1) also interfere with
proper channel function in a similar manner as described above. F747L resides at the intracellular end of IIS6 just 2 amino acids upstream of A749G. When introduced into the long splice variant F747L also strongly shifted the voltage dependence of activation (about −17 mV) to more negative potentials, while the voltage dependence of inactivation remained unaltered (Figure 4(a,b), Table 3). This dramatically broadened the voltage range in which the channel is active and increases the window current. Moreover, F747L currents inactivated more slowly and showed a significantly higher fraction of non-inactivating \( I_{Ca} \) even after prolonged depolarizations (Figure 4(c); Table 4 for statistics). F747L also significantly slowed the time course of activation (time to peak at \( V_{\text{max}} \) [ms]: WT\(_L\): 6.66 ± 0.44, n = 35; F747L\(_L\): 30.1 ± 1.76, n = 21, \( p < 0.0001 \), unpaired Student’s t-test; traces in Figure 4(a), inset). Like for A749G\(_S\), current amplitudes were smaller at positive voltages suggesting overall reduced current through the mutated channel. Unfortunately, quantification of the surface expression of functional F747L\(_L\) was not possible because the mutation eliminated measurable \( Q_{\text{ON}} \) gating charge movement. As evident from the current traces in Figure 4(a) (inset, arrows), in 27 out of 30 recordings the \( Q_{\text{ON}} \) voltage sensor movement, which is present in WT\(_L\) in all cells, was not detectable. We have obtained a similar decrease in \( Q_{\text{ON}} \) relative to ionic current previously for APA mutations I750M, V259D and P1336R [11] and ASD mutation A749G [10] when introduced into the long splice variant. Taken together, F747L exhibits the typical gating changes observed for previously studied mutations clearly identifying
Figure 4. Biophysical properties of Ca\textsubscript{v}1.3 mutant F747L\textsubscript{L}. (a) Current-voltage relationships ($I_{Ca}$, mean ± S.E.M.) of WT\textsubscript{L} and F747L\textsubscript{L} recorded in parallel on the same days (>3-different transfections). Inset (upper): Box plots of peak current densities (median, 5/95 percentile, $[pA/pF]$): WT\textsubscript{L}: −12.7 (−66.1/-5.96), n = 47; F747L\textsubscript{L}: −13.3 (−40.3/-3.97), n = 27). Black circles represent extreme values. Peak values were not statistically different (Mann Whitney test, p = 0.97). Inset (lower): Representative $I_{Ca}$ traces of WT\textsubscript{L} and F747L\textsubscript{L} upon depolarization to $V_{max}$. Note the slower activation time course and the absence of Q\textsubscript{ON} gating charge in F747L\textsubscript{L} (arrows). (b) Steady-state activation (circles, solid lines) and inactivation (squares, dashed lines) of WT\textsubscript{L} (black symbols) and F747L\textsubscript{L} (gray symbols). Gating parameters and statistics are given in Table 3. (c) Normalized inactivation time course of $I_{Ca}$ after 5-s depolarization to $V_{max}$ (n = 10) as compared to WT\textsubscript{L} (n = 28). For statistics, see Table 4.

Table 3. Activation and inactivation parameters for APA mutations F747L and M1354I.

|       | $V_{rev}$ [mV] | Slope [mV] | $V_{0.5}$ [mV] | n  | $I_{Ca}$ [mV] | Slope [mV] | Remaining current [%] | n  |
|-------|----------------|------------|----------------|----|---------------|------------|----------------------|----|
| WT\textsubscript{L} | 70.4 ± 0.67 | 9.06 ± 0.12 | 1.37 ± 0.60 | 63 | −27.0 ± 0.69 | 5.54 ± 0.18 | 14.4 ± 2.02 | 28 |
| FL\textsubscript{L} | 53.8 ± 0.91*** | 6.62 ± 0.22*** | −15.6 ± 0.71*** | 30 | −29.3 ± 0.98* | 4.45 ± 0.32** | 32.2 ± 2.50*** | 19 |
| MI\textsubscript{L} | 69.5 ± 0.69 | 9.01 ± 0.13 | 2.48 ± 0.79 | 40 | −25.0 ± 0.82 | 5.57 ± 0.22 | 19.6 ± 2.26 | 25 |
| WT\textsubscript{S} | 64.9 ± 1.04 | 7.71 ± 0.11 | −9.49 ± 0.42 | 66 | −31.7 ± 0.88 | 4.31 ± 0.12 | 11.5 ± 2.18 | 25 |
| MI\textsubscript{S} | 62.5 ± 1.36 | 7.36 ± 0.18 | −9.01 ± 0.64 | 19 | −30.8 ± 0.86 | 3.92 ± 0.18 | 13.8 ± 2.49 | 13 |

