Highly alternatively spliced genes may provide complex targets for disease mutations. Structural changes created by missense mutations may differentially affect the activity of alternative gene products, whereas missense, silent and non-coding mutations may alter developmental regulation of splice variant expression. \textit{CACNA1H} is a human gene encoding Ca\textsubscript{v}3.2 low-voltage-activated, T-type calcium channels associated with bursting behavior in neurons and has been linked to more than 30 mutations apparently predisposing to childhood absence epilepsy (CAE) and other idiopathic generalized epilepsies (IGEs). Biophysical properties, including the effects of missense mutations, have been evaluated previously for a single splice form of Ca\textsubscript{v}3.2 expressed in transformed cell lines. We here show that \textit{CACNA1H} is alternatively spliced at 12–14 sites, capable of generating both functional and non-functional transcripts. Variable cytoplasmic and extracellular protein domains point to likely differences in gating behavior, sensitivity to neuromodulation and interactions with extracellular matrix. Biophysical profiles of selected physiological Ca\textsubscript{v}3.2 forms reveal variations in kinetics and steady-state gating parameters, most likely to affect membrane firing. These were comparable to or larger than changes reported for previously studied mutations. Missense CAE and IGE mutations were clustered near segments associated with anomalous splicing. Missense and silent mutations were found to destroy, create or change the regulatory specificity of predicted exonic splicing enhancer sequences that may control splicing regulation. We discuss a paradigm for \textit{CACNA1H} expression of Ca\textsubscript{v}3.2 subunits, which may influence future basic and clinical studies.

INTRODUCTION

Elucidating the relationship of mutations to genetic disease requires explicit knowledge of the paradigm for gene expression. This may be complicated for highly alternatively spliced genes capable of expressing any of many different transcripts in different cells or in the same cell at different stages of development (1–4). We here explore alternative splicing of the calcium channel gene \textit{CACNA1H}.

T-type, low-voltage-activated calcium channels appear to play significant and possibly obligatory roles in idiopathic generalized epilepsies (IGEs) (5,6). Recently, a series of 33 mutations located in the T-channel subtype, \textit{CACNA1H}, have been linked to IGEs, including childhood absence epilepsy (CAE) (7–11). It remains unclear whether these are genuine disease-causing or predisposing mutations or simply rare polymorphisms. Contributing to this uncertainty is the fact that although 16 of the mutations are missense changes that alter channel structure and biophysics (8,9,11), the remainder are synonymous polymorphisms found in the ORF or are mutations in non-coding intron sequences. Current models of Ca\textsubscript{v}3.2 expression do not suggest how these silent mutations may contribute to disease.

Nearly all published reports, and all clinical studies of \textit{CACNA1H}, have focussed on a single form of Ca\textsubscript{v}3.2 subunit. This is perhaps surprising that insofar, as the close paralog, \textit{CACNA1G}, has been shown to express a transcriptome of more than 30 variant Ca\textsubscript{v}3.1 transcripts that are expressed in complex, developmentally regulated fashion during human brain development (12–14). The present studies suggest a similar complexity for \textit{CACNA1H} expression, which will influence the analysis of genetic errors.
IGEs include CAE, juvenile absence epilepsy, juvenile myoclonic epilepsy and myoclonic atatic epilepsy (15). A common aspect of the IGEs is the generation of synchronous 3–6 Hz spike-wave-discharges (SWDs) that spread over both cerebral hemispheres. Recent investigations suggest that seizures are initiated in the neocortex, where CACNA1H is expressed, but rapidly engage the thalamus in patterns of anomalous firing that leads to synchronized discharges and generalized seizure (5,16,17). Many lines of evidence suggest a role for T-type channels. SWDs are sensitive to ethosuximide, an anti-epileptic drug that reduces T-currents in thalamic relay neurons (18–20) and also acts on non-inactivating Na⁺ currents and Ca²⁺-activated K currents (6,21). Transgenic mice lacking one subtype of T-type channel, CACNA1G, are resistant to pharmacological induction of seizures and possess thalamic neurons that lack normal SWD firing patterns (22). Knockout mice lacking the P/Q type channels exhibit spontaneous SWDs: these do not occur when CACNA1G is simultaneously inactivated genetically (23). Quantitative increases in T-currents were observed in the coloboma mouse model for absence epilepsy (24,25) and in the genetic absence epilepsy rat from Strasbourg (GAERS) (26). Subsequently, in situ hybridization studies of GAERS brain tissues detected increased transcript abundance for CACNA1G and CACNA1H genes in thalamocortical neurons and neurons of the reticular nucleus (27).

Three genes encode T-type channels, CACNA1G, CACNA1H and CACNA1I, expressing Caᵥ3.1 (α₁C), Caᵥ3.2 (α₁I) and Caᵥ3.3 (α₁I) subunits, respectively (reviewed in 28,29). All three types are expressed in scores of specialized regions of mammalian brain, including neocortex (30). T-channels activate at negative membrane potentials and inactivate at potentials below those that activate L-type channels: they generally exhibit slow gating kinetics, small single channel conductance, low permeation selectivity for Ba²⁺, block by Ni²⁺ and characteristic sensitivity to non-dihydropyridine drugs, including nebivolol. Although capable of regenerative behavior, they typically mediate long-lasting depolarizing plateaus (‘window currents’) that trigger bursts of action potentials by fast-gating sodium and potassium channels (6). T-channels control a wide diversity of electrical events, including slow oscillations of neuronal firing threshold, low threshold spikes and rhythmic burst firing of thalamic relay neurons, pacemaker firing of cardiac myocytes, bursting responses of neurons to EPSPs, paradoxical rebound burst firing in response to IPSPs, synaptic coupling in dendrites, neurosecretion in exocrine cells, and electrical events associated with fertilization in reproductive cells. In addition to being abundant in the CNS (30), CACNA1G and CACNA1H are found in various peripheral tissues (e.g. liver, pancreas, heart, kidney, adrenal cortical cells and vascular smooth muscle).

Caᵥ3.2 cDNA from CACNA1H has been cloned from human heart (31), carcinoma cell line (32) and rat brain (33) and characterized in respect to tissue distribution (30): Caᵥ3.2 expressed in heterologous cell lines has been extensively analyzed by patch-clamp recording (34–36)

Caᵥ3.2 channels are sensitive to second messenger modulation by inhibitory G-protein subunits and calcium calmodulin-dependent protein kinase IIc (CamKIIc) (37–39). Caᵥ3.2 channels are the targets of both anti-convulsant and anti-hypertensive drugs (36,40). In nearly 30 studies, CACNA1H has been represented by a single Caᵥ3.2 splice variant, 545 in our nomenclature. A second form (513), lacking exon 26, has been described in specialized human reproductive cells (41) and rat brain (33): no functional consequences of Caᵥ3.2 splicing have previously been described.

We here find that CACNA1H is alternatively spliced at 12–14 sites within the ORF, with the potential to generate more than 4000 alternative mRNA sequences. Cloning provides evidence for a family of physiological Caᵥ3.2 transcript variations. The most previously studied variant, 545, comprised less than half of transcripts encountered in fetal human brain. We confirm that eight forms of Caᵥ3.2 subunits expressed canonical T-currents in stably transformed mammalian cell lines. Six forms studied systematically show significant differences in gating parameters that are likely sufficient to affect membrane firing.

