A rare schizophrenia risk variant of CACNA1I disrupts CaV3.3 channel activity

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CACNA1I is a candidate schizophrenia risk gene. It encodes the pore-forming human CaV3.3 α1 subunit, a subtype of voltage-gated calcium channel that contributes to T-type currents. Recently, two de novo missense variations, T797M and R1346H, of hCaV3.3 were identified in individuals with schizophrenia. Here we show that R1346H, but not T797M, is associated with lower hCaV3.3 protein levels, reduced glycosylation, and lower membrane surface levels of hCaV3.3 when expressed in human cell lines compared to wild-type. Consistent with our biochemical analyses, whole-cell hCaV3.3 currents in cells expressing the R1346H variant were ~50% of those in cells expressing WT hCaV3.3, and neither R1346H nor T797M altered channel biophysical properties. Employing the NEURON simulation environment, we found that reducing hCaV3.3 current densities by 22% or more eliminates rebound bursting in model thalamic reticular nucleus (TRN) neurons. Our analyses suggest that a single copy of Chr22:39665939G>A CACNA1I has the capacity to disrupt CaV3.3 channel-dependent functions, including rebound bursting in TRN neurons, with potential implications for schizophrenia pathophysiology.

CACNA1I encodes the pore-forming hCaV3.3 α1 subunit, one of three major CaV3 voltage-gated calcium (CaV) channels that contribute to low threshold T-type currents. Compared to CaV channels, CaV3 have low voltage-activation thresholds, rapid inactivation, and slow closing rates1. Of the three-member CaV3 family, CaV3.3 channels have the most depolarized activation thresholds; and they open as well as inactivate and close slowly2.

CaV3.3 channels are expressed in a limited subset of neurons including GABAergic neurons of the thalamic reticular nucleus (TRN) where they support oscillatory activity essential for sleep spindle generation3–6. In TRN neurons, CaV3.3 channels are mostly inactivated at resting membrane potentials and mediate the rebound bursting upon transient membrane hyperpolarizations7,8. Rebound bursting in TRN neurons is largely absent in mice lacking CaV3.3 channels, and sleep spindle generation is disrupted9. Sleep spindle abnormality, as well as altered patterns of neuronal activity in the thalamus, are found in people with schizophrenia, suggesting thalamocortical network dysfunction10–14.

Genetic analyses of large patient cohorts have identified loci associated with the risk of mental illnesses including schizophrenia, autism spectrum disorder and bipolar disorder15–19. Several of these candidate risk genes encode proteins involved in calcium signaling, including voltage-gated calcium channel subunits (CACNA1C, CACNB2 and CACNA1I), that may ultimately converge on a common disease mechanism17,19–23. Biological insight derived from psychiatric-associated common genetic variations is currently limited to a few studies25–29; causative risk alleles can be difficult to localize precisely within GWAS loci, and many map to non-coding regions of as yet undefined functions25,30. Recently, two rare, de novo missense variations, Chr22:39659492C>T and Chr22:39665939G>A, were identified in CACNA1I by exome sequencing of trio samples that include 105 schizophrenia probands. CACNA1I was the only gene in this cohort to harbor missense variations in more than one proband31. The resulting amino acid alterations, T797M and R1346H (numbered according to NM_00100340631), are predicted to reside in extracellular regions of domains II and III of hCaV3.3. CACNA1I was validated as a candidate schizophrenia risk gene, in a genome wide association study by the Schizophrenia Working Group of the Psychiatric Genomics Consortium as one of 108 independent genomic

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loci that exceed genome-wide significance. These studies emphasize the need for functional studies of rare missense, exome variations in CACNA1I that associate with schizophrenia risk.

Here we use biochemical and electrophysiological assays in the HEK293 cell expression system, to show that R1346H hCa3.3 variant has reduced glycosylation and cell surface expression compared to hCa3.3 wild-type, without altered biophysical properties. In contrast, T797M did not affect hCa3.3 channel properties in our assays. Employing NEURON to simulate TRN neuron excitability, we show that R1346H disrupts rebound mediated by CaV3.3 channels.

Materials and Methods

**Mutagenesis.** T797M and R1346H were introduced on an Sbfl-HindIII fragment of FLAG-tagged WT hCa3.3 cDNA (Origene, RC219179) and sub-cloned into pcDNAs5/FRT/TO vector (Thermo Fisher Scientific) to generate full-length FLAG-tagged hCa3.3 cDNAs. Mutagenesis primers were for T797M: 5′-CAAGGACACTGGAGACATGGTGCCCG/5′-TCGGCACCATCTGCCAGTGGTG, and for R1346H: 5′-GCCATGCACTGGAGACATGGTGCCCG/5′-GCCATGCACTGGAGACATGGTGAG. All constructs used in this study were sequence verified.

