Alternative Splicing at C-Terminus of Ca\(\text{v}\)1.4 Calcium Channel Modulates Calcium-Dependent Inactivation, Activation Potential and Current Density*

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*Running title: CDI, activation and current density: Ca\(\text{v}\)1.4 splice variants.

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Background: Alternative splicing diversifies calcium channel structure to change channel properties.

Results: Extensive C-terminal alternative splicing generates channels differing in activation potential and voltage and calcium-dependent inactivation properties.

Conclusion: Diversification of channel function through altered structure is fine-tuned by alternative splicing.

Significance: Ca\(\text{v}\)1.4 C-terminal splice variations recapitulate some aspects of native photoreceptor calcium currents.

ABSTRACT

The Ca\(\text{v}\)1.4 voltage-gated calcium channel is predominantly expressed in the retina and mutations to this channel have been associated with human congenital stationary night blindness type-2 (CSNB2). The L-type Ca\(\text{v}\)1.4 channel displays distinct properties such as absence of calcium-dependent inactivation (CDI) and slow voltage-dependent inactivation (VDI) due to the presence of an autoinhibitory domain (ICDI) in the distal C-terminus (C-ter).

We hypothesized that native Ca\(\text{v}\)1.4 is subjected to extensive alternative splicing, much like the other voltage-gated calcium channels, and employed the transcript scanning method to identify alternatively spliced exons within the Ca\(\text{v}\)1.4 transcripts isolated from the human retina. In total, we identified 19 alternatively splice variations, of which 16 variations have not been previously reported. Characterization of C-ter alternatively spliced exons using whole-cell patch-clamp electrophysiology revealed a splice variant that exhibit robust CDI. This splice variant arose from the splicing of a novel alternate exon (43\(^*\)) that can be found in 13.6\% of full-length transcripts screened. Inclusion of exon 43\(^*\) inserts a stop codon that truncates half the C-ter. The Ca\(\text{v}\)1.4 43\(^*\) channel exhibited robust CDI, a larger current density, a hyperpolarized shift in activation potential by ~10mV as well as slower VDI. Through deletional experiments we showed that the ICDI was responsible for modulating channel activation and VDI, in addition to CDI.

Calcium currents in the photoreceptors were observed to exhibit CDI and are more negatively activated as compared to currents elicited from heterologously expressed full-length Ca\(\text{v}\)1.4. Naturally occurring alternative splice variants may in part contribute to the properties of the native Ca\(\text{v}\)1.4 channels.
CaV1.4 is a member of the L-type family of voltage-gated calcium channels (LTCC) that are predominantly expressed in the rod photoreceptor and retina bipolar synapses. Mutations in the \textit{CACNA1F} gene encoding CaV1.4 channels have been associated with congenital stationary night blindness type-2 (CSNB2), a condition characterized by various visual impairments in addition to night blindness (1-2). Electroretinography of CNSB2 patients suggest that CaV1.4 mediate neurotransmitter release at the synapse (3).