All values are presented as mean ± S.E.M. Number of independent transfections >3. Parameters were obtained after fitting normalized ($I/I_{max}$) I-V relationships or normalized ($I/I_{max}$) steady-state inactivation curves, as described in Methods. Statistical analysis was performed by one-way ANOVA and Bonferroni post-hoc test (long splice variants) or unpaired Student’s t-test (short splice variants). Significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to WT\textsubscript{L}, n, number of experiments; $V_{0.5}$, half maximal activation/inactivation voltage; $V_{rev}$, reversal potential; WT, wild-type.

Table 4. Remaining current upon 5-s depolarization for APA mutations F747L and M1354I.

|       | R50 [%] | R500 [%] | R5000 [%] | n  |
|-------|---------|----------|-----------|----|
| WT\textsubscript{L} | 71.7 ± 2.38 | 29.4 ± 2.11 | 9.83 ± 1.86 | 28 |
| FL\textsubscript{L} | 99.3 ± 0.83*** | 63.4 ± 4.78*** | 32.2 ± 3.52*** | 10 |
| MI\textsubscript{L} | 69.9 ± 3.64 | 29.7 ± 3.81 | 16.1 ± 3.84 | 12 |
| WT\textsubscript{S} | 41.1 ± 1.84 | 20.7 ± 2.05 | 13.5 ± 2.09 | 35 |
| MI\textsubscript{S} | 44.2 ± 3.70 | 23.9 ± 3.99 | 18.4 ± 4.34 | 9 |

All values are presented as mean ± S.E.M. Number of independent transfections >3. $I_{Ca}$ inactivation normalized to $I_{max}$ was obtained 50, 500 and 5000 ms after depolarization to $V_{max}$. Statistical analysis was performed using one-way ANOVA and Bonferroni post-hoc test (long splice variants) or unpaired Student’s t-test (short splice variants). Significance *** $p < 0.001$ in comparison to WT\textsubscript{L}, n, number of experiments; R, remaining current; WT, wild-type.
this mutation as the cause for excess aldosterone production in APAs.

Unlike previously described APA mutations, R990H and M1354I are not located within the activation gate or the S4-S5 linkers [18,21] and may therefore induce different functional changes.

R990H replaces the third (R3) of the five S4 positive charges of the domain III voltage-sensing S4 helix [21]. We have recently shown that, when introduced into the long splice variant, R990H causes gating changes compatible with a loss-of-function phenotype: it tended to reduce inward current at all voltages, and slightly but significantly shifted activation (by about 3 mV) to more positive rather than negative voltages as in all other mutations studied so far (Table 5). For this mutation we have previously provided experimental and molecular modeling evidence for an ω-current through the voltage-sensor. This can be explained by a water wire formed in the voltage-sensor of the R990H mutation, which enables cationic leak current at hyperpolarized resting membrane potentials and therefore can indirectly trigger depolarization-induced Ca²⁺ entry in APAs [21]. However, it is also possible that a gain-of-function phenotype is absent when introduced into the long but present in the short splice variant. We therefore tested this possibility by analyzing R990Hₜ (Figure 5). Like R990Hₐ, R990Hₜ shifted activation voltage to more positive voltages by about 4 mV (Figure 5, Table 5). However, when introduced into the short splice variant R990H also significantly shifted steady-state inactivation by about 6 mV to more negative voltages thus even reducing window current by decreasing channel availability at negative voltages. Current density (Figure 5(a)) and the time course of inactivation (Table 6) were not affected by the mutation in neither of the splice variants. In the absence of any other significant changes observed in R990Hₜ these findings provide additional evidence for a loss-of-function phenotype of this mutation for Ca²⁺ entry through the canonical ion pore of both long and short variants of the channel. This further strengthens the view that the pathogenic gain-of-function phenotype results from its ω-current.

