A common haplotype containing functional *CACNA1H* variants is frequently coinherited with increased *TPSAB1* copy number

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Purpose: *Ca_v3.2* signaling contributes to nociception, pruritus, gastrointestinal motility, anxiety, and blood pressure homeostasis. This calcium channel, encoded by *CACNA1H*, overlaps the human tryptase locus, wherein increased *TPSAB1* copy number causes hereditary α -tryptasemia. Germ-line *CACNA1H* variants may contribute to the variable expressivity observed with this genetic trait.

Methods: Tryptase-encoding sequences at *TPSAB1* and *TPSB2*, and *TPSG1* and *CACNA1H* variants were genotyped in 46 families with hereditary α -tryptasemia syndrome. Electrophysiology was performed on tsA201 HEK cells transfected with wild-type or variant *CACNA1H* constructs. Effects on clinical phenotypes were interrogated in families with *TPSAB1* duplications and in volunteers from the ClinSeq cohort.

Results: Three nonsynonymous variants in *CACNA1H* (rs3751664, rs58124832, and rs72552056) cosegregated with *TPSAB1* duplications in 32/46 families and were confirmed to be in linkage disequilibrium (LD). In vitro, variant *Ca_v3.2* had functional effects: reducing current densities, and altering inactivation and deactivation properties. No clinical differences were observed in association with the *CACNA1H* haplotype.

Conclusion: A previously unrecognized haplotype containing three functional *CACNA1H* variants is relatively common among Caucasians, and is frequently coinherited on the same allele as additional *TPSAB1* copies. The variant *CACNA1H* haplotype, which in vitro imparts partial gain of function, does not result in detectable phenotypic differences in the heterozygous state.

Genet Med advance online publication 21 September 2017

Key Words: *CACNA1H*; hereditary α -tryptasemia; tryptase; *Ca_v3.2*

INTRODUCTION

Increased α -tryptase encoding copy number at *TPSAB1* is linked to elevated basal serum tryptase and is associated with multisystem complaints—a condition called hereditary α -tryptasemia syndrome.¹ A gene-dose effect has been observed in this disorder, where a greater number of α -tryptase encoding copies on a single allele are associated with both increased basal serum tryptase level and prevalence of symptoms. However, the mechanism by which tryptase protein may contribute to clinical symptoms remains unclear. Owing to the structural complexity of the tryptase locus, germ-line sequence of this portion of the linkage region identified in hereditary α -tryptasemia, which includes the extra *TPSAB1* copies, has not been fully ascertained. Given this, and the diffuse nature of clinical phenotypes observed, we hypothesized that coinherited variants may contribute to

one or more of the clinical manifestations observed among affected individuals.

Within the linkage region (Chr16: 556,104–5,653,182; GRCh37/hg19), adjacent to the tryptase locus, exists the *CACNA1H* gene, which encodes the voltage-dependent, T-type, α 1H subunit of the calcium channel, *Ca_v3.2*. While *CACNA1H* is a large and moderately mutable gene (Phred-scaled gene damage index = 11.351),² functional variants in this T-type voltage-gated calcium channel would make a compelling candidate for many clinical phenotypes associated with *TPSAB1* duplications. *Ca_v3.2* signaling has been demonstrated as contributing to irritable bowel syndrome in a rat model of this disorder.³ Heightened anxiety, which can both contribute to and manifest as, symptoms associated with mast cell mediator release, has been reported in animal models with *CACNA1H* null alleles.⁴ While specific effects of

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this channel on autonomic nervous system function have not been reported, gain-of-function mutations have been identified in children with early onset hypertension and absence seizures.^{5,6} Non-mast cell-related, histamine-independent models of pruritus have also been shown to be dependent upon $\text{Ca}_v3.2$ expression.⁷ Finally, in addition to itch, a number of variants in voltage-gated calcium channels, including *CACNG2* and *CACNAD2D3*, have been associated with chronic pain, and $\text{Ca}_v3.2$ -dependent signaling has been shown to enhance pain responses in mice.^{8,9}

We set out to characterize the frequency and effects of *CACNA1H* variants frequently coinherited with additional *TPSAB1* copy, and determine how these affect channel function and clinical phenotypes both among individuals with hereditary α -tryptasemia syndrome and among unselected individuals.

MATERIALS AND METHODS

Subjects

Hereditary α -tryptasemia cohort

Informed consent was provided by all patients and their relatives on NIH IRB-approved research protocols designed to study mastocytosis (NCT00044122 and NCT00001756) and/or atopy (NCT01164241, NCT00852943, and NCT00557895). Family and personal medical histories were obtained, and physical examinations were performed as described.¹

ClinSeq cohort

All study participants provided written informed consent and were actively enrolled in ClinSeq (NCT00410241), an IRB-approved genomic study conducted via the National Human Genome Research Institute.¹⁰ For inclusion in ClinSeq patients had to be: between the ages of 45 and 65 years, tobacco-smoke-free for at least 1 year, able to return frequently at their own expense to the NIH in Bethesda, MD, and have a primary care physician or have access to a community health center. Patients also could not have a nuclear family member concurrently enrolled in ClinSeq.