**Single copy cDNA, stable integration in HEK293 cells.** The Flp-In T-Rex system selects for clones containing single copy integration of cDNAs thereby reducing variability in CaV3.3 protein expression levels among cells and across cell lines. Parental Flp-In T-Rex HEK293 cells (Thermo Fisher, R78007) were transfected with pcDNAs/pFRT/TO vectors containing WT, T797M or R1346H hCa3.3 cDNAs, and pOG44 vector encoding FLRecombinase (Thermo Fisher, K650001) in a 1:9 ratio using Lipofectamine 2000 (Invitrogen). Cells were cultured in DMEM/F12 supplemented with 10% FBS. Single cDNA insertion events were selected after two weeks in 200 μg/mL hygromycin B (Invitrogen) and resistant polyclonal cell lines (10–20 colonies) expanded and maintained with 200 μg/mL hygromycin B and 15 μg/mL blasticidin S (Invitrogen). hCa3.3 channel expression was induced by 1 μg/mL doxycycline and all analyses (including electrophysiology) were performed 72 hours after induction (unless otherwise indicated). At 72 hours Ca3.3 channel protein levels plateaued. cDNA integration in each cell line was validated with Sanger sequencing of the insertion locus.

**Reverse transcription and quantitative PCR.** Total RNA from each cell line was harvested using RNAeasy Plus (Qiagen). 10 μg of total RNA was reverse-transcribed and cDNA synthesized using random hexamer priming (Transcriptor cDNA synthesis, Roche). FastStart Universal SYBR Green 2X Master Mix (Roche) was used to perform quantitative PCR for hCa3.3 (CACNA1I: 5′-CAATGGGACTGGATCTGGTG/5′ATCCAGGGGTGTGGTTG) and β-actin (ACTB: 5′-CCACACGGAGAAGTGGA/5′-CCAGAGGCCTCACGAGT). CACNA1I mRNA in each cell line was analyzed by the relative quantitation of gene expression method and using ACTB that encodes β-actin as the reference control gene (ΔΔCT method). Threshold amplification cycle (CT) values were obtained for target (CACNA1I) and internal control (ACTB) to calculate ACT (ΔCT target–CT reference), and ΔΔCT calculated before and after induction of CACNA1I by doxycycline treatment. We carried out 3–4 technical replicates, from three independent cell culture and dox-induction step (biological triplicates). The biological variability in our RT-qPCR experiments stems primarily from different overall levels of mRNA induction across biological replicates.

**Immunoblotting.** Whole cell lysates of Flp-In T-Rex HEK293 cells expressing WT, T797M or R1346H hCa3.3 channels were prepared as previously described. Electrophoresis samples were prepared in 4x Laemmli buffer (Bio-Rad) and incubated at room temperature for 20 minutes before SDS-PAGE (3–8% tris-acetate gel, Life Technologies). Antibodies: mouse anti-FLAG (1:1000, Sigma F1804); rabbit anti-GAPDH (1:1000, Cell Signaling Technology 14C10); mouse anti-β-actin (1:50000, Sigma A5441); rabbit anti–pan-cadherin (1:1000, Life PA5-17526). Anti-GAPDH or anti-β-actin signals were used as reference signals to normalize across preparations and also as gel loading controls.