The electrophysiological properties of heterologously expressed CaV1.4 included a slow voltage-dependent inactivation (VDI) and a unique absence of calcium-dependent inactivation (CDI) (4-6). CDI is a property well documented in the other LTCCs: CaV1.2, CaV1.3; and, recently, CaV1.1 channels (7). In the CaV1.4 pore-forming \(\alpha1\)-subunit an autoinhibitory domain (ICDI) was recently identified, that resides in the distal end of the C-terminus (C-ter), to profoundly blunts CDI by interacting with the calcium-sensing and inactivation-coupling machinery in the proximal C-ter (8-11). The electrophysiological properties of CaV1.4 channels are supportive of its physiological role as glutamate release at the photoreceptor synapse occurs in a sustained and graded manner. However, the electrophysiological properties characterized using heterologously expressed CaV1.4 channels differed from the properties of the native \(Ca^{2+}\) currents measured at the rod photoreceptors and bipolar cells (12-14). The heterologously expressed channels activated at membrane potentials that were \~30mV more positive compared to the rod \(Ca^{2+}\) currents. In addition, \(Ca^{2+}\) currents measured in rod photoreceptor and bipolar synapses do exhibit CDI (15-17). These discrepancies suggest that the endogenous CaV1.4 channels could be modulated to activate at more hyperpolarized potentials as well as exhibit CDI. This may occur by mechanisms including cytoplasmic \(Ca^{2+}\) protein buffers or posttranscriptional modification such as alternative splicing. Interaction with a calcium binding protein CaBP4 was shown to negatively shift the voltage of activation by 10 mV (18). Additional shifts in activation potential and modulations to other electrophysiological properties may be brought about by other mechanisms. Indeed, in other LTCCs like CaV1.2 and CaV1.3, alternative splicing has been found to modulate their biophysical and pharmacological characteristics (19-21). Alternative splicing in voltage-gated calcium channels is extensive throughout the length of calcium channel coding region. In CaV1.2, at least twelve exon loci in the gene are alternatively spliced with up to twelve different variations of splicing occurring in one locus (compiled in 22). Other examples include seven alternatively spliced loci in CaV2.1 and six alternatively spliced loci in CaV3.1 (23-24).

Here, we systematically screened for alternatively spliced exons in CaV1.4 transcripts from the human retina using the “transcript-scanning method” (23-24,26) and showed that CaV1.4 is extensively alternatively spliced. We characterized the alternatively spliced exons in the C-terminal region and uncovered a population of splice variant that can activate within the photoreceptor operating potentials as well as exhibit CDI. Other electrophysiological properties that were altered include voltage-dependent inactivation (VDI) and current density.

**EXPERIMENTAL PROCEDURE**

\textit{Reference Sequences} – The genomic sequence for CaV1.4 was from GenBank Accession Number AJ006216. The reference CaV1.4 cDNA sequence was from GenBank Accession Number AF201304. This sequence was considered as wild-type, WT, in this work. CaV1.4 exon positions were determined by aligning the cDNA sequence against the genomic sequence (using the MegAlign module of the Lasergene® software suite, DNASTAR, WI, USA), and the exons were numbered in order from 1 to 48. The reference amino acid sequence used was from GenBank Accession Number NM000719.

\textit{Nomenclature For Describing Alternatively Spliced Exon Variant} – Various suffixes and a prefix applied to an exon number are used to describe the type of alternative splicing that occurred at that exon locus (Supplementary Figure 6). ‘∆’ denotes a cassette exon that was skipped in
the course of alternative splicing. ‘a’ or ‘d’ denote the changes to exon length at the acceptor or donor site, respectively. ‘+’ or ‘-’ indicate if the exon is lengthened or shortened, respectively. ‘i’ suffix denote retained intron and ‘x’ denote mutually exclusive exons. ‘*’ denotes a novel exon (not found in public database).

**Transcript-Scanning Method** – To determine the different alternatively spliced exons in the Ca\(_{V}1.4\) gene, we employ the transcript strategy previously described by S. Mittman (24,26) and Soong et al (23). In this method, we first designed PCR primer-pairs that span at least two exons or four splice boundaries along the length of the Ca\(_{V}1.4\) gene (Supplementary Figure 1). Sufficient pairs of primers were made such that amplicons form overlapping segments along the entire Ca\(_{V}1.4\) sequence. The primers, designed using Oligo Primer Analysis Software (Molecular Biology Insights), are given in Supplementary Table 7.

PCR was performed using human retina Marathon®-Ready cDNA (Clontech; Cat. No. 7449-1, Lot Nos. 0120657 and 3060598) as template and Taq DNA polymerase (Promega). Each pair of primer produced amplicons of varying sizes, corresponding to different alternative splice variants (or non-specific products), visualised as multiple bands on an agarose gel (Supplementary Figure 1). Each band was extracted and ligated into pGEM®-T Easy vector and transformed into DH10B E.coli. For every band cloned, eight to thirty positive transformants (indicated by blue/white colony selection) were picked and further PCR screened using specific primers. Colonies yielding different sized PCR products were expanded and the plasmid DNAs extracted for DNA sequencing.