Missense mutations that affect gating may act differently in splice forms that, themselves, exhibit intrinsically variable biophysical properties. In the studies of Vitko et al. (11), 11 of 12 CAE missense mutations were shown to change kinetic and steady-state gating parameters of Caᵥ3.2, sufficiently to alter firing patterns in a computational model of thalamic relay neurons. Gating changes were similarly induced by a common polymorphism (R788C) that occurs in non-afflicted controls. A variant that combined R788C with the CAE polymorphism G773D displayed gating behavior different from either SNP alone, highlighting the influence of the peptide background on the functional manifestation of a mutation.

Both missense and silent mutations have the potential to alter splicing regulation (42). We note that many CAE/IGE mutations cluster in or near two domains whose deletions predict truncated reading frames. We discuss three examples of missense or silent mutations that induced little or no change in channel gating, but destroy, create or change the predicted regulatory specificity of strong candidate exonic splicing enhancer (ESE) sequences that could regulate splicing biases at anomalous splice site boundaries.

RESULTS

CACNA1H transcripts are alternatively spliced at multiple sites in the ORF, each possessing viable donor and acceptor splice sequences (43–47). Variable segments include in-frame cytoplasmic and extracellular domains likely to preserve, but modify channel function (17, i23, 25C, 26, 35A and possibly i3A and i3B). Additional splice sites (9B, 14, 25A, i27B, 31A, 31A' and i32B) are less easily summarized. Most lie in membrane spanning domains and predict premature chain termination (PCT). Several of these are conserved between paralogous genes and have characteristics suggesting they may play roles in the context of regulating gene expression: two of these segments, (9B and 31A), are especially associated with CAE- and IGE-linked mutations. Twelve naturally occurring Caᵥ3.2 splice variants, identified in a survey of 53 fetal clones, included six confirmed to
express T-currents when transfected in stable mammalian cell lines. Two control constructs, not yet encountered physiologically, were similarly active. These were evaluated for variations in activation, inactivation and recovery voltage-dependent gating kinetics and window current magnitude and midpoint voltages at 200 and 5000 ms.

**CACNA1H organization**

**CACNA1H** encompasses >70 kb of human chromosome 16p13.3 (cf. GenBank entries AL031712, AL031703 and AL 031715, compared with cloned cDNA channel sequences AF073931 and AF070604). The ORF includes 36 exons, illustrated in Figure 1A, beginning at nucleotide 249 in exon 2 and ending at nucleotide 7310 in exon 36. In this report, exons are in bold face plain text, whereas splice variants are bold face italics. Variants are named according to a binary nomenclature that encodes variable domain content, described in the legend to Table 1.

**Sites of variable splicing**

Variable sites were identified by short amplicon exon scanning (48) (cf. Materials and Methods) using commercial cDNA libraries from adult and fetal human brain and adult human heart. Ca_v3.2 transcripts were 10–40 times more abundant in fetal than in adult human brain by PCR. Systematic exon scanning throughout the Ca_v3.2 ORF in fetal brain revealed multiple variations: these were subsequently detected in other tissues.

The 12 alternative splice sites in the Ca_v3.2 ORF are shown in Figure 1A and B (see Supplementary Material, Table S4 for sequences). Splice variations included commonly recognized patterns of insertion/deletion (44) (e.g. deletion of cassette exons, mutually exclusive cassette exon splicing, use of alternative donor or alternative acceptor sites within exon sequences and retained introns). Two novel splicing patterns were seen, in which new sequences were introduced by upstream frame-shift deletions coupled to concerted downstream intron-segment insertions that returned the normal reading frame.

Variable exons 14, 17 and 26 are canonical cassette exons. Use of alternative donor or acceptor sequences within exons leads to excision of partial exon segments 9B, 25A, 25C and 35A and two partial deletions in exon 31 (A31A and A31A’); of these, segment 25C was not known to comprise coding sequence for **CACNA1H** and, like 25C in Ca_v3.1, constitutes a retained intron. Other nominal intron segments, i23, i27B and i32B, may also be alternatively retained. Retention of segments i3A and i3C, suggested in published sources (TIGR human EST database NP 336223), was not confirmed in fetal human brain but is provisionally represented in Figure 1A. Of the variations detected, only the deletion of exon 26 had been previously observed in human reproductive cells (41); in rat brain, A26 was the only configuration recovered by McRory et al. (33). As illustrated, after the convention of Nidal Ginard et al. (49) (Fig. 1C), re-assembly of 24 alternative configurations at 12 sites generates 4096 combinatorial transcripts. Future confirmation of i3A and i3C splicing could expand this 4-fold.

An ‘explosion diagram’ of all possible Ca_v3.2 protein coding sequences is depicted in Figure 1D. Many variable sites lie in cytoplasmic regions, including the N- and C-terminus and interdomain loops, ID1–2, ID2–3 and ID3–4. One extracellular variation was contiguous with the ‘pedestal’ that bears multiple consensus sequences for N-linked glycosylation, adjacent to the pore-forming P-loop of D3. When
of consensus phosphorylation sites highly conserved in all three T-channel paralogs. This segment contributes half of the ID2–3 mass (14 kDa) and has been shown by Barrett and coworkers (39) to be the domain that mediates neuro-modulation by inhibitory G-protein heterodimers β2γ2 and β2γ4. This segment also contains serine 1198, the site of phosphorylation by CamKIIc, which strongly modulates gating (37,38). Thus, Δ17 variants may lack neuromodulation by both second messenger pathways.

C-terminal segment 35A, encoding 11 amino acids, can be deleted through the use of an alternative internal acceptor sequence. By comparison, CaV.3.1 C-termini exhibit alternative splicing of segments 34, 35 and 38B: 38B exhibits strong interactions with multiple other domains to affect gating. This raises the possibility that 35A might similarly modulate the behavior of CaV.3.2.

Nominally deleterious variants, including Δ25A, Δ31A and Δ31A’, are found in both CACNA1H and CACNA1G. Consensus splicing sequences for exon 31A, including a long, canonical polypyrimidine element, are well conserved. In CaV3.1, 31A deletion is predicted to create a downstream stop codon and PCT. This is likely to elicit nonsense-mediated decay (NMD), consistent with the low observed frequencies of Δ31A forms. These variants, nevertheless, showed strong evidence of developmental regulation, suggesting that they may provide a means of discontinuing CaV.3.1 expression. Here, in CaV.3.2, Δ31A forms were identified (discussed subsequently), in which the predicted downstream PCT was averted by concerted insertion of a small intron segment 31B, resulting in the insertion of a new coding segment in the region D4S5 (Fig. 1D, orange segments).