Genotyping and DNA sequence analysis

For the hereditary α -tryptasemia cohort, exome sequencing was performed on eight families using TruSeq (Illumina, San Diego, CA) capture kits and a custom analysis pipeline as described.¹¹ Genome sequencing was performed as described¹² on nine families (five of which previously had exome sequencing performed) using the HiSeq platform (Illumina) with the Burrows-Wheeler Aligner and Picard (<http://broadinstitute.github.io/picard/>) used for basic alignment and sequence quality control. For the ClinSeq cohort, exome sequencing was performed as described.¹³ The gene damage index for *CACNA1H* was obtained using the publicly available website <http://pec630.rockefeller.edu:8080/GDI/>.² Linkage disequilibrium calculations were performed using Haploview (Broad Institute) as described.¹⁴ Recombination rate distribution in chromosome 16 near *CACNA1H* and *TPSG1* was generated based on 1000 Genomes-phased OMNI data for

CEU population (ftp-trace.ncbi.nih.gov/1000genomes/ftp/technical/working/20130507_omni_recombination_rates/CEU_omni_recombination_20130507.tar).

TPSAB1 and *TPSB2* genotyping and *CACNA1H* haplotype determination

Allelic α - and β -encoding tryptase copy number at *TPSAB1* was determined using digital droplet PCR (ddPCR) with use of custom primers and probes as described.¹ *TPSG1* and *CACNA1H* variant genotyping was performed using validated primer/probe sets for rs143120059 (C16107914), rs113856625 (C15283055), and rs72552056 (C99547390), rs3751664 (C25746770), rs58124832 (C90589833), respectively (ThermoFisher, Waltham, MA) by both RT-PCR and ddPCR; the results were validated by exome and genome sequence variant calls. Only individuals with all or none of the three *CACNA1H* variants were included in the phenotypic analysis.

ClinSeq individual selection and clinical phenotyping of all subjects

For clinical phenotypic analysis, haplotype carriers were defined as individuals with at least one allelic copy each of three different variants in *CACNA1H* passing quality filters, which were observed in strong linkage disequilibrium across the cohort: rs3751664 (NM_001005407:c.C2362T:p.(R788C)), rs58124832 (NM_001005407:c.G5594A:p.(R1865Q)), and rs72552056 (NM_001005407:c.C5995T:p.(R1999C)). Wild-type (WT) individuals were defined by homozygosity of the dominant allele at all three variant loci; these samples also met quality filters and had a minimum $10\times$ coverage depth at all three variant loci. Basal serum tryptase levels were measured in identified individuals, and the *TPSAB1* copy number was determined among those with tryptase >8 ng/ml; those identified with increased *TPSAB1* copy number were excluded from further evaluation.

A standardized questionnaire was administered to assess common clinical features associated with increased *TPSAB1* copy number, seen in hereditary α -tryptasemia syndrome as described.¹ Included in the survey were previously validated question sets from COMPASS 31 and Rome III.^{15,16} Patients were contacted by phone in random order by blinded interviewers, and the data were subsequently assigned to the appropriate genotype groups.

Site-directed mutagenesis

The variant *CACNA1H* construct (*CACNA1H*^{VAR}) was created using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) from a wild-type (WT) *CACNA1H* (*CACNA1H*^{WT}) cDNA (transcript variant 2, NM_001005407.1) pcDNA3.1 vector (a kind gift from G.W. Zamponi, University of Calgary, Calgary, Alberta, Canada)). Mutation primers were designed using NEBaseChanger and obtained from UCDNA Services DNA/RNA Synthesis Laboratory (University of Calgary) as follows: R788C forward: GCGGCAAGCTtcgctGCATCGTGGA and reverse: TGAAG GTAACCCAGAGGCGGC; R1865Q forward: AAGGAGGCA

CaGGAGGATGCG and reverse: GTTGCTCTCTCCAGG TG; R1999C forward: CTCGGGGCACgacctGCTCCCCAG and reverse: GGGACAGGGCGTGGAGGG. cDNAs were purified from transformed DH5 α bacteria (MiniPrep kit; Qiagen, Hilden, Germany). Site-directed mutagenesis products and *CACNA1H*^{VAR} were sequence verified (University Core DNA Services, University of Calgary).

Transfection and cell culture

tsA201 human embryonic kidney (HEK) cells were transiently transfected with 3 μ g of plasmid and 0.5 μ g of yellow fluorescent protein (YFP) plasmid using Lipofectamine 3000 (Life Technologies, Carlsbad, CA). Six hours after transfection, tsA201 HEK cells were plated on glass coverslips at 20% confluence and cultured for another 18 hours before patching.

Electrophysiology

Transfected tsA201 HEK cells were patched in the whole-cell configuration. WT and variant *Ca_v3.2* currents were recorded in voltage-clamp mode using a Multiclamp 700B amplifier, a Digidata 1440A digital-to-analog converter, and pClamp10.4 software (Molecular Devices, Palo Alto, CA). Data were sampled at 10 kHz and filtered at 2 kHz. External modified Ringer's solution contained (in mM): 105 NaCl, 1 MgCl₂, 2 CaCl₂, 5 KCl, 10 HEPES, and 10 Glucose, pH 7.4 with NaOH and 320 mOSM with D-sorbitol. Internal cesium gluconate pipette solution contained (in mM): 130 CsOH, 10 EGTA, 2 MgCl₂, 7 NaCl, 10 HEPES, 3 ATP and 0.6 GTP, pH 7.25, and 300 mOsm with gluconic acid. Borosilicate glass pipettes were pulled with a PC-10 puller (Narashige) to a resistance of 4 m Ω . Access resistance was compensated to 80% and the capacitance of each cell was measured for current normalization.