**Cell-Surface Biotinylation.** hCav3.3 protein expression was induced in transformed Flp-In T-Rex HEK293 cells with 4 day treatment of 1 μg/mL doxycycline. Cells were then washed three times with solution A (see below) and incubated with 1.5 mg/mL EZ Link Sulfo-NHS-SS Biotin (Life Technologies) for 30 min at 4 °C. The labeling reaction was quenched with 3 x 5 min BSA/L-lysine solution, and the cell preparation washed twice at 4 °C for 5 min with solution A. Cells were lysed on ice with 200 μL of PB/SDS and 1 mL of PB/Triton, sonicated for 30 min at 30 on 30 off intervals in a Bioruptor bath sonicator (Diagenode), and centrifuged at 13000 rpm for 15 minutes. Biotinylated lysates were incubated with NeutraAvidin beads (Thermo Fisher Scientific) under constant rotation for 2 hours, centrifuged at 2000 rpm for 30 s, and then beads were washed sequentially with 2 x 1 mL PB/Triton, 2 x 1 mL PB/Triton/600, and 2 x 1 mL of PB. Protein bound to NeutraAvidin beads was eluted for 20 minutes at RT in 2 x 1 mL of 0.5 M NaH2PO4 and 1.5 M NaOH. Input and eluate samples were analyzed by immunoblotting. Solution A: 7.74 mM Na2HPO4, 2.26 mM NaH2PO4, 2.7 M KCl, 137 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2. BSA/lyase quenching solution: 0.5% BSA, 50 mM L-lysine-H2O in PBS/Ca/Mg, pH 7.4; PB: 7.74 mM NaH2PO4, 2.26 mM NaH2PO4, 50 mM EDTA, 50 mM EGTA, 100 mM NaCl, 10 mM Na2PO4, 50 mM NaF, 1 mM NaVO3, PB/SDS: PB, 3% SDS; PB/Triton: PB, 1% Triton; PB/Triton/600: PB, 1% Triton, 600 mM NaCl.

The protein measurements for T797M and R1346H channels have similar dispersion, and scales proportionally to the absolute values. The coefficient of variation (CV) for whole cell protein level for R1346H is 40% compared to 31% for T797M. Using 1000 bootstrapping samples we estimated that the 95% confidence interval for T797M CV is (0.21,0.42), while the 95% CI for R1346H CV is (0.26, 0.54). The 95% confidence interval for the difference in CV between T797M and R1346H is (~0.26, 0.09)—not different from 0—indicating that the data dispersion between T797M and R1346H is statistically equivalent.
Deglycosylation. Deglycosylation was performed on input or eluate samples with Protein Deglycosylation Mix (New England Biolabs). Briefly, 60 μg of total protein (input) or biotin labeled membrane protein (eluate) was denatured in Glycoprotein Denaturing Buffer for 20 minutes at RT. The denatured protein was then treated with either buffer alone (control) or with the Deglycosylation Enzyme Cocktail (PNGase F, α-Glycosidase, Neuraminidase, Galactosidase, and β-N-acetylglucosaminidase) for 1 hour at 37 °C in a solution of 50 mM sodium phosphate and 1% NP-40. Samples were analyzed by immunoblotting.

Conventional electrophysiology. Voltage-gated calcium currents (CaV) were only resolved in Flp-In T-REx HEK293 cells after, and not before, doxycycline induction of wild-type or mutant hCaV3.3 cDNA. Whole-cell patch clamp recordings were used to compare hCaV3.3 channel currents in doxycycline treated Flp-In T-REx HEK293 cells carrying a single copy of exogenous wild-type or mutant hCaV3.3 cDNA. Recordings were performed as previously reported. The macroscopic CaV3.3 currents recorded in this expression system originate from the gating of CaV3.3 channels: we have never recorded CaV currents in untransfected tsA201, HEK293 or Flp-In T-REx HEK293 cells under the recording conditions used in this study (see also refs 35, 37–39). Wild-type macroscopic CaV3.3 currents shown here have all the properties of CaV3.3 currents and the peak current is about 60 pA/pF, equivalent to ~700–750 pA, with only 2 mM calcium as the charge carrier. The properties of the macroscopic as well as single CaV3.3 channel currents are completely consistent with previously published data e.g.2,35.

Whole-cell external recording solution contained: 2 mM CaCl2, 10 mM HEPES, 140 mM NaCl, pH adjusted to 7.2 with NaOH and the intracellular pipette solution contained: 126 mM CaCl2, 10 mM EGTA, 1 mM EDTA, 10 mM HEPES, 4 mM MgATP, pH 7.2 with CsOH. Whole-cell hCaV3.3 currents were evoked by square step depolarizations from a holding potential of ~100 mV. Currents were leak subtracted online using a P/4 – protocol. Currents recorded with Axopatch 200B amplifier (Molecular Devices, LLC) were sampled at 20 kHz and filtered at 2 kHz. For single-channel recordings, we used the HEK293-derived cell line tsA201 transiently expressing wild-type or mutant hCaV3.3 cDNA for 48 hrs. We used a pipette solution for single channel recording which is optimized to isolate CaV currents: 110 mM BaCl2, 2 mM CsCl, 10 mM HEPES, pH adjusted to 7.2 with Ba(OH)2 and the extracellular bath solution contained: 145 mM potassium aspartate, 10 mM EGTA, 5 mM HEPES, 5 mM KCl, pH adjusted to 7.2 with KOH (see refs 35, 37–39). The high potassium extracellular solution effectively clamps the membrane potential to 0 mV eliminating contributions of the membrane potential to the transmembrane patch voltage. To reduce electrode capacitance in single channel recording, pipettes were coated with Sylgard 184 (Dow Corning, Co) and polished to resistances of 5–8 MΩ. Single channel currents recorded using Axopatch 200B amplifier (Molecular Devices, LLC) were sampled at 20–100 kHz and filtered at 1 kHz. Clampln software (Molecular Devices, LLC) was used for single channel analyses. Leak subtraction was performed offline using a noiseless stimulated null trace. All recordings were obtained at room temperature.