Splicing of 25A is conserved in both genes: although the specific acceptor splice sites differ slightly in sequence, they lie in the same location and flanking sequences are identical. In CaV.3.1, 25A splicing is in frame and is strongly developmentally regulated. In CaV.3.2, 25A leads to a frame shift. As for Δ31A, Δ25A forms were identified, in which downstream PCT was averted by insertion of a partial intron segment 27B, which returns the normal reading frame, inserting a new sequence in the region D3S6–D3D–4. These forms were detected by short amplicon PCR of library cDNAs; one example of both variations occurred in the survey of longer cDNAs from an independent cDNA library. Δ9B variations were of the order of >10% of PCR products from each of the tissues examined (cf. Fig. 3). Splicing at this site involves conventional U2 boundaries. Segment 9B encompasses 26 amino acids of cytoplasmic ID1–2, beginning at amino acid 462 and ending at 489: this deletion changes the reading frame and is predicted to elicit downstream PCT in exon 10 at amino acid 695. Interestingly, a cryptic in-frame translation initiation site lies at nucleotide 2571 (amino acid 775), 57 nt (19 amino acids) before S1 of D2, resulting in a second ORF encompassing the last homology domains and C-terminus. This splicing pattern, which does not create a chain termination codon immediately 5′ to a normal splice site, may escape the NMD pathways for degrading invalid mRNA (51,52), perhaps contributing to the relative abundance of Δ9B transcripts.

Dolphin and coworkers (53,54) have demonstrated dominant negative suppression by truncated constructs, similar to

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**Table 1. Fetal splice variant distributions**

<table>
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<th>26</th>
<th>i27</th>
<th>31A</th>
<th>31A'</th>
<th>i32B</th>
<th>35A</th>
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<td>53</td>
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</table>

Splice variant inventory: variants were numbered according to a binary nomenclature as previously describe (12). In this convention, each site of variation (v1, v2, ..., vN) is listed in the order in which it occurs, 3′ → 5′, and is assigned a position in a binary number (2^1, 2^2, ..., 2^n).

Each site is given a bit value of 0 or 1 depending on whether it is retained or deleted, according to the following convention: (a) for alternative sites (17, 25C, 26 and 35A): retained, 1; deleted, 0; (b) for intron sequences (3A, 3B, i23, i27B and i32B): retained, 1; deleted, 0; for ‘conservative’ exon segments (14, 25A and 31A/A’): retained, 0; deleted, 1. [Conservative segments are those segments likely to be required (e.g. expected to be constitutive) for channel function, such as membrane spanning helices.]

A clone is thus assigned a binary number such as ...0011001, reflecting variable domain content. This may be evaluated numerically (25 in the example here) for a unique designation. Names and domain compositions may be inter-converted with a table or simple Java program (http://www.spliceprint.com/tools.html). Hexadecimal synonyms may be useful.

Clone names are in bold face italics; exon segments are in bold face. ORF exons are in bold face.

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- Clone names are in bold face italics; exon segments are in bold face. ORF exons are in bold face.
those predicted by $\Delta 9B$ splicing, for several genes including CACNA1H. This suggests that activation of 9B splicing could terminate Ca$_{\alpha 3.2}$ expression and prevent synthesis from normal transcripts in the expressing cell. Mutations influencing $\Delta 9B$ formation could be deleterious (cf. Discussion). There is some possibility that both peptide segments could be synthesized and assemble to form functional bi-molecular $\alpha_1$ subunits (54,55).

**Multiple tissues exhibit Ca$_{\alpha 3.2}$ splice variations**

Ca$_{\alpha 3.2}$ alternative splicing was not limited to fetal brain. Figure 3 illustrates short amplicon PCR of representative tissues in human fetal and adult brain, heart, kidney and liver cDNA libraries. Splicing variants at all identified sites were evident, varying in relative abundance with tissue source, especially noticeable for $\Delta \Delta$, 25C and 26 configurations of ID3–4, 35A and $\Delta 9B$. $\Delta 17$ forms were reproducibly detected at low levels in both adult and fetal brain tissues (data not shown).

**Evidence for naturally occurring splice variants**

To search directly for physiological Ca$_{\alpha 3.2}$ variants, we scored variable sites in fifty-three 3.3 kb Ca$_{\alpha 3.2}$ cDNAs encompassing exons 19–36, inclusive.

Figure 4 illustrates a gallery of 12 splicing patterns detected among 53 Ca$_{\alpha 3.2}$ cDNAs. Relative abundance and splice site compositions are given in Table 1. Of these clones, 30% possessed the 3′-configurations of variant 545: as these clones lack 5′-coding exons 2–18, containing five additional sites of variation, this may overestimate 545 abundance. Of the remaining 70% of transcripts, variants 511, 522, 544 and 577 were each 3.8%, with the remaining forms (640, 672, 688 and 800) represented by a single clone (1.9%). Full-length cDNAs in Figure 4B were constructed from the isolated variants with 545 3′-configurations. Of the 12 variants detected, six were functional when expressed in HEK293/FLP cells with the 545 configuration of exons 2–18 (544, 545, 512, 513, 577 and 800), as described subsequently: synthetic variant 576 was functional, as were constructs lacking exon 17. PCR and preliminary cloning data suggested that variants with 25C replacing 26 are more abundant in adult tissues, a pattern also seen for Ca$_{\alpha 3.1}$ (12).

This inventory of Ca$_{\alpha 3.2}$ variants does not include variations in coding exons 2–18. Full-length cloning of this gene is hindered by the presence of GC-rich regions in the 5′-ORF, which will necessitate stringent methods to overcome: in addition, transcript abundance in other tissues, including adult brain, is one to two orders of magnitude lower than fetal tissues. Our previous survey of nearly 1600 CACNA1G transcripts suggests that marked differences in forms and relative abundance of Ca$_{\alpha 3.1}$ transcripts could occur in different tissues, such as adult and fetal brain. A similar large-scale survey of full-length cDNAs may thus be justified to determine the complete inventory and the regional and developmental specificity of Ca$_{\alpha 3.2}$ variant expression.

**Alternative splicing alters Ca$_{\alpha 3.2}$ biophysics**

As many as 32 in-frame variants of Ca$_{\alpha 3.2}$ could arise from splicing of segments 17, 123, 25C, 26 and 35A. No biophysical studies have been reported on the effects of splicing at any of these sites. To examine a systematic subset of Ca$_{\alpha 3.2}$ variants involving segments 25C, 26 and 35A, analogous to parallel studies of Ca$_{\alpha 3.1}$, we prepared six full-length cDNAs for expression in mammalian cell lines. These were selected...
i.e. the half-points of peak conductance only varied between forms of peak current–voltage relationships, as described in behavior. Activation was evaluated by simple conductance trans-
and possible inserts i3A

![Image](image_url)

**Figure 3.** Splicing patterns by gel electrophoresis: (A) Gel patterns for multiplex short amplicon PCR illustrate alternative configurations of variable sites in 12 types of splice variant detected among 53 fetal brain cDNAs. The ‘bar code’ pattern for each variable domain is specified at the right side. Domain composition and the variant nomenclature is provided in Table 1. (B) Variable splicing of Cav3.2 in multiple tissues: short amplicon patterns are depicted for 9B, 25A, 25C, 26 and 35A in human fetal (F) and adult (A) brain, heart (H), kidney (K) and liver (L) cDNA libraries.

because of their structural parallels with variations of Ca-v3.1 that are prominently regulated during development and that induce diverse changes in Ca-v3.1 gating (12).

These were 545 (canonical Ca-v3.2), 544, 512, 513 and 577. Variant 576, not yet found as a natural form, was also prepared. These were expressed in stable lines of HEK/FLP cells (Materials and Methods); all of these clones expressed currents well in this cell background. Data for configurations 25C, 26 or ΔΔ are often presented in pairs, +/− 35A.