Voltage data acquisition and analysis

A current-voltage (I-V) step protocol was used to determine voltage-dependent activation, current densities, activation rate, and inactivation rate properties for the *Ca_v3.2*^{WT} and *Ca_v3.2*^{VAR} channel populations, expressed in tsA201 HEK cells. The cell membrane potential was stepped from a resting voltage (V_r) of -90 mV to a test potential (V_t) of -70 mV for 150 ms then returned to the V_r for a 10-second interval to allow complete recovery from inactivation. The V_t was increased in 10-mV increments over a series of 11 steps; the last step was to $+30$ mV. Peak inward Ca^{2+} currents (I_{Ca}) were plotted as a function of the V_t to generate I-V relations. The I_{Ca} was also normalized by the individual cell capacitance measurement for the comparison of current densities. Individual I-V and mean current-density-V relations were fit with the Boltzmann equation, where $I_{peak} = (V_t - E_{rev}) G / (1 + \exp((V_{a,1/2} - V_t)/S))$, and E_{rev} is the reversal potential, $V_{a,1/2}$ is the half-activation potential, G is the maximum slope conductance, and S is the slope factor that is inversely proportional to the effective gating charge. The I_{Ca} activation and inactivation phases at $V_t = -30$ mV were fit with single

exponential functions to determine the tau of activation (τ_{act}) and the tau of inactivation (τ_{inact}).

A steady-state inactivation protocol was used to assess parameters of voltage-dependent inactivation; a V_r of -100 mV was stepped to a conditioning potential (V_c) between -100 and 0 mV for a duration of 1 second before a V_t of -30 mV was applied for 150 ms. V_c steps increased in 10-mV increments; between runs the cell membrane potential was returned to V_r for 10 seconds to allow for complete recovery from inactivation. I_{Ca} during V_t for each V_c was normalized to the peak I_{Ca} and fit with the Boltzmann relation: $I/I_{max} = 1/(1 + \exp(z^*(V_c - V_{i,1/2})/25.6))$, where $V_{i,1/2}$ is the voltage of half-inactivation and z is the slope factor.

A tail-current protocol was used to determine deactivation rates; a short 2-ms prepulse to $+45$ mV from a V_r of -100 mV was given to maximally open WT and mutant *Ca_v3.2* channels without evoking inactivation before stepping the membrane potential of the cell back to a V_t of -100 mV. The deactivation phase of the large I_{Ca} evoked by V_t was fit with a single exponential function to determine the tau of deactivation (τ_{deact}).

Statistical analyses

Data obtained with Clampex 10.4 were analyzed with Clampfit 10.4 (Molecular Devices, Sunnyvale, CA). Fits of the I-V and steady-state inactivation curves were done in SigmaPlot 12.5 (Jandel Scientific, San Rafael, CA). Student's *t*-tests were employed to test significances of associations as indicated. Fisher's exact test was used to test the association of carrier status and clinical phenotypes. The Mann-Whitney test was applied to compare COMPASS 31 scores between common haplotype carriers and noncarriers. Data are presented as the mean $\pm$ standard error. Statistical analysis was performed in SigmaPlot 12.5 (Jandel Scientific) and Prism (Graphpad, La Jolla, CA). Correction for multiple comparisons was not performed, given the small sample sizes.

RESULTS

Three *CACNA1H* variants form a haplotype that frequently cosegregates with increased allelic *TPSAB1* copies encoding α -tryptase

We first examined exome and genome sequences obtained from 12 families (87 individuals) with hereditary α -tryptasemia syndrome; all individuals in these families had increased allelic *TPSAB1* copy number and multisystem complaints as described.¹ All genes containing coding nonsynonymous segregating variants with a minor allele frequency (MAF) of at least 0.1 (in 1000 Genomes v3) in each family were identified, and compared to variant-containing genes that segregated in other families (GQ filter 20, allelic balance > 25). Only six genes (*CACNA1H*, *KCNJ12*, *MUC16*, *MUC6*, *PRAMEF1*, and *TPSG1*), in addition to α -encoding variants mapping to *TPSAB1* and *TPSB2*, were identified with segregating variants in five or more of the sequenced families

(Supplementary Table S1 online). Of these, variants in *TPSG1* and *CACNA1H* were identified in six families, with the same variants cosegregating in all individuals. The remaining variants were common in other databases or present in highly mutable genes (e.g., *MUC16*, *MUC6*) with little predicted effect on clinical phenotype.

The MAF of the identified *CACNA1H* variants rs3751664 (NM_001005407:c.C2362T:p.(R788C)), rs58124832 (NM_001005407:c.G5594A:p.(R1865Q)), and rs72552056 (NM_001005407:c.C5995T:p.(R1999C)) were 0.089, 0.098, 0.091 in the ExAC database and 0.047, 0.031, 0.032 in the 1000 Genomes v3 database, respectively; the MAF for those identified in *TPSG1*, rs143120059 (NM_012467.3:c.G578A:p.(R193H)) and rs113856625 (NM_012467.3:c.C413T:p.(S138F)) were 0.053, 0.054 in the ExAC database and 0.025, 0.027 in the 1000 Genomes v3 database, respectively (Supplementary Table S2). Similar MAFs for variants within each gene, and our observation of coinheritance in all six families, suggested a larger shared haplotype was often co-inherited with *TPSAB1* duplications or triplications (Figure 1a). Indeed, performing linkage disequilibrium analysis in 880 ClinSeq self-reported European-American individuals, we found that all five variants were present as a haplotype in 3.0% and were in linkage disequilibrium (Figure 1b, Supplementary Table S2); *TPSAB1* duplications cannot be assessed using this method. Analysis of recombination rates at the tryptase locus confirmed two of the variants (rs58124832, rs72552056) in *CACNA1H*, and all tryptase encoding genes (*TPSG1*, *TPSB2*, *TPSAB1*, *TSPD1*) are within one large region with recombination rates of less than 3 cM/Mb, flanked by two relatively high recombination rate regions (left > 25 cM/Mb and right > 65 cM/Mb). These data suggest that this large region is more likely to be inherited as one haplotype in this population as well.