Properties of single CaV3.3 channel currents. The estimated reversal potential for current flow through single CaV3.3 channels was positive to 0 mV; the single channel conductance estimated from the single channel current-voltage relationship was consistent with single channel conductance measurements for CaV3, and CaV3.3 specifically e.g.2,35; single CaV3 channel currents exhibited classic slow closing kinetics during the repolarization step; and we have never resolved single CaV channel currents in tsA201 cells in un-transfected cells35,37.

Experimental Design. All data were acquired without knowledge of clone identity, all analyses were done before revealing clone identity, and the experimenter interleaved recordings from cells expressing different clones on each experimental day. To compare current amplitudes across cells, we converted to current density (pA/pF) to normalize for cell size. In high throughput electrophysiology, cell capacitance is not measured but the much larger sample sizes (>100) add statistical power and compensate for the relatively small variation in size among HEK293 cells. Peak currents evoked by depolarizing pulses were used to generate current voltage relationships. These were fit with the sum of a Goldmann–Hodgkin–Katz flux equation (I) and an exponential function to model the shape of inward and outward unitary conductance, multiplied by a single Boltzmann function to summarize gating.

\[
I = (\phi + ae^{\frac{V_m + c}{V_c}}) * \frac{1} {1 + e^{\frac{V_m - V_c}{V_c}}} \\
\phi = \rho * zF^2 I^{1/2} \exp\left(\left[\frac{\mu_m}{S_o} - \frac{\mu_m}{S_i} e^{-zF^{1/2} V_m/RT}\right]\right) / (RT (1 - e^{-zFV_m/RT}))
\]

The following were held constant: R, the gas constant; F, Faraday’s constant; [S]o, internal Ca2+ (100 mM); [S]i, external Ca2+ (2 mM); z, valency of permeant ion (2); T, temperature (18 °C). We measure total whole-cell capacitance and estimate cell size using 1 μF/cm2.

High throughput electrophysiology. Recordings from doxycycline-induced Flp-In T-REx HEK293 cells expressing hCav3.3 channels were performed using Ion-works Barracuda by ChariTest, Charles River41. Briefly, cells were harvested, washed and re-suspended in HEPES-buffered physiological saline before adding to the single-hole 384-well patch plate. External recording solution contained: 137 mM NaCl, 4 mM KCl, 7 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with NaOH. The internal solution contained 90 mM CsF, 50 mM CsGlu, 2 mM MgCl2, 0.5 mM EGTA, and 10 mM HEPES, pH 7.2 adjusted with CsOH. Membrane currents were recorded with on-board patch clamp amplifiers, after establishing whole-cell configuration using amphotericin B (100 μg/ml). Peak current amplitudes measured from cells expressing hCaV3.3 WT, T797M and R1346H were fit to a bimodal distribution comprising two log-normal functions (sub-distributions) of different scale and shape factors. Descriptors of interest, the Bernoulli parameter and the median of the larger sub-distribution were determined by finding the parameters that maximize the likelihood of the data.
T797M were not significantly different from WT (Fig. 2c, lanes 1 and 2; Fig. 2d, of surface hCaV3.3 is glycosylated. We analyzed CaV3.3 mRNA levels in Flp-In T-REx HEK293 cells from three