The DNA sequences were analysed, by comparison with the Ca\(_{V}1.4\) genomic and cDNA sequences, to identify the type of alternative splicing that had occurred and to determine the exact location of the alternative exon-intron splice junctions as well as their adherence to the “gt...ag” rule. In order to transcript scan exon 1, we made use of the Marathon® adaptors that were ligated to the ends of Marathon®-ready cDNAs. The adaptor primer was provided by the manufacturer and this was paired with a reverse primer residing in exon 2 or exon 3. To scan exon 48, we used a forward primer from exon 47 or 46 paired with an oligo dT that annealed to the poly-A tail. As one primer in each pair was non-specific (i.e. adaptor primer, oligo dT), the PCR reactions yielded diffused and multiple bands when separated on agarose gel. These were extracted, cloned and rigorously screened in the same manner as described above.

**Construction of Cloned Full-Length Ca\(_{V}1.4\) Library** – Primers that reside in the 5’ and 3’ UTR (un-translated region) of the Ca\(_{V}1.4\) gene were employed for long PCR amplification using human retina cDNA as template and the Elongase® Enzyme Mix (Invitrogen), a polymerase cocktail having proof-reading function and capable of long PCR. The resultant ~6 kb amplicons were sub-cloned into pCR®-XL-TOPO® vector (Invitrogen) and transformed into DH10B bacteria. Positive transformants were then picked and grown in 96-well plates. Random clones were picked and verified by restriction digest profiling as well as DNA sequencing.

**Screening Full-Length Library to Determine Abundance Of Splice Variants** – Pairs of primers were selected that flank exons shown by transcript scanning to have alternatively spliced variants. The clones in the full-length library were then screened using each selected pair of primers by PCR. The clones that produced PCR products and migrated with the expected sizes (WT or alternative splicing) on the agarose gel were counted. Clones were picked at random for sequencing to verify the validity of the screen. The quantity of alternatively spliced exon variants were expressed as a percentage of the total number of clones counted (Supplementary Figure 3).

**Generation of Expression Constructs for Electrophysiology Characterisation** – Ch-WT is a chimera consisting of a Ca\(_{V}1.2\) backbone and the entire C-terminus from Ca\(_{V}1.4\) cloned in a mammalian expression vector. The Ca\(_{V}1.2\) fragment was excised using HindIII and BclI restriction sites from the α1C77WT clone (kindly provided by Dr Roger D. Zühlke, University of Bern, Switzerland). The Ca\(_{V}1.4\) C-ter fragment was PCR-amplified from the human Ca\(_{V}1.4\)-pCDNA3.1 clone (kindly given by Dr J. E. McRory, University of British Columbia, Canada),
with the addition of a NotI site after the stop codon. The PCR also introduced a silent mutation to remove the second of the two BclI sites. The CaV1.2 fragment was ligated to CaV1.4 at the remaining BclI site; within a region of sequence identity in the IVS6 domain. The final chimeric construct was assembled in the pCDNA3 vector (Invitrogen) between HindIII and NotI sites.

Subsequent constructs containing the CaV1.4 C-ter alternatively spliced exons were generated by appending the C-ter of Ch-WT. DNA fragments of CaV1.4 C-ter that contained either Δ37, 42d+, 43* or 45a- splicing were obtained by PCR or restriction digest from CaV1.4 clones in the full-length library. These were substituted into Ch-WT using various restriction sites: Δ37 – NsiI/NotI, 42d+ – SbfI/SbfI, 43* and 45a– – BamHI/XhoI.

For the deletion constructs, different fragments of CaV1.4 C-ter were first PCR amplified with the insertion of stop codon to terminate at H1718, R1755, Q1835 and L1878 for the clones Ch-e43, Ch-e44, Ch-e45, Ch-e46 and Ch-ΔICDI respectively. These were substituted into Ch-WT using BamHI and XbaI sites.