Figure 5A and B illustrates that these variable domains are not obligatory for channel activity and show the properties of variant 512, lacking 25C, 26 and 35A. Segments 17 and 123 and possible inserts i3A and i3B are also not required (data not shown): that is, the core subunit, lacking these cytoplasmic and extracellular domains, exhibits canonical T-channel behavior. Activation was evaluated by simple conductance transforms of peak current–voltage relationships, as described in Materials and Methods. All six variants activated similarly, i.e. the half-points of peak conductance only varied between −39 and −44 mV (Table 2). Activation was not affected by segment 35A. When data were combined on the basis of the exon composition in the ID3–4 linker (25C/26/ΔΔ), there was a small although significant, dependence on the linker (P = 0.01, Student’s t-test). This is evident in Figure 5D.

The functional window supported by each variant was evaluated by measuring functional availability after 200 ms at various conditioning potentials. Typical data for 512 are shown in Figure 5C, and summary data for all of the variants are shown in Figure 5D and E. For Ca-v3.2, activation typically begins near −70 mV, with conductance midpoints between −45 and −48 mV and peak currents at −30 mV. Differences in activation were modest, varying over a 5 mV range, compared with an 11 mV range for Ca-v3.1. The midpoints of availability (200 ms) varied over a 10 mV range and were strongly, linearly correlated with the midpoint for activation (Fig. 5D). As found for Ca-v3.1, variants that gated at the more negative potentials had smaller functional windows than those gating at positive voltages (Fig. 5E). Functional window magnitude (200 ms) varied from a minimum of 22 to >30% and the voltage for peak window currents varied over a 9 mV range. Variants possessing exon 26 fell at the most negative potentials, ΔΔ forms at the most positive potentials and 25C forms in between; this is opposite to the finding with the paralog gene CACNA1G, in which windows for forms expressing 25C were displaced −11 mV from forms lacking inserts, with 26 forms falling in between. This suggests that a broad shift with brain maturation toward Ca-v3.1 forms gating at more negative potentials may not occur for Ca-v3.2 variants.

In contrast to the small, although orderly, differences in midpoints and windows, there were larger differences in the time courses of the variants. Similar to all T-type channel data previously reported, all variants displayed little voltage dependence in the decay of current at positive potentials. Figure 6A illustrates a representative voltage-clamp time course for wild-type variant 545. Currents are relatively slow to develop: at negative potentials, peak currents occurred between 50 and 100 ms, whereas at positive potentials, current peaked at ∼6 ms. Figure 6B and C illustrates voltage dependence of τm and τh for all six variants; the data are well described by Hodgkin–Huxley formalism. The voltage ranges depicted lie between the approximate midpoint for activation (−45 mV) and a potential that elicits maximum conductance (−10 mV). τh approached a constant value at more positive potentials, as expected for a voltage-independent inactivation process. There was no significant difference in time constants between variants that contained and was missing exon segment 35A; therefore, for the determination of the potential roles of segments 25C and 26, data +/− 35A were pooled. Exon 26 produced the fastest currents (544 and 545), both for τh and τm. Conversely, in ΔΔ forms, both τh and τm were slowest (512 and 513). Interestingly, however, when 25C was present, the activation time constant τm was similar to when 26 was present, but current decay, τh, was slow, similar to when neither 25C or 26 was present.

None of the variants inactivated appreciably from closed states over the first 200 ms, i.e. there was virtually no reduction in peak current magnitude when cells were depolarized to voltages negative to those at which currents were

Figure 5A and B illustrates that these variable domains are not obligatory for channel activity and show the properties of variant 512, lacking 25C, 26 and 35A. Segments 17 and 123 and possible inserts i3A and i3B are also not required (data not shown): that is, the core subunit, lacking these cytoplasmic and extracellular domains, exhibits canonical T-channel behavior. Activation was evaluated by simple conductance transforms of peak current–voltage relationships, as described in Materials and Methods. All six variants activated similarly, i.e. the half-points of peak conductance only varied between −39 and −44 mV (Table 2). Activation was not affected by segment 35A. When data were combined on the basis of the exon composition in the ID3–4 linker (25C/26/ΔΔ), there was a small although significant, dependence on the linker (P = 0.01, Student’s t-test). This is evident in Figure 5D.

The functional window supported by each variant was evaluated by measuring functional availability after 200 ms at various conditioning potentials. Typical data for 512 are shown in Figure 5C, and summary data for all of the variants are shown in Figure 5D and E. For Ca-v3.2, activation typically begins near −70 mV, with conductance midpoints between −45 and −48 mV and peak currents at −30 mV. Differences in activation were modest, varying over a 5 mV range, compared with an 11 mV range for Ca-v3.1. The midpoints of availability (200 ms) varied over a 10 mV range and were strongly, linearly correlated with the midpoint for activation (Fig. 5D). As found for Ca-v3.1, variants that gated at the more negative potentials had smaller functional windows than those gating at positive voltages (Fig. 5E). Functional window magnitude (200 ms) varied from a minimum of 22 to >30% and the voltage for peak window currents varied over a 9 mV range. Variants possessing exon 26 fell at the most negative potentials, ΔΔ forms at the most positive potentials and 25C forms in between; this is opposite to the finding with the paralog gene CACNA1G, in which windows for forms expressing 25C were displaced −11 mV from forms lacking inserts, with 26 forms falling in between. This suggests that a broad shift with brain maturation toward Ca-v3.1 forms gating at more negative potentials may not occur for Ca-v3.2 variants.

In contrast to the small, although orderly, differences in midpoints and windows, there were larger differences in the time courses of the variants. Similar to all T-type channel data previously reported, all variants displayed little voltage dependence in the decay of current at positive potentials. Figure 6A illustrates a representative voltage-clamp time course for wild-type variant 545. Currents are relatively slow to develop: at negative potentials, peak currents occurred between 50 and 100 ms, whereas at positive potentials, current peaked at ∼6 ms. Figure 6B and C illustrates voltage dependence of τm and τh for all six variants; the data are well described by Hodgkin–Huxley formalism. The voltage ranges depicted lie between the approximate midpoint for activation (−45 mV) and a potential that elicits maximum conductance (−10 mV). τh approached a constant value at more positive potentials, as expected for a voltage-independent inactivation process. There was no significant difference in time constants between variants that contained and was missing exon segment 35A; therefore, for the determination of the potential roles of segments 25C and 26, data +/− 35A were pooled. Exon 26 produced the fastest currents (544 and 545), both for τh and τm. Conversely, in ΔΔ forms, both τh and τm were slowest (512 and 513). Interestingly, however, when 25C was present, the activation time constant τm was similar to when 26 was present, but current decay, τh, was slow, similar to when neither 25C or 26 was present.

None of the variants inactivated appreciably from closed states over the first 200 ms, i.e. there was virtually no reduction in peak current magnitude when cells were depolarized to voltages negative to those at which currents were
frame. At 5000 ms, the availability midpoint has shifted from −55 to −76 mV, with an increase in slope factor. This most likely results from slow steps of activation at negative potentials that, absent closed state inactivation, delay gating equilibrium. A small ‘true’ window current persists after 5000 ms, (<1−2% of I_{\text{max}}) centered ∼−60 mV.