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**Figure 2.** R1346H affects surface expression of hCaV3.3. All membranes were cut in two; upper membranes were probed with anti-FLAG to measure hCaV3.3 levels and lower membranes with control antibodies. (a) Anti-FLAG signals from Flp-In T-REx HEK293 cell lysates after induction for 24, 48, and 72 hrs with (lanes 1–3), and without (lanes 4–6), 1 μg/ml doxycycline. (b) Anti-FLAG signals in whole cell lysates from cells expressing WT-hCaV3.3, untreated (1), with glycosidase (2), and same as (2) but lacking glycosidase (3). (a,b) Compiled figures from 4 digital images of the same gel. Protein ladder images are juxtaposed to the immunoblots; ladder lane is colored in blue. Dotted lines indicate the spliced borders of two immunoblots. (c) Anti-FLAG hCaV3.3 levels in whole cell lysate (1–3) and biotin-surface fraction (4–6) from cells expressing WT (1, 4), T797M (T/M) (2, 5), and R1346H (R/H) (3, 6). (d) Anti-FLAG signal in whole cell and biotinylated (surface) preparations from cells expressing T/M and R/H shown relative to WT and normalized to controls (cadherin and β-actin). Mean ± SE values for T/M were 1.12 ± 0.08 (n = 18, whole cell) and 0.87 ± 0.13 (n = 7, surface); for R/H were 0.29 ± 0.03 (n = 18, whole cell) and 0.12 ± 0.03 (n = 7, surface). Coefficient of variation: T/M, 31% and R/H, 40% (1000 samples bootstrapping). (e) Anti-FLAG signals in biotinylated surface protein from cells expressing WT-hCaV3.3, untreated (1), glycosidase exposure (2) and, conditions same as (2) but lacking glycosidase (3). (f) Average fraction of upper MW band relative to total hCaV3.3 for WT: 0.80 ± 0.02 (n = 8); T/M: 0.80 ± 0.02 (n = 8); and R/H: 0.62 ± 0.05 (n = 8) from data in (c,d). (g) RT-qPCR analysis of hCaV3.3 mRNA 72 hr after doxycycline induction expressed as fold-change relative to non-induced. Each individual point is a separate qPCR measure for 3 biological replicates and 3–4 technical replicates. Lines connect the relative levels of mRNA for three genotypes within each biological experiment. Data do not violate D’Agostino-Pearson test for normality.

Our data demonstrated that hCaV3.3 protein levels are substantially lower in cells expressing R1346H compared to WT and T797M. The glycosylated R1346H hCaV3.3 protein fraction (>250 kDa) is preferentially reduced relative to WT (Fig. 2f), suggesting that R1346H might interfere with glycosylation and surface trafficking of hCaV3.3.

To compare rates of hCaV3.3 protein decay in cells expressing WT, T797M and R1346H, we treated cells with 0.8 μg/mL puromycin to inhibit protein translation and measured levels of hCaV3.3 protein at different time points. At this concentration and duration of exposure, puromycin had no obvious cellular toxicity. The 250 kDa lower molecular weight hCaV3.3 signal decayed relatively rapidly after protein translation was inhibited and was not detectable after 6 hours of puromycin exposure (Fig. 3a). By contrast, the glycosylation-associated >250 kDa hCaV3.3 signal decayed more slowly and incompletely (Fig. 3a,b). After 48 hrs of exposure to puromycin, the >250 kDa hCaV3.3 signal was reduced ~50% of control levels similarly among three genotypes (Fig. 3b, 48 hr data point, F(2,20) = 2.675, p = 0.0935, one-way ANOVA with Dunnett’s post test, p<0.05). In the first 2 hrs of puromycin treatment, the >250 kDa hCaV3.3 signal increased ~40% and ~30% in cells expressing WT and T797M respectively and the levels were not different between the two conditions (Fig. 3b, 2 hr data point, F(2,20) = 17.6, p < 0.001, one-way ANOVA with Dunnett’s post test, $p_{\text{WT,T797M}} = 0.540$). We also showed that GAPDH levels did not decrease following puromycin treatment in cells expressing different CaV3.3 clones over the time course of the experiment (Fig. 3c). Our data suggest that R1346H reduces the net accumulation of glycosylated hCaV3.3 compared to wild-type and does not impact the rates of channel protein decay.