Mutation M1354I is located in a highly conserved region at the extracellular end of segment IVS5 closely interacting with segment IIIS1 and the pore loop in domain IV [33], interactions that may be involved in coupling the voltage-sensing domain to the pore domain [34]. However, in contrast to the other mutations, M1354I neither altered the voltage dependence of activation or inactivation (Figure 6(a-c), Table 3) nor the time course of inactivation during 5-s depolarizing pulses to Vₘₐₓ (Figure 6(f,g), Table 4). In addition M1354Iₜ did not change the Iₜₐᵢₜ/Qₐₜₐₜ ratio as an indirect measure of open probability ((Iₜₐᵢₜ/Qₐₜₐₜ [ms⁻¹]): WTₜ: 15.8 ± 1.77, n = 16; M1354Iₜ: 18.3 ± 1.70, n = 17, p = 0.3067, unpaired Student’s t-test). Current densities were reduced at all voltages in both long (Figure 6(a)) and short Ca₁.3 variants (Figure 6(b)), which could be explained by less surface expression of the mutant as evident from a significantly lower Qₐₜₐₜ as compared to WTₜ ([Qₐₜₐₜ [pA*ms⁻¹]]: WTₜ: 144.7 ± 29.8, n = 16; M1354Iₜ: 60.0 ± 7.38, n = 17, p = 0.008, unpaired student’s t-test).

A more detailed characterization of its gating properties further revealed no changes in recovery from inactivation determined by 10-ms test pulses to Vₘₐₓ at different time-points after a 5-s conditioning pulse to Vₘₐₓ (Figure 6(d)). Accumulation of

| Table 5. Activation and inactivation parameters for APA mutation R990H. |
|---|
| **activation** | **inactivation** |
| Vₐₜₜ [mV] | Slope [mV] | Vₐₜₜ [mV] | n | Vₐₜₜ [mV] | Slope [mV] | Remaining current [%] |
| WTₜ | 64.9 ± 1.04 | 7.71 ± 0.11 | −9.49 ± 0.42 | 66 | −31.7 ± 0.88 | 4.31 ± 0.12 | 11.5 ± 2.18 | 25 |
| RHₜ | 60.0 ± 1.73 | 7.55 ± 0.19 | −5.37 ± 0.63*** | 11 | −37.1 ± 0.57** | 5.26 ± 0.53* | 3.92 ± 2.42 | 8 |
| WTₙ | 62.2 ± 1.10 | 9.02 ± 0.23 | −0.65 ± 1.27 | 29 | −30.8 ± 1.14 | 3.38 ± 0.30 | 11.3 ± 2.35 | 16 |
| RHₙ | 69.7 ± 1.45 | 8.57 ± 0.14 | 2.61 ± 0.69⁹ | 33 | −32.5 ± 1.12 | 6.76 ± 0.39⁹ | 11.3 ± 2.05 | 18 |

All values are presented as mean ± S.E.M. Number of independent transfections >3. Parameters were obtained after fitting normalized (I/Iₘₐₓ) I-V relationships or normalized (I/Iₘₐₓ) steady-state inactivation curves, as described in Methods. Statistical analysis was performed by unpaired Student’s t-test. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001 in comparison to WTₜ (*) or WTₙ (*). n, number of experiments; Vₐₜₜ, half maximal activation/inactivation voltage; Vₐₜₜ, reversal potential; WT, wild-type. Reference data, which were already published previously, are indicated in italics (taken from reference 21).
inactivated states induced by repetitive 3-Hz stimulation (30 100-ms steps to \( V_{\text{max}} \)) also did not differ from WT (Figure 6(e)).

Taken together, our data clearly show that APA mutations F747L and R990H can lead to functional changes permitting enhanced voltage-dependent \( Ca^{2+} \) entry into APAs through different molecular mechanisms. They can therefore explain the excessive aldosterone production in these tumors. In contrast, a pathogenic role of mutation M1354I was not evident from our study.