These properties vary dramatically with splicing, as illustrated in Figure 7B. Variant 512, lacking 25C, 26 and 35A, exhibits a much smaller shift in the midpoint voltage for availability after 5000 ms (from −46 to −58 mV); thus, the availability midpoint for 512 at long times is 18 mV more positive than that for 544 (+26/−35A). A pronounced anomaly in the availability curve, a shoulder of ∼20% of the maximum current, is present, with a midpoint voltage of −100 mV. This profile results in a much larger (6–10-fold) persistent window current (∼12% of I_{\text{max}} at 5000 ms), with midpoint voltage −50 mV, 10 mV more positive than for 544. This rise could also be detected at 200 ms for availability measured between −80 and −110 mV: currents increased ∼10% over this range, small enough not to be detected easily in Boltzmann fits to the 200 ms availability data. It is likely that the window was slightly underestimated as a result. Similar behavior was observed in five cells expressing 512: for other variants lacking 26, it was much less common (Fig. 7).

Figure 5D shows that the 200 ms window was smallest in magnitude, and most negative, when 26 was present, and largest and most positive in ΔΔ forms. Given that there were no statistical differences in conductance between the variants, it must be the case that recovery from inactivation was most rapid in ΔΔ forms and slowest when exons 26 and 35A were present, with other variants likely in between. This trend was generally observed. Figure 7C illustrates the time course of recovery from inactivation by the six tested variants. When segment 26 is present, recovery satisfies a mono-exponential time course with time constant of ∼1 s; when 25C is present, a similar mono-exponential recovery is well fit with a time constant from 0.9 to 1 s. When both segments are deleted (ΔΔ), we found a nominally 3-fold overall faster recovery, following a bi-exponential time course with one time constant (30–40% of current) of 0.1 s and a second (60–70%) varying between 0.6 and 0.7 s. Variations of 35A had minimal impact on these profiles. Interestingly, those variants that recovered from inactivation most rapidly (ΔΔ forms) also had the longest time constants for inactivation from the open state, taken as the time constant of decay at positive potentials. This suggested that 25C and 26 stabilized the inactivated configurations.

**DISCUSSION**

The present studies indicate that CACNA1H is extensively alternatively spliced and generates a family of variant transcripts. The emerging paradigm for CACNA1H expression can provide an informative context for considering disease mutations (7−11).

The number and pattern of CACNA1H alternative splice sites are similar to that previously reported for CACNA1G. A schematic for regulated splice variant expression, adapted from that proposed for CACNA1G (12), may be considered.
Figure 5. Activation and availability: (A) example data from a cell expressing variant 512. Cell was held at −110 mV and stepped for 200 ms to potentials between −80 and +20 mV. Inset shows raw currents, digitally filtered at 2 kHz for cell A4319008. Peak currents are shown in the inset. \(G_{\text{max}}\) for this cell was 21.8 nS with a half-point of conductance at −42 mV and a slope factor of 4.8 mV. (B) Availability determined after 200 ms conditioning at potentials between \(V_{\text{h}}\), −110 and −20 mV. Cells were held for 200 ms and the stepped to 0 mV to determine availability. Cells were allowed to recover at −120 mV for 8 s between conditioning steps. Peak currents were normalized to the maximum current and fit with a Boltzmann function. Half-point of availability for this cell was −51 mV with a slope factor of 4.3 mV. (C) Mean values of grouped data for variant 512 (see Supplementary Material, Table S4 for Boltzmann values). (D) Comparison of midpoint of conductance and availability at 200 ms for all variants (cf. data from Table 2). There was a strong linear relationship between conductance and availability (see text). (E) Functional window. The overlap between conductance and availability is shown for all variants. Window magnitude was larger for variants that activated/inactivated at more positive potentials. The voltage range for gating was more positive than previously reported for Cav3.1 (12). This was a consequence of intracellular solutions used in these experiments, chosen to match those used by Barrett et al. (38). When solutions like those for our Cav3.1 experiments were used, the midpoint of conductance for the parent construct was 5 mV more negative, −49 mV (\(n = 4\)), close to the most positive of the Cav3.1 variants (12).
sequences, which typically reside near or overlap with 5'- and 3'-boundaries of each alternative segment. Several SR factors may act competitively or synergistically to influence the bias toward retaining or skipping the alternative segment (3). To select a particular transcript, the ensemble of variable states must be regulated in concert. In this model, transcription factors may promote the activation of gene transcription and may simultaneously activate a cascade of SR factors that select the variant for a particular cell lineage. Members of the various classes of SR factors may themselves be subject to regulated alternative RNA splicing (3).

In this model, splicing at each site in the precursor mRNA is modulated by trans-acting splicing regulatory factors, typically serine/arginine (SR)-rich proteins that bind to cis-ERE sequences, which typically reside near or overlap with 5'- and 3'-boundaries of each alternative segment. Several SR factors may act competitively or synergistically to influence the bias toward retaining or skipping the alternative segment (3). To select a particular transcript, the ensemble of variable states must be regulated in concert. In this model, transcription factors may promote the activation of gene transcription and may simultaneously activate a cascade of SR factors that select the variant for a particular cell lineage. Members of the various classes of SR factors may themselves be subject to regulated alternative RNA splicing (3).

In this model, missense mutations may alter the function of expressed channels: such effects may be permuted among the various alternative subunits and differentially disturb disparate electrophysiological phenomena (e.g. synaptic coupling, firing threshold oscillations, SWDs and rebound burst firing). Missense, silent and even non-coding mutations that produce little or no direct changes in channel function may nevertheless perturb ESE regulatory sites and thus alter normal patterns of splice variant expression. Both of these phenomena are suggested by the distribution and properties of mutations that have been implicated in CAE and IGE.

First, we here find that Ca,3.2 expression is diversified; only one of these variations has previously been detected. The principal form previously studied, variant 545, comprised a minority of the forms detected among 53 cDNAs from fetal human brain. Moreover, splicing of 25C, 26 and 35A shifted the voltage-domain for gating, the kinetics of activation, inactivation and recovery from inactivation and the magnitude and voltage midpoints for functional window currents (e.g. persistent currents in the 200 ms time frame). The magnitude and voltage-dependence of steady-state window currents (>5 s) were strongly affected: when accompanied by deletion of 35A in the C-terminus, deletion of both 25C and 26 resulted in a 6–12-fold increase in peak steady-state window current and a −12 mV shift in the voltage midpoint. This was accompanied by a relatively enormous +18 mV change in the availability midpoint. Among the six forms described here, we observed a continuum of inactivation and availability midpoints (cf. Fig. 5D). Splicing variations that involve segments 17, 23, 25C, 26 and 35A, as well as possibly 13A, 13B and 9B (discussed subsequently), could generate 32 or more variants, which may differ in regard to gating, localization or sensitivity to neuromodulation. Thus, both differential modification of function by missense mutations and altered patterns of expression of otherwise physiologically variants by mutations of regulatory sequences could underlie pathological manifestations of CAE or IGEs more generally.