Collectively, our data are consistent with the hypothesis that R1346H interferes with surface trafficking of hCaV3.3 by a mechanism that may involve glycosylation. Two putative N-linked glycosylation sites are within 1 and 4 amino acids of R1346 (N1345 and N1342). Mutations of putative glycosylation sites, N192Q and N271Q in S3-S4 and S5-S6 linkers of domain I of CaV3.2 protein are associated with reduced levels of biotinylated, functional channels on the cell membrane surface. The glycosylation states of auxiliary subunits of CaV channels have also been reported to influence protein function.
hCa$_{3.3}$ channel current densities are lower in cells expressing R1346H. We next tested if T797M and R1346H affect the functional properties of hCa$_{3.3}$ channels using whole-cell recording from the same Flp-In T-REx HEK293 cells expressing FLAG-tagged hCa$_{3.3}$ as described above. We used 2 mM Ca$^{2+}$ as the charge carrier for conventional whole-cell recording and resolved large Ca$_{3.3}$ currents that peaked at ~60 pA/pF in cells expressing WT channels. Consistent with our biochemical analyses, hCa$_{3.3}$ channel current densities in doxycycline-induced HEK293 cells expressing R1346H, but not T797M, were smaller relative to those in cells expressing WT hCa$_{3.3}$, independent of membrane voltage (Fig. 4a). To compare hCa$_{3.3}$ currents across a range of voltages, we estimated permeability rates from Boltzmann-GHK fits of individual current voltage data sets from cells expressing WT, R1346H and T797M hCa$_{3.3}$ (Fig. 4b; left). Permeability rate was used as a measure of the overall current flow in a cell for a range of voltages (see methods). Permeability rates were ~43.6% lower for hCa$_{3.3}$ currents in cells expressing R1346H compared to WT (Fig. 4b; left; $P_{\text{WT,R1346H}} = 0.00054$, Kolmogorov-Smirnov test followed by Bonferroni correction), whereas permeability rates calculated from cells expressing T797M were similar to WT (Fig. 4b; left; $P_{\text{WT,T797M}} = 0.923$, Kolmogorov-Smirnov test followed by Bonferroni correction). Estimates of the membrane potential at which hCa$_{3.3}$ currents reverse direction were not different in cells expressing WT, R1346H and T797M (Fig. 4b; right, $P_{\text{WT,T797M}} = 0.74$, Kolmogorov-Smirnov test followed by Bonferroni correction) suggesting that ion selectivity in hCa$_{3.3}$ channels is unchanged by R1346H and T797M.

We next contracted ChanTest to perform unbiased, high throughput electrophysiology using the Flp-In T-REx HEK293 cells expressing hCa$_{3.3}$ channels, and data are shown in Fig. 4c (IonWorks Barracuda; Molecular Devices). The large sample sizes, possible from high throughput automated whole-cell analyses, allowed for population analyses of hCa$_{3.3}$ current densities in cells expressing WT, R1346H or T797M (Fig. 4a,b). Population data, displayed in bee swarm and cumulative frequency plots, illustrate that hCa$_{3.3}$ currents in cells expressing R1346H hCa$_{3.3}$ are on average 2-fold smaller compared to WT (Fig. 4c). The cumulative frequency relationships for each condition were bimodal, consistent with two populations of Flp-In T-REx HEK293 cells: one expressing and a smaller fraction not expressing hCa$_{3.3}$ currents (Fig. 4c). Parameterization of each distribution, allowed by the larger sample size, showed that the percentage of cells not expressing hCa$_{3.3}$ current was similar among all variants (WT: 16%, T797M: 16%, R1346H: 20%) and that the median hCa$_{3.3}$ current of cells expressing R1346H was ~2-fold relative to WT (Fig. 4c, $P_{\text{WT,R1346H}} < 0.0001$, Kolmogorov-Smirnov test followed by Bonferroni correction). These data are consistent with our findings from conventional whole-cell recording.

Lower hCa$_{3.3}$ current densities in cells expressing R1346H could originate from fewer hCa$_{3.3}$ channels on the cell surface, from reduced current flow through individual hCa$_{3.3}$ channels, when open, or a combination of both. We used high-resolution, low-noise cell-attached patches to measure the rate of ion flow through single hCa$_{3.3}$ channels directly (Fig. 5a–c). The amplitude of single hCa$_{3.3}$ channel currents was consistent with that of individual current voltage data sets from cells expressing WT, R1346H and T797M (Fig. 4b; left), but importantly, the permeability rates were ~43.6% lower for hCa V3.3 currents in cells expressing R1346H compared to WT (Fig. 4b; right). This finding is consistent with a decrease in the number of CaV3.3 channels on the cell surface, from reduced current flow through individual CaV3.3 channels, when open, or a combination of both. We used high-resolution, low-noise cell-attached patches to measure the rate of ion flow through single CaV3.3 channels directly (Fig. 5a–c). The amplitude of single CaV3.3 channel currents was consistent with that...
reported previously for single CaV3.3 currents and indistinguishable among WT, R1346H, and T797M hCaV3.3 recordings over a range of test potentials (~13 pS, 110 mM barium as charge carrier; Fig. 5c, \( p_{\text{WT,R1346H}} = 0.85 \), and \( p_{\text{WT,T797M}} = 0.53 \), Kolmogorov-Smirnov test followed by Bonferroni correction). We measured single channel currents from tail currents to generate the single channel I-V relationship because of the larger current amplitudes (greater driving force) at negative voltages. The slow gating kinetics typical of CaV3.3 currents is illustrated in Fig. 5 from individual traces as well as captured in the ensemble averages. We conclude that the smaller CaV3.3 current densities in cells expressing R1346H reflect reduced numbers of CaV3.3 channels on the cell surface relative to WT, but the amount of current that flows through individual hCaV3.3 channels is unaffected by R1346H.