The ICDI-mCherry construct consists of the ICDI domain of CaV1.4 fused in-frame to a red fluorescent protein, mCherry. ICDI was PCR-amplified from CaV1.4-pcDNA3.1 template with the addition of kozak sequence and start codon. An XbaI restriction site replaced the endogenous stop codon. The mCherry fragment was amplified from the pRSET-B mCherry clone (gift from Dr Roger Y. Tsien, University of California, San Diego, USA) with the addition of a kozak sequence and start codon. The mCherry fragment was amplified with the insertion of a stop codon to terminate at H1718, R1755, A1797, Q1835 and L1878 for the clones Ch-e43, Ch-e44, Ch-e45, Ch-e46 and Ch-ΔICDI respectively. These were substituted into Ch-WT using BamHI and XbaI sites.

The integrity of all constructs was validated by DNA sequencing.

**Transient expression of calcium channels in HEK-293 cells** – HEK-293 cells were co-transfected with 1.75 μg of α (Ch-WT or variants), 1.25 μg each of β2a and α2δ-subunits (kind gifts of Dr Terry P. Snutch, University of British Columbia, Canada) and 0.2 μg of T-antigen plasmids using the calcium phosphate method. The cells were then incubated for at least 36 hrs in a water-saturated 5% CO2 incubator at 37°C before whole-cell patch clamp recordings were made.

**Whole-cell patch-clamp electrophysiology** – Whole-cell patch clamp recordings were performed on transfected cells between 36-72 hrs after transfection. The external bath solution was comprised of (in mM): 10 HEPES, 140 TEAMS, 5 BaCl2, or CaCl2, pH 7.4, 300-310 mOsm (adjusted with glucose). The pipette internal solution contained (in mM): 138 Cs-MeSO3, 5 CsCl, 0.5 EGTA, 10 HEPES, 1 MgCl2, 2 mg/ml Mg-ATP, pH 7.3, 290-300 mOsm (adjusted with glucose). The junction potential was determined to be -11 mV and the voltages reported here are uncorrected for junction potential. True voltages may be calculated by subtracting 11 mV from the reported values. Whole-cell currents were obtained under voltage-clamp with either the Axopatch 200A or Axopatch 200B amplifier, operated using pClamp software (Molecular Devices). The signals were filtered at 1-5 kHz and sampled at 5-50 kHz. Series resistance was typically 1.2-1.8 MΩ after 80% compensation. A P/4 protocol was applied on-line to subtract leak and capacitive transients.

To assess the current-voltage (I-V) relationship of the channels, transfected cells were depolarised to a family of test potentials of -60 to 60 mV, in steps of 10 mV increments, from a holding potential of -90 mV. The peak current evoked by each voltage was normalised to the maximal current obtained for each cell recorded and fitted with the following equation:

\[ I = G_{max}(V - E_{rev})/(1+\exp[V - V_{1/2}\ act]/k_{act}) \]

where \( G_{max} \) is the maximum conductance, \( E_{rev} \) is the reversible potential, \( V_{1/2}\ act \) is the half-activation potential, and \( k_{act} \) is the slope.

For steady-state activation, we analysed the tail currents (\( G \)) obtained at the end of a short depolarising pulses. Cells were held at -90 mV before depolarising to a family of voltages ranging from -60 to 100 mV, in steps of 10 mV
increments, for 20 ms. Following which, a repolarisation to -50 mV for 10 ms evokes the tail currents that are measured. The peak of each tail current was normalised to the maximum obtained for each cell recording and fitted with a dual Boltzmann equation: 

\[ G/G_{\text{max}} = F_{\text{low}}/(1+\exp((V_{1/2, \text{low}}-V)/k_{\text{low}})) \] + \[ (1-F_{\text{low}})/(1+\exp((V_{1/2, \text{high}}-V)/k_{\text{high}})) \]; where \( G \) is the tail current and \( G_{\text{max}} \) is the maximum tail current, \( F_{\text{low}} \) is the fraction of low threshold component, \( V_{1/2, \text{low}} \), \( k_{\text{low}} \), and \( k_{\text{high}} \) are the half-activation potentials and slope factors for the low and high threshold components, respectively, and \( V_{1/2, \text{act}} \) may be calculated when 

\[ G=0.5G_{\text{max}} \].