Although ion channel mutations unequivocally account for some monogenic diseases, the disease significance of mutations identified in genetic surveys of CAE and IGE patient cohorts remains to be fully established. The distributions and properties of some of the CAE and IGE associated mutations may be considered in relation to the emerging picture of CACNA1H alternative splicing. Thirty-three such mutations have been identified: Chen et al. (7) reported 29 mutations, including 12 missense polymorphisms, among a cohort of 118 Chinese CAE patients; none of these was found in a normal population of 230. Eleven of these were subsequently found to alter gating parameters of human variant 545 sufficiently to change firing patterns in a computational model of thalamic relay neurons (11). In addition, Heron et al. (10) identified four missense mutations in a cohort of 192 patients displaying a variety of IGEs; none of the four mutations occurred in a set of 92 healthy controls. The association with disease, however, was ambiguous: no variant segregated with a single syndrome; in two instances, unaffected siblings were encountered which harbored the mutation; in two cases, siblings without the mutations

Table 2. Splice variant biophysical parameters

<table>
<thead>
<tr>
<th>Splice variant</th>
<th>$G_{\text{max}}$ (nS)</th>
<th>$V_{\text{half}}$ (mV)</th>
<th>$d_{\text{half}}$ (mV)</th>
<th>$d_{\text{ availability}}$ (mV)</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>545</td>
<td>17.8 ± 3.9</td>
<td>−44.0 ± 1.8</td>
<td>−5.4 ± 0.3</td>
<td>−55.5 ± 3.5</td>
<td>4</td>
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<tr>
<td>544</td>
<td>17.7 ± 2.5</td>
<td>−43.4 ± 0.7</td>
<td>−5.0 ± 0.1</td>
<td>−55.6 ± 1.3</td>
<td>10</td>
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<tr>
<td>577</td>
<td>32.0 ± 3.8</td>
<td>−41.9 ± 1.4</td>
<td>−4.9 ± 0.2</td>
<td>−50.7 ± 2.2</td>
<td>9</td>
</tr>
<tr>
<td>576</td>
<td>18.4 ± 2.7</td>
<td>−41.1 ± 1.2</td>
<td>−5.6 ± 0.2</td>
<td>−52.0 ± 1.8</td>
<td>12</td>
</tr>
<tr>
<td>513</td>
<td>22.9 ± 2.1</td>
<td>−38.6 ± 1.4</td>
<td>−5.4 ± 0.1</td>
<td>−46.9 ± 2.1</td>
<td>11</td>
</tr>
<tr>
<td>512</td>
<td>28.2 ± 3.3</td>
<td>−39.9 ± 1.1</td>
<td>−4.9 ± 0.2</td>
<td>−49.5 ± 5.3</td>
<td>13</td>
</tr>
<tr>
<td>545</td>
<td>WinLocmax (mV)</td>
<td>Window size</td>
<td>Half $\Delta$ (mV)</td>
<td>$N$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−50.7 ± 3.1</td>
<td>0.27 ± 0.03</td>
<td>9.7 ± 1.2</td>
<td>4</td>
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</tr>
<tr>
<td>544</td>
<td>−49.6 ± 1.0</td>
<td>0.22 ± 0.01</td>
<td>12.2 ± 0.8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>577</td>
<td>−46.0 ± 2.5</td>
<td>0.27 ± 0.02</td>
<td>10.5 ± 1.5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>576</td>
<td>−46.8 ± 1.4</td>
<td>0.27 ± 0.02</td>
<td>10.7 ± 1.2</td>
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<td></td>
</tr>
<tr>
<td>513</td>
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<td>8.7 ± 1.0</td>
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</tr>
<tr>
<td>512</td>
<td>−44.5 ± 1.2</td>
<td>0.26 ± 0.02</td>
<td>10.4 ± 0.8</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Splice variant biophysical parameters: summary data (mean ± standard error for N cells) of Boltzmann fit parameters and derived data. For conductance, peak currents as a function of potential were fit with $I_{\text{Na}}/(V - V_{\text{rev}}) = G_{\text{max}}/(1 + \exp ((V - V_{\text{half}})/d_{\text{half}}))$, where $G_{\text{max}}$ is the maximum conductance (nS), $V_{\text{half}}$ the half-point and $d_{\text{half}}$ the slope factor of the relationship in mV. To test for availability (fraction of channels available for activation), cells were depolarized from −120 mV to a range of potentials for 200 ms and subsequently depolarized to 0 mV (8 s between trials). Peak $I_{\text{Na}}$ as a fraction of the maximum $I_{\text{Na}}(F)$ was fit according to $F = 1/(1 + \exp ((V - V_{\text{half}} at 200 ms)/d_{\text{half}}))$; $V_{\text{half}} at 200 ms$ is the midpoint of the relationship and $d_{\text{half}}$ the midpoint in mV. For calculation of windows, pairs of measurements of conductance and availability at 200 ms gathered close in time were used; $\text{WinLocmax} = (dx_{\text{half}} + dx_{\text{half}} at 200 ms)/(dx_{\text{half}} - dx_{\text{half}})$ and Window size $= 1/(1 + \exp (\text{WinLocmax} - V_{\text{half}} at 200 ms)/d_{\text{half}})$. $d_{\text{half}}$ is the difference (in mV) between $V_{\text{half}} and V_{\text{half}} at 200 ms for the same cells.
nonetheless exhibited the disorder. The loose association with disease could in part reflect the polygenic nature of IGEs (6,56). Functional analyses of three of the four Heron mutations identified two, in which small, but statistically significant, gain of function changes were identified, also in the background of human variant 545 (9). The fourth mutation produced a frame shift in ID1–2, generating a truncated, non-functional product.

Figure 6. Time course of currents for splice variants: (A) representative voltage-clamp records from wild-type variant 545 of the type fit to the Hodgkin–Huxley formalism (fit = m h). (B) Voltage dependence of variations of activation (τm): +35A forms are depicted as filled symbols and bars; Δ35A, open symbols and bars; Δ35C, circles; ΔΔ (513 and 512), diamonds. Inset shows mean and standard error of data at −10 mV. Star indicates data significantly different at P < 0.05 using the Bonferroni correction for repeated measures. (C) Time constants of current decay (τh). Symbols are the same as in (B). τh divergence at negative potentials near the activation midpoint likely reflects delay due to slow steps of activation; closed resting state inactivation appears to be minimal.