### Discussion

We and others, have previously discovered an important pathophysiological role of gating changes of \textit{de novo} \( CACNA1D \) mutations, which are capable of enhancing \( Ca^{2+} \) influx through \( Ca_{v} \) 1.3 channels. Such mutations occur as somatic mutations in APAs (resulting in treatment-resistant hypertension) and, if present germline, cause a neurodevelopmental disorder with a wide symptomatic spectrum. From a diagnostic point of view, it is important to distinguish pathogenic mutations causing the disease from other rare polymorphisms with low disease risk or no disease relevance at all.

Here we further expand our collection of functionally verified pathogenic mutations by demonstrating that APA mutation F747L exhibits very similar gating changes as previously observed for other mutations [9–12,15, Table 7], all characterized by a strong negative shift of activation and inactivation voltage. This facilitates channel opening by weaker depolarizations from negative

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**Table 6.** Remaining current upon 5-s depolarization for APA mutation R990H.

|       | R50 (%)  | R500 (%) | R5000 (%) | n  |
|-------|----------|----------|-----------|----|
| WT    | 41.1 ± 1.84 | 20.7 ± 2.05 | 13.5 ± 2.09 | 35 |
| RH    | 46.6 ± 3.45 | 19.5 ± 3.73 | 15.1 ± 3.54 | 9  |
| WT    | 69.7 ± 3.94 | 24.8 ± 3.50 | 11.1 ± 2.94 | 10 |
| RH    | 67.1 ± 3.59 | 21.6 ± 3.75 | 13.6 ± 3.62 | 12 |

All values are presented as mean ± S.E.M. Number of independent transfections >3. \( I_{\text{Ca}} \) inactivation normalized to \( I_{\text{max}} \) was obtained 50, 500 and 5000 ms after depolarization to \( V_{\text{max}} \). Statistical analysis was performed using unpaired Student’s t-test in comparison to WT or WT, n, number of experiments; R, remaining current; WT, wild-type.
Figure 6. Biophysical properties of Ca,v1.3 mutant M1354I. (a) Current-voltage relationships ($I_{Ca}$, mean ± S.E.M.) of WT L and M1354I L recorded in parallel on the same days (>3-different transfections). Inset (upper): Box plots of peak current densities (median, 5/95 percentile, [pA/pF]): WT L: $-10.9$ ($-72.3/-5.86$) n = 38; M1354I L: $-8.33$ ($-25.3/-4.44$), n = 40) were significantly smaller for the mutant (Mann Whitney test, $p = 0.029$). Inset (lower): Representative $I_{Ca}$ traces of WT L and M1354I L upon depolarization to $V_{max}$. (b) Current-voltage relationships ($I_{Ca}$, mean ± S.E.M.) of WT S and M1354I S recorded in parallel on the same days (>3-different transfections). Inset (upper): peak current densities (median, 5/95 percentile, [pA/pF]): WT S: $-7.50$ ($-28.5/-3.63$), n = 30; M1354I S: $-5.89$ ($-12.6/-3.00$), n = 19) were significantly smaller for the mutant (Mann Whitney test, $p = 0.037$). Inset (lower): Representative $I_{Ca}$ traces of WT S and M1354I S upon depolarization to $V_{max}$. (c) M1354I steady-state activation (circles, solid lines) and inactivation (squares, dashed lines) of WT L (filled black symbols), M1354I L (filled blue symbols), WT S (open black symbols), and M1354I S (open blue symbols). No significant changes were observed in mutant channels. Gating parameters and statistics are given in Table 3. (d) Recovery from $I_{Ca}$ inactivation (mean ± S.E.M.) of WT L and M1354I L. First 300 ms are magnified in the inset. Curves were fitted using a mono-exponential function; there was no difference in the time constants ($\tau$ as mean ± S.E.M [ms]: WT L: $166.1 ± 20.6$, n = 5; M1354I L: $154.5 ± 42.5$, n = 5; unpaired Student’s t-test). E: Fraction of $I_{max}$ remaining after repetitive depolarizations to $V_{max}$ at 3 Hz (mean ± S.E.M.) of WT L (n = 10) versus M1345I L (n = 5). (f) Normalized inactivation time course of $I_{Ca}$ after 5-s depolarization to $V_{max}$ (mean ± S.E.M.) of WT L (n = 35) and M1354I L (n = 9). For statistics, see Table 4.
resting membrane potentials and enables window current at subthreshold voltages. Although the time course of $I_{Ca}$ inactivation during long depolarizations is variable, several of the mutants (G403D, G403R, G407R) are characterized by a very strong loss of slow inactivation giving rise to a large fraction of non-inactivated current even after 5-s depolarizations to $V_{max}$. Interestingly, in contrast to G403D and G403R [15], G407R caused no negative shift in activation voltage when introduced into the C-terminally long Ca$_{1.3}$ α1 splice variant [10]. However, our present data show that such a negative shift (of about −6 mV) is observed when introduced into the short splice variant. Thus, more negative activation voltage range seems to be the common hallmark of the majority of pathogenic gating-modifying CACNA1D mutations analyzed so far.