Using real-time and digital droplet PCR (ddPCR), 46 families with hereditary α -tryptasemia syndrome (confirmed by clinical phenotype, elevated basal serum tryptase, and ddPCR demonstrating increased monoallelic α -tryptase encoding *TPSAB1* copy number) were evaluated for the presence of the *CACNA1H* variant haplotype (*CACNA1H*^{VAR}). A total of 32/46 families were found to have *CACNA1H*^{VAR} cosegregating with increased *TPSAB1* copy numbers. Eleven families did not carry any of the *CACNA1H* variants, and three families had two of the three variants (rs58124832 and rs72552056) segregating with increased *TPSAB1* copy number. The *TPSG1* variants were not detected in exome or genome sequence data in the absence of *CACNA1H* variants, and all 11 families without the haplotype were confirmed as carrying the major alleles at both *TPSG1* loci. Only one individual carried the haplotype in trans with an α -tryptase duplication allele. Given the putative role of *Ca_v3.2* in a number of clinical phenotypes of interest, we next focused our efforts on characterizing the *CACNA1H* variants and their effect on channel physiology.

The cosegregating *CACNA1H* haplotype variants impart partial gain of function in vitro

Expression of *CACNA1H*^{VAR} results in smaller current densities than *CACNA1H*^{WT}

The voltage-gated T-type calcium channel *Ca_v3.2*, encoded by *CACNA1H*, contains four transmembrane domains with interposed linker regions. The p.(R788C) amino acid substitution occurs in the domain I-II linker just proximal to the domain II transmembrane α -helix, and both p.(R1865Q) and p.(R1999C) substitutions are present in the carboxy terminus just distal to the domain IV transmembrane α -helix (Figure 1c). Both the domain I-II linker and the carboxy terminus contribute to voltage-dependent inactivation of T-type calcium channels;^{17–20} thus we hypothesized that the loss of positively charged residues in these intracellular segments could alter fundamental channel properties.

Current densities, whole-cell *I_{Ca}* normalized for cell-membrane capacitance (pA/pF), differed between tsA201 HEK cells expressing wild-type *Ca_v3.2* (*Ca_v3.2*^{WT}) and variant (*Ca_v3.2*^{VAR}) channels ($P = 0.05$; Figure 2a). At the peak of the I-V curve (–30 mV), current densities for *Ca_v3.2*^{WT} were -27.5 pA/pF $\pm$ 5.1, $n = 17$, while *Ca_v3.2*^{VAR} current densities were -17.1 pA/pF $\pm$ 3.3, $n = 19$. *Ca_v3.2*^{VAR} may destabilize functional channel formation, resulting in the observed lower current densities. Differences in maximal conductance (G) measures obtained from I-V curve Boltzmann fits are also best explained as a consequence of reduced channel expression; *Ca_v3.2*^{WT} G was 8.0 pS $\pm$ 1.1 ($n = 17$) while *Ca_v3.2*^{VAR} G was 4.3 pS $\pm$ 0.6 ($n = 19$; $P = 0.009$) (Table 1).

Properties of channel activation, namely the voltage sensitivity of activation ($V_{a,1/2}$) and the rate of activation (τ_{act}), remained unaltered by the presence of *Ca_v3.2*^{VAR}. *Ca_v3.2*^{WT} $V_{a,1/2}$ was -40.0 ± 2.0 mV, while *Ca_v3.2*^{VAR} $V_{a,1/2}$ was -38.9 ± 2 mV ($P = 0.35$). τ_{act} , determined through a single exponential fit of *I_{Ca}* development evoked by a voltage step from -90 to -30 mV, was 4.2 ± 0.4 ms for *Ca_v3.2*^{WT} ($n = 17$) and 4.7 ± 0.7 ms for *Ca_v3.2*^{VAR} ($n = 19$; $P = 0.9$).