Figure 7. Activation, availability and recovery: (A) voltage-dependent availability for conditioning times of 200 ms and 5 s for variant 544: conductance (open squares); availability at 200 ms (half-solid squares); availability after 5000 ms (solid squares) (n = 5). Recovery between sweeps was 5, 8 and 15 s, respectively. As has been reported for the wild-type 545, with 35A, availability after long time shifts ~20 mV negative. (B) Voltage-dependent availability and conductance for conditioning times of 200 ms and 5 s for variant 512. Symbols are filled as in (A). For this cell, a second conductance determination was made following the determining 5 s availability (solid diamonds). Lines are fits to data by a Boltzmann function up to −85 mV. The anomalous shoulder for 512 (vertical arrow) was seen with forms lacking 26 and could suggest that inactivation is not entirely complete at the lowest voltages tested. Long-lasting window currents at −65 mV reach 10–12% of maximum, ~10-fold greater than for 544 (arrows at left). Similar data were obtained in four cells; level of availability at 290 mV was 0.79 ± 0.01. (C) Time course of recovery at −100 mV from 5 s of inactivation at −10 mV. Inset illustrates protocol. Symbols for variants are as in Figure 4. Note that at this potential, fastest recovery was exhibited by the ΔA variants (t ~ 400 ms) (see text for discussion of recovery kinetics). Data are mean values and standard error of 4–7 cells.
nearly 90\% in the experimental cell lines. It was further observed that D1 truncation forms of Ca\(_{\text{v}2.1}\), Ca\(_{\text{v}2.2}\), Ca\(_{\text{v}2.3}\) and Ca\(_{\text{v}3.2}\) suppressed expression of full-length constructs in a sub-class-specific fashion. This inhibition depended on activation of the endoplasmic reticulum-resident RNA-dependent kinase PERK. Thus, activating 9\(\text{B}\) deletion could silence expression of function by generating a truncated variant and prevent synthesis of active channels from normal transcripts in the expressing cell. The frame-shift mutation identified by Heron (10) (V621fsX654) predicts downstream PCT at amino acid 654, thus mimicking deletion of 9\(\text{B}\) by regulated alternative splicing. It could therefore prevent synthesis of the mutant allele and suppress expression from wild-type alleles in heterozygotic individuals. Because of these twin effects, modest changes in steady-state levels of \(\Delta9\text{B}\) transcripts could lead to significant increases in Ca\(_{\text{v}3.2}\)-mediated T-currents (if 9\(\text{B}\) deletion were diminished) or to decreases (if 9\(\text{B}\) deletion were enhanced).

As discussed by Mount (43), it is not possible to reliably identify physiological splice sites by any strategy other than direct examination of cDNAs. Splicing can involve any number of nucleotides, including 0 (e.g. ‘resplicing’) and both conventional and non-canonical spliced donor or acceptor sites. He concludes that physiological patterns of alternative splicing can only be determined by direct examination of cDNAs. Rapid advances have been made, however, in predictive algorithms for locating candidate ESE sequences (46,47) specific for different classes of SR factors. In at least three instances, CACNA1H mutations were noted in previous studies to elicit minimal or no changes in Ca\(_{\text{v}3.2}\) variant 545 function (C456S, D1463N and A1765A). We examined these mutations in relationship to candidate ESE sequences using the web-based programs RESCUE-ESE (46) and ESE-Finder (47).

Figure 9B indicates that six candidate ESE hexamer consensus sequences are located in the middle of exon 9, within a few base pairs of the 9\(\text{B}\) splice site. As noted by Fairbrother et al. (46), strong ESE activity is often associated with clusters of three to six sites that neighbor or overlap the consensus splice site. Using the alternative criteria of Cartegni et al. (47), we identified three strong SC35 specific sites, displaced slightly 5’ from the clusters of six sites. This program distinguishes between four classes of SR factors. We note that for mutation C456S, a single base change (1455T→A) eliminates one of the overlapping SC35 sites and converts the specificity of the remaining site to SRp55. These changes in ESE specificity are perhaps as likely to be pathogenic as the minimal changes noted for the channel biophysics. According to Vitko et al. (11) and Khrosvani et al. (9), this mutation produced minimal changes in Ca\(_{\text{v}3.2}\) channel gating in the human and rat forms of the 545 variant. If 9\(\text{B}\) splicing is subject to SR factor regulation, perhaps to silence gene expression, mutation C456S, which lies in 9\(\text{A}\), just 5’ to the 9\(\text{B}\) splice site, could cause 9\(\text{B}\) skipping or, alternatively, interfere with normal regulation of 9\(\text{B}\) splicing.

In Figure 9C, we make a similar analysis of the silent mutation A1765A, associated with the 31\(\text{A}\)A splice junctions: this mutation does not change the ORF and thus cannot elicit a direct change in the biophysics of Ca\(_{\text{v}3.2}\). However, this segment is associated with two strong candidate
Figure A: Diagram of a gene with protein interactions indicated.

Figure B: Comparison of wild-type (WT) and mutant (MUT) sequences for Exon 9B.

Figure C: Comparison of wild-type (WT) and mutant (MUT) sequences for Exon 31.

Figure D: Comparison of wild-type (WT) and mutant (MUT) sequences for Exon 23.
ESE sequences for SR factors SF2/ASF and SRp55. The G→A change results in a slight increase in the predicted quantitative strength of the SF2/ASF splice site and introduces a third very strong overlapping site specific for SRp40. These changes could alter the sensitivity of 31A and/or 31A’ splicing biases to expressed patterns of splicing regulatory factors. Insofar as these deletions appear likely to prevent normal channel expression, changes in regulation could result in either loss or gain of function by activating or silencing 31A/31A’ splicing.

In Figure 9D, we illustrate candidate splice sites near the i23/23 splice junction. By the criteria of Fairbrother et al., we identify a cluster of three overlapping hexamer candidate ESE sequences, together with two additional flanking sequences. These are consistent with similar findings by the criteria of Carneci et al., which identify three clusters of overlapping sites of 14 candidate sequences, selective for four SR factors, SF2/ASF, SC35, SRp55 and SRp40. The most complex of these clusters involves eight ESEs of the four types. Such clustering may potentiate competitive or synergistic interactions in regulating splicing at this boundary. The mutation DI463N was reported by Vitko et al. (11) to cause no detectable changes in Ca,3.2 gating parameters in the 545 variant background. As illustrated, the G→A base conversion has several effects on the third ESE cluster. Three ESE sequences are eliminated altogether, and the strength of two remaining SR35 sites are altered. We have encountered cDNAs in which intron 123 is retained, creating an additional extracellular loop with significant homologies to collagen. These ESE sequence alterations at the i23/23 boundary could alter retention of 123 or the constitutive exon 23 itself, depending on SR factors active in the expressing cell.

Heterologous expression of functional splice variants provides a useful screen for altered channel function, as used here. However, transfected cDNAs are not typically subject to RNA splicing. Development of model systems involving mini-gene expression in cell lines with defined SR factor profiles will be required to reveal whether mutations noted here perturb splicing and may thereby provide a mechanism for disease potentiation (42).

Collectively, the observations described here indicate that CACNA1H is an extensively alternatively spliced gene, with 12 or more sites of transcript variation within the ORF.

A preliminary survey indicates that transcript variation is extensive. Eight structural variants, including six found naturally in a survey of fetal brain cDNAs, gave rise to canonical T-type currents in transformed mammalian cell lines. Variations in splicing induce changes in parameters likely to be crucial to T-channel function. Spliced domains include conservative variations that preserve membrane topology and channel function and anomalous variations that likely generate truncated products. Such truncated forms have been encountered in every calcium channel α1 subunit gene so far examined and may play a physiological role in regulating gene expression. Interestingly, one of these anomalous variable domains, 9B, encompasses or lies adjacent to 11 of 16 missense mutations, as well as one silent and one frame-shift polymorphism, reported to predispose for CAE or IGE. The model proposed for CACNA1H expression suggests that function-perturbing missense mutations could generate permutations of changes among variants likely to be expressed in different cells. Both missense and silent polymorphisms could alter the regulation of splicing, perturbing the normal timing and sites of Ca,3.2 splice variant expression.

Taken together, these studies begin to outline a more complex context for the examination of disease mutations for CACNA1H than has been previously considered. Variant proteins could provide differentiated targets for therapeutic agents. Further studies may thus be warranted to identify the full physiological inventory of CACNA1H transcripts and determine the regional specificity of their expression with brain development.