Steady-state inactivation properties were determined by comparing the test current obtained after a long depolarising pulse to an initial pre-pulse. For the -90 mV holding potential, a pre-pulse current was evoked by stepping to 10 mV for 30 ms. This was followed by a family of 15 s-long depolarising pulses ranging from -120 to 20 mV. Next, a test-pulse current was evoked by stepping the cell to 10 mV for 100 ms. The peak current evoked by each test pulse was divided by the peak current evoked by the pre-pulse to obtain the normalised current. The values were fitted with a single Boltzmann equation: 

\[ I_{\text{relative}} = I_{\text{min}} + (I_{\text{max}}-I_{\text{min}})/(1+\exp(V_{1/2, \text{inact}}-V)/k_{\text{inact}}) \]; where \( I_{\text{relative}} \) is the normalised current; \( V_{1/2, \text{inact}} \) is the potential for half-inactivation, and \( k_{\text{inact}} \) is the slope value.

The strength of calcium dependent inactivation (CDI) exhibited by the channel is expressed by the \( f \)-value (28). To determine the \( f \)-value, first, the current amplitude that remained at a given time point (i.e. 30 ms, 50 ms, 100 ms, 200 ms and 300 ms) after depolarisation was measured and normalised against the peak current to obtain the residual current (i.e. \( r_{30} \) and \( r_{300} \) for 30 and 300 ms, respectively). Next, the \( f \)-value was calculated by subtracting the residual \( I_{\text{Ba}} \) with the residual \( I_{\text{Cu}} \). An \( f \)-value of 0 indicates no CDI while the maximum \( f \)-value of 1 indicates complete CDI. For voltage-dependent inactivation (VDI), cells were placed in the external solution with \( \text{Ba}^{2+} \) as the charge carrier and depolarised to \( V_{\text{max}} \) from a holding potential of -90 mV. As time elapsed, the current evoked by \( V_{\text{max}} \) decayed and the amplitude was measured at different time points. These were subtracted from the peak current evoked to obtain the inactivated current. This was then expressed as a percentage of the peak current (percentage inactivation).

Fractional recovery from inactivation was determined using a two-pulse protocol whereby following a 2-sec depolarising pre-pulse to \( V_{\text{max}} \) (determined from the I-V properties of the transfected cell), a test-pulse is applied after a certain time period, \( \Delta T \), has elapsed. The peak \( I_{\text{Ba}} \) measured during the test pulse was expressed as a fraction of peak \( I_{\text{Ba}} \) obtained at the beginning of the pre-pulse to obtain the fraction of recovery. This was plotted against \( \Delta T \). The curve was obtained by fitting the values with a double exponential equation: 

\[ Y = Y_{\text{min}} + A_1[1-\exp(-t/\tau_1)] + A_2[1-\exp(-t/\tau_2)] \]; where \( Y \) is the fraction of recovery, \( A_1 \) and \( A_2 \) are the maximum values of the fast and slow component, and \( \tau_1 \) and \( \tau_2 \) are their time constants, respectively.

**Data and statistical analyses** – All values presented are mean ± SEM (standard error of mean). Electrophysiology data were analysed using Clampfit software (Molecular Devices). Data values were exported to Microsoft® Office Excel® software (Microsoft Corporation) for calculations. Data plotting, curve fitting and statistical analyses were performed using GraphPad Prism® software (GraphPad Software, Inc.). Statistical significance of the difference between means was determined using Student’s t test or one-way ANOVA. Statistical significance was accepted at \( p < 0.05 \).