Ca_v3.2^{VAR} has altered inactivation properties compared to *Ca_v3.2*^{WT}

τ_{inact} , the rate at which channels move into a non-conducting inactivated state, was determined through an exponential fit of the declining *I_{Ca}* evoked by membrane depolarization to -30 mV. *Ca_v3.2*^{VAR} τ_{inact} was 25.8 ± 2.2 ms ($n = 19$), significantly slower than *Ca_v3.2*^{WT} 20.1 ± 1.2 ms ($n = 17$; $P = 0.03$) (Figure 2b). A slower τ_{inact} would allow for greater calcium influx into neurons through conducting channels. *Ca_v3.2*^{VAR} also altered parameters describing the voltage dependence of channel inactivation: the voltage of half-inactivation ($V_{i,1/2}$) and slope factor (z). $V_{i,1/2}$ was -60.6 ± 2.4 mV for *Ca_v3.2*^{WT} ($n = 13$) and -55.3 ± 1.6 mV for *Ca_v3.2*^{VAR} ($n = 14$; $P = 0.04$) (Figure 2c). Thus *Ca_v3.2*^{VAR} would be predicted to allow greater calcium influx, as it would activate at the same low potentials as the *Ca_v3.2*^{WT} population, but a greater depolarization would be required to

place it in the nonconducting inactive state. z also differed significantly; it was 5.2 ± 0.4 for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 13$) and 6.8 ± 0.4 for $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 14$; $P = 0.005$). The steeper

$\text{Ca}_v3.2^{\text{VAR}}$ z indicates that inactivation proceeds to completion over a smaller range of voltages than for $\text{Ca}_v3.2^{\text{WT}}$ channels.

Figure 1 Location and protein changes in the *CACNA1H* variant haplotype near the tryptase locus. (a) Schematic of the 105 kb region (GRCh38.p7) at 16p13.3, containing the tryptase locus (*TPSG1*, *TPSB2*, and *TPSD1*) and *CACNA1H*. Haplotype variants identified in *CACNA1H* and *TPSG1* are indicated; *TPSAB1* is duplicated as seen in hereditary α -tryptasemia. (b) Recombination rate distribution for all variants in chromosome 16 (top) and of the region containing *CACNA1H* and *TPSG1* (bottom) based upon 880 European-Americans in ClinSeq. *CACNA1H*, *TPSG1*, *TPSB2*, *TPSAB1*, *TPSD1* regions are indicated in red, blue, pink, green, and purple, respectively. Haplotype variants (rs3751664, rs58124832, rs72552056) in *CACNA1H* are indicated by red vertical lines, and (rs143120059, rs113856625) in *TPSG1* are blue vertical lines. (c) Schematic of $\text{Ca}_v3.2$ demonstrating locations of the amino acid substitutions (Δ) resulting from the three *CACNA1H* variants (red text in a).

Figure 2 Altered kinetics of $\text{Ca}_v3.2^{\text{VAR}}$. (a) $\text{Ca}_v3.2^{\text{VAR}}$ has a smaller current density. Average current densities (pA/pF) as a function of voltage and fit using Goldman–Hodgkin–Katz (GHK) equation (values shown in Table 2). Current densities at -30 mV for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 17$) and $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 19$) were -27.5 ± 5.1 pA/pF, $n = 17$ and -17.1 ± 3.3 pA/pF, respectively; $*P = 0.05$. Data are shown as mean $\pm$ SEM, Student’s t -test. (b) $\text{Ca}_v3.2^{\text{VAR}}$ inactivates more slowly than $\text{Ca}_v3.2^{\text{WT}}$. Voltages (top) used to evoke representative current (I) traces normalized to peak I at -30 mV (bottom); $\tau_{\text{inactivation}}$ obtained from single exponential fits of the inactivation phase was 20.1 ± 1.2 ms for $\text{CACNA1H}^{\text{WT}}$ ($n = 17$) compared to 25.8 ± 2.2 ms for $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 19$); $\tau_{\text{activation}}$ was 4.2 ± 0.4 ms for $\text{CACNA1H}^{\text{WT}}$ ($n = 17$) and 4.7 ± 0.7 ms for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 19$). $*P = 0.03$, Student’s t -test. (c) $\text{Ca}_v3.2^{\text{VAR}}$ shifts voltage-dependent inactivation positively. Open probability-voltage relationships with Boltzmann fit indicated in solid lines; voltage of half-inactivation ($V_{1/2}$) were -60.6 ± 2.4 mV for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 13$) and -55.3 ± 1.6 mV for $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 14$), $*P = 0.04$; and slope factors (z) were 5.2 ± 0.4 for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 13$) and 6.8 ± 0.4 for $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 14$), $P = 0.005$. Data are shown as mean $\pm$ SEM, Student’s t -test. (d) $\text{Ca}_v3.2^{\text{VAR}}$ deactivates faster than $\text{Ca}_v3.2^{\text{WT}}$. Voltages used (top) to evoke representative current (I) traces normalized to peak I at -100 mV (bottom); $\tau_{\text{deactivation}}$ obtained from single exponential fits of the deactivation phase was 1.9 ± 0.2 ms for $\text{Ca}_v3.2^{\text{WT}}$ ($n = 11$) and 1.4 ± 0.08 ms for $\text{Ca}_v3.2^{\text{VAR}}$ ($n = 12$); $*P = 0.02$, Student’s t -test.

Table 1 Conductance is significantly decreased in $\text{Ca}_v3.2^{\text{VAR}}$ compared to $\text{Ca}_v3.2^{\text{WT}}$

	$V_{a,1/2}$ (mV)	E_{rev} (mV)	S	G_{max} (pS)	n
$\text{Ca}_v3.2^{\text{WT}}$	-40.0 ± 2.0 (-42.9)	31.0 ± 2.6 (39.1)	5.7 ± 0.5 (6.2)	8.0 ± 1.1 (0.5 pS/pF)	17
$\text{Ca}_v3.2^{\text{VAR}}$	-38.9 ± 2 (-40.0)	29.5 ± 2.8 (38.9)	5.4 ± 0.5 (8.2)	$4.3 \pm 0.6^*$ (0.3 pS/pF)	19

Individual current–voltage (I–V) relationships were fit with the Goldman–Hodgkin–Katz (GHK) equation to determine parameters of voltage-dependent activation. Only the conductance (G_{max}) was found to differ significantly between $\text{CACNA1H}^{\text{WT}}$ and $\text{CACNA1H}^{\text{VAR}}$. Values in parentheses are GHK equation fits of the current density–voltage relationships shown in Figure 2.