**MATERIALS AND METHODS**

**Exon scanning**

Primer pairs were designed to generate fragments spanning a single exon or a set of tandem exons. Constitutive exons yield a single PCR product, whereas alternatively spliced segments typically produce multiple bands that may be resolved electrophoretically. Products were isolated, subcloned and DNA sequenced. Variants were compared with genomic data to confirm consensus splice donor and acceptor sequence (44,45,58). Primer sequences are presented in Supplementary Material, Tables S2 and S3. Clonetech (Palo Alto, CA,
USA) provided the human fetal brain, adult brain and adult heart Marathon-Ready™ cDNA libraries (Clontech lot nos 639300, 639302, and 639304). All primers were designed with the OLiGO Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO, USA) on the basis of Ca,3.2 cDNA sequence (NM_021098) from NCBI GenBank, each pair of primers amplifying one or sets of tandem exons (cf. Supplementary Material, Table S1). Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The thermal profile for short amplification was as follows: initial denaturation 94 °C for 1.5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and ending with a final extension at 72 °C for 2 min. Each thin-walled 200 μl tube contained 1 μl of cDNA in a total volume of 25 μl with 200 μM dNTPs, 400 μM each sense and antisense primers, 1× PCR buffer and 1 U of hotstart Taq Polymerase (Qiagen Hotstart kit). PCR product was run on 2–4% agarose gel. Multiple bands were subject to sequencing with BigDye terminators (Applied Biosystems, Foster City, CA, USA) at the local Biosynthesis and Sequencing Facility.

**Ca,3.2 library construction**

ORF sequences containing exon 19 to the 3′-UTR of CACNA1H message were amplified from human Marathon-Ready fetal brain cDNA library (Clontech) with Elongase amplification system (Life Technologies). Primers were designed: anti-sense primer: 5′-GCCTACAGGCCCACTGTG GT-3′; sense primer: 5′-CAGGTCATGCAGGTCTTC-3′. Wax beads (AmpliWax PCR Gem 100, Perkin–Elmer Applied Biosystems) were employed, following the manufacturer instructions, to perform a hot start to increase the specificity of the reaction. The reaction volume was 50 μl, containing 2 mM magnesium (10× Elongase buffer), 200 μM each dNTP (Life Technologies), 200 nM each primer, 2 μl cDNA and 1.4 μM Elongase enzyme mix (1 U/ml). The thermal program was: 94.5 °C for 45 s, 94.5 °C for 30 s/65 °C for 30 s/68 °C for 4 min for 24 cycles and a final extension at 68 °C for 6 min. Ten microliter of each PCR product was run on an agarose gel adjacent to a DNA ladder to assess specificity and to verify that the product concentration did not exceed a 100 pM in order to keep template switching below a desired level (59). The ~3.4 kb PCR product was excised after electrophoresis on 0.8% agarose, purified from the gel slice on a Qiaquick column (Qiagen) and cloned into the gel slice on a Qiaquick column (Qiagen) and cloned into plasmid vector (Invitro-gene). White colonies were picked into 200 μl pCR-XL-TOPO with the TOPO-XL PCR cloning kit (Invitrogen). The lysates, for PCR screening reactions, were store frozen at −80 °C in LB with 20% glycerol.

**Splicing variant screening**

Alternative exon combinations for full-length clones were detected by short-amplicon PCR screening with primer pairs surrounding sites of alternative splicing. The reaction volume was 25 μl, containing 5 μl of bacterial lysate as template and other reaction components as for exon scanning earlier (for primer pairs, see Supplementary Material, Table S2). Splice variants were identified by electrophoresis of PCR products on 4% agarose gel after band validation by sequencing. Clones with Δ31A and Δ31A’ required direct sequencing.

**Splicing in different tissues**

Tissue comparisons of splice site configurations were made by amplification of Ca,3.2 from Clontech Marathon-Ready human fetal brain, adult brain, heart, kidney and liver cDNA libraries (nos 639302, 639300, 639304, 639305 and 639303) using the primer pairs as Supplementary Material, Table S1. Amplification conditions were the same as earlier. PCR products were resolved on 1 or 4% agarose gel.

**Electrophysiology**

Cav3.2 splice variant cDNAs were subcloned into pcDNA5/pFRT/Zeocin, and stable cell lines in HEK293/FLP cells were created (Invitrogen, Carlsbad, CA, USA) for each of the variants. Cell lines were maintained with Dulbecco’s eagle medium (GibcoInvitrogen) in 100 mM Corning culture dishes. Aliquots were lysed for 20 min at 100 °C in 96-well tissue culture dishes. Ten microliter of each PCR product was used 48 h and cells were passaged weekly. Whole-cell voltage clamp was performed on trypsinized cells (0.25% Trypsin–EDTA, Invitrogen) 3–6 days after plating. Whole-cell voltage clamp recordings were made using glass pipettes made from alumina silicate capillary tube (Fisherbrand) pulled to resistances of 1.0–2.0 MΩ with a Flaming/Brown micropipette puller (Model 97, Sutter Instruments, Novato, CA, USA). The pipette solution (24) contained (in mM) 115 CsCl, 1 tetrabutylammonium chloride, 1 MgCl₂, 5 Mg-ATP, 1 Li-GTP, 20 HEPES, pH 7.2 (adjusted with CsOH), and 11 BAPTA, with 0.9 mM CaCl₂ to set the free Ca²⁺ at 27 mM. HEK293 cells were superfused with a solution containing the following (in mM): 127 TEA-Cl, 2 CaCl₂, 0.5 MgCl₂, 10 HEPES, 5 n-glucose and 32 sucrose, pH 7.4 (adjusted with CsOH). Recordings were made at room temperature (22–24 °C) using pCLAMP8 and an Axopatch 1D amplifier with a DigiData 1322A Series Interface digital-to-analog converter. The recording chambers were held at −110 mV, and for current–voltage relationship, determinations stepped to various potentials for 200 ms each 5 s. Steady-state inactivation protocols used 200 ms prepulse steps and a 0 mV test step, with 8 s between trials at a holding potential of −120 or −110 mV. Data were low-pass filtered at 5 kHz and sampled at 20 kHz during recording and digitally filtered at 2 kHz during analysis. Specifics of other protocols are given in Results.
activation slope factors steeper than 4 mV, or with $G_{\text{max}} < 5$ nS were excluded from analysis. Conductance and availability curves were constructed from normalized data, which were then fit using a Boltzmann equation, i.e. fraction $= 1/(1 + \exp(V_{1/2} - V_\text{r}(t))dx)$, in order to estimate $V_{1/2}$, the midpoint of the relationship, and dx, the slope factor, i.e. the number of millivolts over which the relationship changed $e$-fold. Hodgkin–Huxley formalism was used to estimate the time constants of activation ($\tau_\text{a}$) and inactivation ($\tau_\text{i}$) ($I = m^3h$) from capacity corrected data, digitally filtered at 2 kHz excluding the first millisecond after the step to avoid filtering artifacts. Statistical tests for differences used $t$-tests carried out in Origin or with, when appropriate, Boneferroni correction to minimize false positives from repeated measures in SAS (Cary, NC, USA). All data are reported as mean ± SEM.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** The authors declare that they have no conflicts of interest or competing financial interests.

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