We completed our assessment of R1346H and T797M hCaV3.3 channel properties not captured by analyses of peak current-voltage relationships, using a series of voltage protocols to evaluate whole-cell currents. We assessed: voltage-dependence from tail current analyses (Fig. 6a-c, \( V_{1/2\text{negative}}, p_{\text{WT,R1346H}} = 0.32 \), and \( p_{\text{WT,T797M}} = 0.91 \), V_{1/2\text{positive}}, \( p_{\text{WT,R1346H}} = 0.6 \), and \( p_{\text{WT,T797M}} = 1.00 \), Slope (k), \( p_{\text{WT,R1346H}} = 0.52 \), \( p_{\text{WT,T797M}} = 0.19 \), Kolmogorov-Smirnov test followed by Bonferroni correction), kinetics of channel activation from −50 to 20 mV voltages (Fig. 6d,e), rate of channel closing as derived from the time constant of the tail current decay (Fig. 6f, \( \tau_{\text{closing at−60 mV}}, p_{\text{WT,R1346H}} = 0.43 \), \( p_{\text{WT,T797M}} = 0.97 \), Kolmogorov-Smirnov test followed by Bonferroni correction), voltage-dependence of channel inactivation (Fig. 7a,b, \( V_{1/2\text{inactivation}}, p_{\text{WT,R1346H}} = 0.12 \), \( p_{\text{WT,T797M}} = 0.76 \), Slope (k), \( p_{\text{WT,R1346H}} = 0.52 \), \( p_{\text{WT,T797M}} = 0.19 \), Kolmogorov-Smirnov test followed by Bonferroni correction), and time course of channel inactivation from −50 to 0 mV (Fig. 7c,d). Tail current kinetics provide a measure of the overall rate of channel closing, because the tail potential is below the threshold for channel opening and the slow closing kinetics
is a hallmark feature of CaV3 channels, compared to other CaV channels. We conclude from these extensive analyses, that R1346H and T797M do not affect the biophysical properties of hCaV3.3 channels as assessed in human cell lines, including the time course of recovery from inactivation (T797M; data not shown). It is important to note that our analyses, which find T797M has no measurable effect on the basic features of hCaV3.3 channels including expression levels and biophysical properties, do not rule out a potential effect of T797M that depends on the presence of cofactors in the native environment.

**R1346H impacts rebound bursting in a model of TRN neuron.** In TRN neurons, transient membrane hyperpolarizations can trigger bursts of action potentials, called rebound bursting, immediately upon membrane hyperpolarizations.
ophrenia pathophysiology31, is damaging. We did not find any evidence that the

(iii) firing of TRN neurons evoked by depolarizing current injections is insensitive to changes in dendritic CaV3.3

channel density is reduced to 78% or less of initial WT values (Fig. 8b; black line shows WT relationship); and

recruited from a previously inactivated state when the membrane is hyperpolarized. CaV3.3 underlies 90% of

the low threshold, voltage-gated calcium channel expressed in TRN9 and rebound bursting is absent in TRN neurons

bursting regardless of hyperpolarization magnitude (Fig. 8a, top three rows). In contrast, firing of TRN neurons,

evoked by depolarizing current injections is insensitive to changes in dendritic CaV3.3 current densities to 40% of initial WT values (Fig. 8a, bottom two rows). The results from NEURON simulation are consistent with the notion that Depolarization-induced firing is primarily mediated by activation of voltage-gated ion channels other than CaV3.3 in TRN8,9.