$*P = 0.009$, Student’s t -test.

Ca_v3.2^{VAR} has altered deactivation properties compared to Ca_v3.2^{WT}

τ_{deact} , the rate at which channels move from the open to the closed state, differed significantly between Ca_v3.2^{WT} and Ca_v3.2^{VAR} channels ($P = 0.02$; **Figure 2d**). Ca_v3.2^{WT} τ_{deact} was 1.9 ± 0.2 ms ($n = 11$), while Ca_v3.2^{VAR} τ_{deact} was 1.4 ± 0.08 ms ($n = 12$); a faster τ_{deact} attenuates calcium influx and promotes rapid neuronal repolarization.

Functional CACNA1H variant haplotype does not affect phenotypes among individuals with allelic TPSAB1 duplications

To determine potential clinical implications of the presence of the heterozygous CACNA1H partial gain-of-function variant allele, 57 individuals with TPSAB1 duplications were clinically phenotyped as described, with validated questionnaires for irritable bowel syndrome (Rome III criteria) and orthostatic symptoms (COMPASS 31, a validated measure of dysautonomic symptoms) being employed.¹ Individuals with TPSAB1 triplications, those harboring two TPSAB1 duplication-carrying alleles, or those carrying the haplotype in trans with TPSAB1 duplications, were excluded from the analysis. Individuals with the CACNA1H^{VAR} allele ($n = 35$), were compared to those found to carry the major allele for the three CACNA1H variants (CACNA1H^{WT}; $n = 22$). Clinical phenotypic data were analyzed to assess potential impact upon the clinical phenotypes associated with TPSAB1 duplications. None of the features described in association with TPSAB1 duplications were significantly increased by the presence of CACNA1H^{VAR} (**Table 2**; **Supplementary Figure S1**). CACNA1H^{VAR} was associated with a modest but significant reduction in prevalence of systemic venom reactions ($n = 2$ v. 6; $P = 0.04$). Post-hoc analysis revealed that despite a small sample size, these data were powered to detect an approximately 30% difference for most parameters, thus if heterozygous expression of the CACNA1H^{VAR} allele had an effect on clinical phenotype, it did not appear to be a major contributor.

In the absence of hereditary α -tryptasemia, the CACNA1H variant haplotype does not alter the prevalence of symptoms associated with TPSAB1 duplications

It remained possible that any modifying effect of CACNA1H^{VAR} on assessed clinical phenotypes was not detected because of the dominant effect of increased TPSAB1 copy number, as suggested by the observed gene-dose effect in hereditary α -tryptasemia syndrome.¹ Therefore, we next looked for an effect of the CACNA1H^{VAR} haplotype (defined by the presence of the three CACNA1H variants) on the same set of clinical symptoms, within the ClinSeq cohort, an unselected population of healthy unrelated adults.¹⁰ After stringent filtering was applied, requiring at least $10\times$ coverage over all three variants, 22 individuals were identified as having the CACNA1H^{VAR} allele. Some individuals with the variant haplotype may have been classified as carrying only one or two variants, owing to inadequate exon capture in some

samples; however, based upon the calculated frequency of the haplotype, we expected to identify only 26 individuals with the haplotype (**Supplementary Table S2**). Using the same selection strategy, an additional 50 individuals with wild-type CACNA1H sequence at these loci were also selected at random, and attempts were made to contact all individuals for a blinded phone interview.

Basal serum tryptase levels were obtained on all individuals to screen for hereditary α -tryptasemia, and ddPCR was performed to confirm genetic diagnosis in all individuals with tryptase > 8 ng/ml. Of the 22 individuals with CACNA1H^{VAR}, six were subsequently excluded, owing to the identification of increased monoallelic TPSAB1 copy, and three were not available for phone interview. Four of the randomly selected CACNA1H^{WT} individuals were likewise unavailable for interview. The remaining 13 individuals with the CACNA1H^{VAR} haplotype were then compared to the 46 randomly selected individuals who both had wild-type CACNA1H sequence (CACNA1H^{WT}) and did not harbor TPSAB1 duplications. The age and demographic characteristics did not differ significantly between these two populations. No association was seen between the variant haplotype and any of the clinical features associated with TPSAB1 duplications: including irritable bowel syndrome (by Rome III criteria), orthostatic symptoms (by COMPASS 31) or systemic reactions to venom (**Table 3**, **Supplementary Figure S1**).

DISCUSSION

A previously unrecognized haplotype containing three functional variants in LD within CACNA1H is frequently coinherit on the same allele as additional α -tryptase-encoding TPSAB1 copies. This haplotype appears to be common in Caucasians and results in a partial gain of function in vitro.