**RESULTS**

**Transcript-scanning of \( \text{Ca}_v1.4 \)** from human retinal cDNAs – To determine the different alternatively spliced exons in the \( \text{Ca}_v1.4 \) gene, we employed the transcript scanning strategy that has been previously reported (23-24,26). As the \( \text{Ca}_v1.4 \) transcripts are predominantly expressed in the retina (1-2,4), we used human retina cDNA in PCR reactions to amplify across all the splice
junctons of the Cav1.4 gene; and we generated at least fifteen overlapping PCR amplicons (Supplementary Figure 1). Bacterial colonies obtained from plasmids subcloned with each of the 15 PCR amplicons were screened and the individual clones harboring different size PCR products were subject to automated DNA sequencing (Supplementary Figure 2). The identity and mechanism of the alternative splicing for each splice locus was identified by inspection of the exon-intron boundaries and determined by analysis of the DNA sequences in comparison with the Cav1.4 genomic sequence (Figure 2; GenBank accession number AJ006216).

Transcript-scanning of Cav1.4 from human retina cDNA revealed that 16 exons are alternatively spliced, of which some may undergo more than one mode of alternative splicing with some adjacent exons alternatively spliced in tandem (Figure 2); resulting in 19 different exon variants. These include cassette exons that can be fully excluded by alternative splicing, alternate use of splice donor/acceptor junctions that resulted in lengthening or shortening of the exons and mutually exclusive exons. Two of the cassette exons are novel and have not been predicted in the public database. In all cases the canonical “gt...ag” splice junctions were preserved at the intron-exon junctions of the alternative splice sites (P. A. Sharp and C. B. Burge, 1997). Not all alternative splicing were productive as some resulted in frame-shifts that gave rise to pre-mature stop codons. The relative positions of the alternatively spliced loci along the entire length of the Cav1.4 open-reading frame are illustrated in Figure 1. Alternative splicing at the C-ter produced 4 splice variants (Δ37, 42d+, 43* and 45a-) that contained different lengths of the c-tail. The Δ37 splice variant is truncated of the entire C-ter while in contrast the use of alternate donor site in 42d+ adds an additional 13 a.a. to the region downstream of the IQ-motif. The inclusion of the novel alternative exon 43* adds a novel 19 a.a. peptide past exon 42 but contains a stop codon that resulted in premature termination of the C-ter with a truncation of the last 257 a.a. On the other hand, 21 a.a. are removed in the 45a- variant by the use of an alternative acceptor site with no frameshift of downstream codons. This occurs in a region of the C-ter that has poor conservation among LTCCs (Supplementary Figure 4). Notably, the splice variants 16a-Δ17a+, Δ16&17a+ and Δ17&18a, are pairs of adjacent alternatively spliced exons that when spliced in tandem gave rise to transcripts coding for functional Cav1.4 channels. Here, alternative splicing of the first exon generated a frame-shift that was corrected by the alternative splicing in the second exon. Had alternative splicing occurred independently in one locus frameshift would result in a premature, stop codon. This mode of splicing is interesting and unusual because it manages to alter the gross peptide sequence without relying on a different set of nucleotide sequences.

We also transcript-scanned the bracketing exons (i.e. exon 1 and exon 48) by using Marathon®-ready human retinal cDNA (Clontech; 29) as template in PCR reactions that contained a primer that targets the sequence in the Marathon® adapter and a Cav1.4-specific primer. Based on the results from the PCR screen, coupled with DNA sequencing of clones, we found no additional alternate exons to exon 1 or exon 48.