To simulate heterozygosity—equal contribution of WT and R1346H alleles—we reduced CaV3.3 current den-
sity to 72.5% of WT levels and showed that this reduction in CaV3.3 current density fails to support rebound

bursting regardless of hyperpolarization magnitude (Fig. 8a, top three rows). In contrast, firing of TRN neurons,

evoked by depolarizing current injections is unaffected by R1346H (Fig. 8a, bottom two rows). The results from

NEURON simulation are consistent with the notion that Depolarization-induced firing is primarily mediated by

activation of voltage-gated ion channels other than CaV3.3 in TRN8,9.

Discussion

CACNA1I has been identified as a candidate schizophrenia risk gene based on genome-wide association studies,

and on the identification of de novo, rare missense variations in CACNA1I from exome sequencing of schiz-

ophrenia proband trios19,31. Our study is the first to assess the functional impact of two missense CACNA1I

variants found in schizophrenia patients, but not in unaffected family members31. Based on our analyses, we

find that the de novo coding variant Chr22: 39665939G > A of CACNA1I31, is sufficiently disruptive to CaV3.3 in

the heterozygous condition to impact rebound bursting in a TRN model neuron. Our data lend support to the

proposal that CaV3.3 R1346H, which is proposed as one of several de novo risk variations that contribute to schiz-

ophrenia pathophysiology31, is damaging. We did not find any evidence that the de novo coding variation Chr22:
39659492C > T of CACNA1I (T797M CaV3.3) is disruptive in biochemical and electrophysiological HEK cell assays. However, we cannot rule out the possibility that T797M impacts CaV3.3 channel function by mechanisms that are not reconstituted in HEK cells.

Our findings are interesting in light of documented functional associations between reduced CaV3.3 expression, rebound bursting of TRN neurons, sleep spindle oscillations, and sleep spindle coherence across cortex in schizophrenia^4,10,14,49–51. Moreover, reduced spindle activity is a heritable component of the sleep electroencephalogram patterns detected in the 1st degree relatives of people with schizophrenia^52.

Our data are consistent with a mechanism by which R1346H interferes with CaV3.3 glycosylation and plasma membrane trafficking leading to reduced CaV3.3 current density. An extracellular N-linked glycosylation motif N1345 (N-x-S/T) encompasses R1346 residue and glycosylation of proteins in the endoplasmic reticulum (ER) is known to influence the rate of protein transport from the ER through the Golgi apparatus to the plasma membrane^53. Our studies add to reports that glycosylation of many ion channel proteins, including CaV3.2, regulates their levels of surface expression^54–58. Several human diseases are known to arise from defects in glycosylation. For example, in cystic fibrosis causal mutations in human CFTR lead to altered glycosylation patterns of CFTR channel protein and channel trafficking defects in lung epithelial cells, and in long QT syndrome, coding mutations in human KCNE and KCNQ lead to reduced potassium ion channel glycosylation and reduced expression in the heart^59–62.

CaV3.3 is essential for rebound bursting in TRN neurons, and we show that reduced CaV3.3 current density in model TRN neurons expressing R1346H is sufficient to disrupt this phenomenon. CaV3.3 is expressed in other brain regions including the cortex and in mitral cell dendrites of the olfactory bulb^53. In mitral cells, CaV3.3 contributes to modulation of evoked and asynchronous release, and it mediates rebound bursting^63. Deficits in olfaction have, for several years, been described in people with schizophrenia^64.

It is widely accepted that schizophrenia disease risk depends on the accumulated effect of multiple or many common risk loci^65, but the relative contribution of each individual rare variant to the disease risk is not known^17. Rare, coding variations have the greatest potential to disrupt protein function and are likely to make greater contribution to complex common disease risk including schizophrenia^17,19,25,30,65. Our experiments were designed to assess the functional consequences of T797M and R1346H on CaV3.3 channel activity in a robust expression system, and they demonstrate that R1346H disrupts CaV3.3 channel trafficking to the plasma membrane. We did
not identify a phenotype associated with T797M in our assays of CaV3.3 function and, as discussed above, we cannot rule out the possibility that T797M will be disruptive to CaV3.3 signaling in the native environment, but our analyses should help guide future studies designed to assess the potential contribution of R1346H-CaV3.3 to schizophrenia risk.

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**Author Contributions**

A. Andrade, J.H., A. Allen and V.Y. performed research. A. Andrade, J.H. and A. Allen, V.Y., D.L. and J.Q.P. designed research. A. Andrade, D.L. and J.Q.P. wrote the manuscript.

**Additional Information**

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