Many clinical symptoms associated with hereditary α -tryptasemia are variably expressed. We had speculated that in the syndromic presentation, a second coinherit genetic variant might contribute to the observed differences in expressivity, as may occur relatively commonly in single gene disorders.¹⁷ Given the frequent coinheritance of this functional CACNA1H^{VAR} haplotype and the physiology attributed to Ca_v3.2 signaling, a compelling case can be made for a contribution to the multisystem complaints seen in this syndrome. Despite this, in the heterozygous state, we were unable to detect any clinical effects of the CACNA1H^{VAR} haplotype on manifestations of hereditary α -tryptasemia syndrome among individuals with TPSAB1 duplications or from the ClinSeq cohort. However, it remains possible that individuals homozygous for this functional haplotype may display a clinical phenotype.

The potential clinical importance of the newly discovered CACNA1H^{VAR} haplotype beyond symptoms associated with increased TPSAB1 copy number remains unexplored. The p.(R788C) and p.(R1865Q) amino acid substitutions have been linked to neurological disorders—childhood absence epilepsy^{6,18} and autism spectrum disorders,¹⁹ respectively—and a distinct gain-of-function mutation resulting from the missense c.4645A $>$ G:p.(M1549V) substitution has been

Table 2 Effect of *CACNA1H* variant haplotype on clinical phenotypes among individuals with *TPSAB1* duplications

	<i>CACNA1H</i> ^{VAR} (N = 35)	<i>CACNA1H</i> ^{WT} (N = 22)			P value
Serum tryptase , ng/ml median (interquartile range)	15.6 (9.4–31.5)	13.5 (9.6–19.2)			0.12
Manifestation	N (%)	N (%)	OR	RR	
Anaphylaxis ^a	8 (23)	8 (36)	0.5 (0.2–1.6)	0.6 (0.3–1.4)	0.37
Systemic venom reaction^b	2 (6)	6 (27)	0.2 (0.0–0.8)	0.2 (0.1–0.8)	0.04
Flushing/pruritus	12 (34)	6 (27)	1.4 (0.4–4.9)	1.3 (0.6–2.9)	0.77
IBS (Rome III)	11 (31)	5 (23)	1.6 (0.4–4.9)	1.4 (0.6–3.5)	0.56
Chronic gastroesophageal reflux symptoms	18 (51)	14 (64)	0.6 (0.2–1.7)	0.8 (0.5–1.3)	0.42
Congenital skeletal abnormality ^c	11 (31)	3 (14)	2.9 (0.8–11)	2.3 (0.8–7.2)	0.21
Retained primary dentition	4 (11)	7 (32)	0.3 (0.1–1.1)	0.4 (0.1–1.0)	0.09
Hypermobility (Beighton score ≥ 4) ^d	9 (26)	4 (18)	1.6 (0.4–5.1)	1.4 (0.5–4.0)	0.75
COMPASS 31 ^e	27 (11–47) ^f	30 (15–43) ^f	—	—	0.84
Positive tilt-table test	3 (≥ 9)	1 (≥ 5)	—	—	NA
Arthralgia	13 (37)	12 (55)	0.5 (0.2–1.4)	0.7 (0.4–1.2)	0.27
Body pain/headache	13 (37)	12 (55)	0.5 (0.2–1.4)	0.7 (0.4–1.2)	0.27
Sleep disruption	16 (46)	6 (27)	2.2 (0.7–7.7)	1.7 (0.8–3.7)	0.26

IBS, irritable bowel syndrome.

^aSystemic immediate hypersensitivity reaction consistent with immunoglobulin E-mediated reactions to protein antigen or to ^bstinging insect. ^cDefined as the presence of a congenital skeletal malformation or diagnosis of Ehlers–Danlos syndrome. ^dOnly individuals over 12 years of age and who could be directly visualized were assessed and reported. ^eComposite autonomic symptom score, 31 questions (COMPASS 31). ^fValues are median composite scores with the interquartile range provided in parentheses. OR, odds ratios; RR, relative risk; bold indicates statistical significance.

Table 3 Self-reported clinical features among ClinSeq participants carrying *CACNA1H*^{VAR} compared to those wild-type *CACNA1H* sequence (*CACNA1H*^{WT})

	<i>CACNA1H</i> ^{VAR} (N = 13)	<i>CACNA1H</i> ^{WT} (N = 46)			P value
Serum tryptase , ng/ml median (interquartile range)	4.2 (2.5–5.4)	4.3 (3.8–8.6)			0.25
Manifestation	N (%)	N (%)	OR	RR	
Anaphylaxis ^a	1 (8)	4 (9)	0.9 (0.1–6.4)	0.9 (0.1–5.1)	1.00
Systemic venom reaction ^b	0 (0)	0 (0)	—	—	1.00
Flushing/pruritus	1 (8)	8 (17)	0.4 (0.0–2.6)	0.4 (0.1–2.3)	0.67
IBS (Rome III)	1 (8)	2 (4)	1.8 (0.1–17)	1.8 (0.2–12)	0.53
Chronic gastroesophageal reflux symptoms	5 (38)	24 (52)	0.6 (0.2–1.9)	0.7 (0.3–1.4)	0.53
Scoliosis	1 (8)	8 (17)	0.4 (0.0–2.6)	0.4 (0.1–2.3)	0.67
Retained primary dentition	0 (0)	3 (7)	0.0 (0.0–4.1)	0.0 (0.0–4.0)	1.00
COMPASS 31 ^c	15 (8–25) ^d	15 (11–23) ^d	—	—	0.98
Arthralgia	4 (31)	15 (33)	0.9 (0.3–3.2)	0.9 (0.4–2.1)	1.00
Body pain/headache	4 (31)	4 (10)	4.7 (1.1–18)	3.5 (1.1–11)	0.06
Sleep disruption	2 (16)	15 (33)	0.4 (0.1–1.7)	0.5 (0.1–1.5)	0.31

IBS, irritable bowel syndrome; OR, odds ratio; RR, relative risk.