Abundance of each alternative splice locus within Cav1.4 transcripts – Proteomic variations generated by alternative splicing may alter the electrophysiological properties of Cav1.4 channels. The relative abundance of each Cav1.4 splice variant would no doubt influence the overall property of the native Cav1.4 currents. To determine the levels of occurrence of the splice variants, we first PCR amplified and cloned full-length Cav1.4 cDNAs from human retina to obtain a pool of full-length Cav1.4 clones. Using primer-pairs that target alternatively spliced loci we characterized the full-length Cav1.4 clones by PCR-screening to identify the combinatorial splicing pattern of each clone. Clones producing PCR product size alterations that tallied with the expected changes were counted and expressed as a percentage of the total population (Figure 2, left column). Random clones were selected for DNA sequencing to validate the PCR screen. The most abundant splice variant we have found was 16a- (61.7%). Here the alternative use of an alternative splice acceptor site at exon 16 resulted in a frame shift with a consequent stop codon at IIS6 domain. This variant may give rise to the formation of a hemi-channel. The most abundant productive
splice variant we detected was Δ32 (17.9%). The skipping of exon 32 shortens the IVS3-S4 linker by 7 a.a. residues. Shortening of the IVS3-S4 linker by alternative splicing may be a common mechanism for diversity among LTCC. In Cαv1.1, alternative splicing at this locus can comprise 10% of transcripts in adult muscle and more than 66% in regenerating muscle (30). In Cαv1.2, alternative splicing of exons encoding IVS3 and the IVS3-S4 linker could produce up to 12 variations (22). Although the a.a. content of the IVS3-S4 linker varies greatly between the four LTCC, the length of the linker, however, may be important in modulating channel activation (22).

Electrophysiological characterizations of Cαv1.4 c-terminal splice variants – Alternatively splicing at 4 loci produced splice variants Δ37, 42d+, 43* and 45a- that have various lengths of the C-ter. Reports from previous investigations into the biophysical properties of full-length Cαv1.4 channels have shown that these channels expressed relatively small currents (about 3-times lower than Cαv1.2; 5), despite the use of high barium concentration in the external solution (15-30 mM), and tend to have a low transfection efficiency (22%; 6). In order to screen, in a more efficacious manner, the C-ter splice variants for distinct electrophysiological properties we chose the chimera approach. We generated a chimeric reference construct (Ch-WT) switching the full-length C-ter of human Cαv1.4 into the human Cαv1.2 channel backbone (Figure 3A). The Cαv1.2 was selected because it is a well characterized L-type subtype and has an almost identical I-V relationship to Cαv1.4 (5). Similar chimera have been successfully used in a robust manner to investigate the effects of the Cαv1.4 C-ter on channel properties and the mechanisms involved (9-11).

The Δ37 splice variant is truncated of the entire cytosolic tail and may be non-functional. However, because a CSNB2 Cαv1.4 mutant with no cytosolic tail was previously reported to mediate current (4), we attempted to characterize this splice variant as well but we were not able to evoke any current in the Δ37 chimeric channel.

43* splice variant inactivated more rapidly in Ca** and activated in more hyperpolarized potential – To assess the current-voltage (I-V) relationship of the splice variants, transfected cells were depolarized to a family of test potentials from -60 to 70 mV, in steps of 10 mV increments, from a holding potential of -90 mV (Figure 3B). The current trace profile for Ch-43* displayed a much slower inactivating I_{Ca} compared to Ch-WT or the other two functional C-terminal splice variants (Figure 3C, grey traces), but showed an early and more pronounced inactivation of I_{Ca} (Figure 3C, black traces). These inactivation properties of the channels in Ba** compared to Ca** will be further analyzed in later sections.

Ch-43* also activated at a more negative potential with a hyperpolarized shift in voltage for half-maximal activation, V_{1/2_{act}}, by 21.0 mV in Ba** (Supplementary Table 1) and 19.4 mV in Ca** (Supplementary Table 2) compared to Ch-WT (grey trace; both p < 0.001; unpaired t-test). Such changes in channel activation are slight and less apparent in Ch-42d+ and Ch-45a-. The V_{1/2_{act}} of Ch-42d+ hyperpolarized-shift by 2.6 mV in Ba** and 2.0 mV in Ca** (p<0.001; unpaired t-test), while the V_{1/2_{act}} of Ch-45a- depolarized-shift by 1.2 mV in Ba** and 0.5 mV in Ca** (p<0.001; unpaired t-test). We then appended the Cαv1.4 full-length channel with the 43* exon and found that in a similar manner, exon 43* mediated a pronounced hyperpolarized shift in activation (12.5 mV; p<0.0001; unpaired t-test) in the native channel (Figure 4B). Also, Cαv1.4 4.3* mediated a rapidly inactivating I_{Ca} while the WT displayed little inactivation (Figure 4A).