Splice-variant dependent gating changes are of particular interest for germline mutations, since the relative expression of Ca$_{1.3}$ splice variants differs across different tissues and within brain regions and might therefore result in tissue-specific phenotypes. For example, a particularly high relative expression of short splice variants is observed in dopamine neurons in the Substantia nigra [35]. Splice-variant dependent mutational effects have also been reported for other voltage-gated Ca$_{2+}$ channels. A single missense mutation in exon 24 (III-IV cytoplasmic linker) in the α1-subunit of Ca$_{3.2}$ T-type channels (CACNA1H), which confers part of the risk for epilepsy in GAERS rats, speeds recovery from inactivation and thereby increases Ca$_{2+}$ currents during repetitive depolarizations [36]. This phenotype was only observed in channels also containing a short exon 25. The inclusion of this exon slowed recovery from inactivation as compared to channels devoid of exon 25, and the mutation offset this effect. Likewise, alternative splicing in the C-terminal tail of Ca$_{2.1}$ α1-subunits of P/Q-type channels (CACNA1A) affected the functional changes of several gain-of-function missense mutations causing Familial Hemiplegic Migraine Type 1 [8], including S218L. It is possible that this mechanism also contributes to the differential effects of this mutation on neurotransmission at cortical pyramidal (gain-of-function of excitatory neurotransmission) and multipolar interneurons (unaltered inhibitory neurotransmission) in S218L knockin mice [37].

In addition, our data further strengthen our previous finding that $\omega$-currents through the voltage sensor could represent an alternative molecular mechanism how CACNA1D mutations could drive depolarization and subsequent activation of voltage-gated Ca$_{2+}$-entry [21]. APA mutation R990H affects the basic charge R3 in the voltage-sensor of domain III of the Ca$_{1.3}$ α1-subunit and allows permeation of cation flux at resting

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**Table 7. Classification of CACNA1D missense mutations by characteristic functional changes.**

| Type | Mutation | Disease phenotype | Characteristic functional changes | References |
|------|----------|--------------------|-----------------------------------|------------|
| 1    | G403D    | APAs; hyperaldosteronism or hyperinsulinism with seizures and developmental delay | ● Inactivation almost abolished (voltage-dependence of inactivation not measurable) | [15] |
|      | G403R    | APAs               | ● Voltage-dependence of activation shifted to hyperpolarized voltages or unchanged | [15] |
|      | G407R    | ASD (and ID)       |                                   | [10] |
| 2    | V259D    | APAs               | ● Voltage-dependence of activation strongly shifted to hyperpolarized voltages | [11] |
|      | V401L    | ASD, ID and seizures | ● Inactivation not abolished$^*$ with voltage-dependence of inactivation strongly shifted to hyperpolarized voltages or unchanged | [11,16] |
|      | F477L    | APAs               | $^*$ may be faster, slower, more or less complete after 5 s depolarizations to $V_{max}$ | [11,15] |
|      | A749G    | ASD (and ID)       |                                   |           |
|      | I750M    | APAs; hyperaldosteronism with seizures and developmental delay | ● Slower and less complete inactivation during 3–5 s depolarizations to $V_{max}$ and no changes in voltage-dependence of gating | [14] |
|      | V1153G   | APAs               |                                   | [11] |
| 3    | Q558H    | ID, seizures, hearing impairment | ● Mutation-induced (depolarizing) $\omega$-current | [11,21] |
|      | P1336R   | APAs               |                                   |           |
| 4    | R990H    | APAs               |                                   |           |
membrane potentials compatible with the formation of a water wire as predicted by molecular dynamics simulations [21]. However, to support the hypothesis of a pathogenic role of this mechanism the absence of typical gating changes as described for other mutations needs to be demonstrated. While we have shown this earlier for the long splice variant of Ca_{1.3} (R990H_{L}), we here complement our findings by also demonstrating that gain-of function gating changes are also absent in R990H_{S}.