^aSystemic immediate hypersensitivity reaction consistent with immunoglobulin E-mediated reactions to protein antigen—foods ($n = 3$) and environmental allergen immunotherapy ($n = 2$) or ^bstinging insect. ^cComposite autonomic symptom score, 31 questions (COMPASS 31). ^dValues are median composite scores with the interquartile range shown in parentheses. OR, odds ratios; RR, relative risk.

associated with primary hyperaldosteronism.⁵ While these phenotypes were not reported in the small sample we studied, it remains possible that this *CACNA1H*^{VAR} haplotype may have significant impacts on these or other clinical phenotypes.

It also is important to note that in the model system employed to study Ca_v3.2 channel physiology, only wild-type or variant proteins were overexpressed. Thus, homozygosity of the variant allele might be required in vivo to impart a clinical phenotype; we have confirmed only seven such individuals, all in association with increased *TPSAB1* copy number. Of these, four individuals each had two alleles with duplications, and three each had one duplicated and one

triplicated allele, making any comparison between groups anecdotal, and any rational statistical analysis impractical. All individuals homozygous for increased *TPSAB1* copy number ($n = 7$) identified to date have been symptomatic and also homozygous for the *CACNA1H*^{VAR} haplotype.

Two coding nonsynonymous *TPSG1* variants were also present exclusively with the *CACNA1H*^{VAR} haplotype in all 46 families. These variants are frequently encountered as part of this haplotype as well, and may also have a functional effect on mast-cell physiology. γ -Tryptase, encoded by *TPSG1*, has a similar structure and is believed to have a similar proteolytic function to α - and β -tryptases, albeit a more limited one. It is

not known to be secreted, rather transiently expressed on the surfaces of mast cells following degranulation.²⁰ While potential effects of these variants have not yet been investigated in vitro—a considerable challenge in the human system, given the limited data on the physiology of γ -tryptase and the confounding presence of secreted α - and β -tryptases in these same systems—any major contribution to common clinical phenotypes associated with hereditary α -tryptasemia syndrome, if present, would also have been detected in this analysis. Much additional work is required to understand the functional role of wild-type γ -tryptase in human mast cells before functional analysis of these variants can be undertaken.

The remaining four genes excluded from functional analysis were not found to have any variant shared by more than three families within the cohort (**Supplementary Table S1**). Further, tissue expression of these genes is highly restricted: *KCNJ12* has been reported only in skeletal muscle,²¹ *MUC6* and *MUC16* encode mucins restricted to the gastric mucosa²² and ovarian/ocular tissues respectively,^{23,24} and *PRAMEF1* is a cancer/testis antigen.²⁵ Of these, only *KCNJ12* variants might be predicted to result in a phenotype associated with hereditary α -tryptasemia syndrome. *KCNJ12* encodes an inward rectifying potassium channel, mutations in which can lead to periodic paralysis.²¹ However, the rarest *KCNJ12* variant in our cohort was common (ExAC MAF 0.18), making any variant unlikely to be damaging or result in observed clinical phenotypes.

As additional patients with hereditary α -tryptasemia are identified, expanded interrogation of phenotypes among individuals homozygous for the *CACNA1H*^{VAR} haplotype may yet unmask clinical effects of associated alterations in Cav3.2 signaling.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

ACKNOWLEDGMENTS

This study was supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases and of the National Human Genome Research Institute, National Institutes of Health, the NIAID Clinical Genomics Program, and funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. Funding to J.J.L. was also provided in part by ARTrust/The Mastocytosis Society Research Award in Mastocytosis and/or Mast Cell Activation Syndrome. Funding to M.A.C. was provided by the Canadian Institutes of Health Research. L.G.B. and K.L.L. were supported by the Intramural Research Program of the National Human Genome Research Institute, grants HG200359 08 and HG200387 03. The investigators thank the patients, their families, and healthy volunteers who contributed to this research, as well as the clinical staff of the LAD and NHGRI for their contributions.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

AUTHOR CONTRIBUTIONS

J.J.L., S.C.S., M.A.C., and J.D.M. designed the study. J.J.L., J.D.M., C.N., T.D., N.J., all recruited subjects to the study. J.J.L., J.D.M., and J.C., collected and/or analyzed clinical data. X.Y., J.D.H., C.H., Y.Z. A.J.O., J.J.M., L.G.B., and J.D.M. performed and supported genomic sequencing and X.Y., J.D.H., C.H., Y.Z., and A.J.O. performed the bioinformatics analyses. J.C. and Y.L. performed, and J.J.L. designed and performed PCR assays. S.C.S. and M.A.C. designed and performed the functional electrophysiology studies. K.L.L. and L.G.B. facilitated all ClinSeq related study data and H.S. performed LD calculations and analysis. S.C.S., J.J.L., M.A.C., and J.D.M. prepared the draft manuscript. All authors contributed to discussion of the results and to manuscript preparation.

DISCLOSURE

L.G.B. is an uncompensated adviser to Illumina, receives royalties from Genentech and Amgen, and receives honoraria from Wiley-Blackwell. The other authors declare no conflict of interest.

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