We were unable to demonstrate any functional consequences for APA mutation M1354I despite an extensive analysis of its gating properties and characterization of the mutation in both splice variants. The only significant change was a small reduction in maximal current amplitude compared to WT in both splice variants. However, based on findings in Ca_{1.3} knockout mice and humans with CACNA1D mutations [19, 20], a loss-of-function phenotype by a heterozygous de novo mutation is unlikely to be pathogenic. At present, we cannot rule out the possibility that a special combination of accessory β- and αδ-subunits, other splice variants or a yet unknown molecular mechanism not active in our expression system can unmask functional changes in M1354I channels. An alternative possibility is that this rare variant is not responsible for excess aldosterone production in APAs. This is supported by the fact that high-confidence somatic nonsynonymous CACNA1D variants that passed stringent in silico filtering have recently been described in APCCs [22] that are also unlikely to be pathogenic. This includes two premature stop codons. One of these (R510X) must result in a truncated non-functional channel. Another APA mutation, V728I, reported previously only once in an APA of a Chinese patient [18, 38] is frequently present germline in healthy individuals [39 heterozygous alleles in EXAC database]. This also questions if it alone can account for excessive aldosterone production. Since this mutation is located on the extracellular end of helix IIS6 it is outside the regions highly vulnerable to mutational gating defects [S4, S4-S5-linker, cytoplasmic end of S6; 18]. Functional characterization of this mutation must therefore address its potential pathogenic role.

Our work also has important diagnostic consequences. First, by extending the list of APA mutations for which we can demonstrate functional changes compatible with a pathogenic phenotype, we facilitate future genetic diagnosis in patients with neurodevelopmental syndromes of unknown cause. If any of these APA mutations were identified germline in such patients, this would provide robust evidence for a causal role in their disease, even if endocrine symptoms were absent. Although APA mutations can drive excess aldosterone production in these benign tumors, hyperaldosteronism has so far only been reported for two mutations in three (two with G403D, one with I750M) of the patients with germline missense mutations. No endocrine abnormalities were reported in the clinically well-studied patient carrying the V401L APA mutation [9]. At present the overall number of patients with germline mutations is too small for a reliable genotype-phenotype correlation.

Second, we show that the typical (“diagnostic”) gating changes, despite significant differences, are found in long and short Ca_{1.3} splice variants. Therefore, for further routine diagnostics, analysis in one of the splice variants is sufficient if the diagnostic hallmarks can be demonstrated therein. Characterization of a larger set of mutations now allows us to propose four different characteristic types based on functional criteria (Table 7): Type 1 are the inactivation-deficient mutations, in which most of the Ca_{1.3} current fails to inactivate in Ca_{1.3}. These comprise G403D, G403R, G407R. They could also be classified as “Timothy Syndrome- like” mutations because they are very similar to the Ca_{1.2} α1-subunit (CACNA1C) mutations diagnostic for Timothy Syndrome [40, 41]. Type 2 mutations still inactivate to variable extents but are characterized by pronounced negative shifts of activation voltage with or without a strong negative shift also of inactivation voltage (A749G, I750M, V401L, V1153G, V259D and, as reported here, F747L). Type 3 is characterized only by slower and less complete inactivation after 3–5 s at V_{max} which should favor persistent current in cells during prolonged depolarizations. Type 4 are mutations 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 [39, 42–44]. Mutations without a functional phenotype in vitro as shown here for M1354I should be considered rare non-pathogenic variants until further functional or clinical proof for their pathogenicity can be obtained.
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