43* splice variant exhibited a negative shift in window current – We have shown that exon 43* caused a pronounced shift of the I-V relationship in the hyperpolarized direction, indicating a more negatively activating channel. To enable a more accurate assessment of the voltage activation of these channels, we analyzed the tail currents (G) obtained at the end of short depolarizing pulses to various potentials (Figure 5A). Also, to determine the inactivation properties of the channels under steady-state conditions, transfected cells were held at various potentials for 15-s and currents evoked before and after each inactivating pulse were compared (Figure 5B).

Compared to Ch-WT, the voltage of half-maximal activation in Ch-43* was shifted by -29.9
mV (Figure 5C; \( V_{1/2,\text{act}} \), Ch-WT, 21.9 ± 5.0 mV, \( n = 11 \); Ch-43*, -8.1 ± 2.8 mV, \( n = 16 \); \( p < 0.0001 \), unpaired \( t \) test). There was no change in the lower part of the slope \( k_{\text{low}} \), however an increase was detected in \( k_{\text{high}} \), although the difference was not statistically significant due to large error. Likewise, exon 43* mediated a hyperpolarized shift in \( V_{1/2,\text{act}} \) of the native channel by 7.9 mV (Figure 5E; \( V_{1/2,\text{act}} \), Ca\textsubscript{v}1.4 WT, -18.1 ± 1.8 mV, \( n = 12 \); Ca\textsubscript{v}1.4 43*, -26.7 ± 1.1 mV, \( n = 11 \); \( p < 0.0001 \), unpaired \( t \) test), with no significant changes in the slope values. Alternative splicing of exon 43* in the chimera resulted only in a minor shift in the voltage for half-maximal steady-state inactivation (Figure 5D; \( V_{1/2,\text{inact}} \), Ch-WT, -45.1 ± 0.2, \( n = 11 \); Ch-43*, -43.8 ± 0.1 mV, \( n = 8 \); \( p < 0.0001 \), unpaired \( t \) test) but gave rise to a significantly steeper SSI slope by decreasing the \( k_{\text{inact}} \) by 5.7 (\( k_{\text{inact}} \), Ch-WT, 10.8 ± 0.1; 43*, 5.1 ± 0.1; \( p < 0.0001 \), unpaired \( t \)-test). In the native Ca\textsubscript{v}1.4 channel, both WT and 43* displayed similar SSI at more negative membrane potentials. However beyond -40 mV Ca\textsubscript{v}1.4 43* could not be further inactivated (Figure 5F; \( V_{1/2,\text{act}} \), Ca\textsubscript{v}1.4 WT, -36.5 ± 1.0 mV, \( n = 16 \); Ca\textsubscript{v}1.4 43*, -44.5 ± 1.8 mV, \( n = 5 \); \( p < 0.0001 \), unpaired \( t \) test). \( k_{\text{inact}} \), Ca\textsubscript{v}1.4 WT, 9.5 ± 0.9 mV, \( n = 16 \); Ca\textsubscript{v}1.4 43*, 7.1 ± 1.5 mV, \( n = 5 \); \( p = 0.17 \), unpaired \( t \)-test). Taken together, exon 43* supported a window current that is more hyperpolarized than WT (Supplementary Figure 4A) but with a reduced SSI at higher potentials.

**Calcium-dependent inactivation (CDI) is restored by 43* splicing** – Rapid inactivation of activated channels due to Ca\textsuperscript{2+} is a phenomenon displayed by many high-voltage activated channels. Interaction of Ca\textsuperscript{2+}-bound calmodulin (CaM) with the IQ-domain triggers CDI, however in the Ca\textsubscript{v}1.4 channel the ICDI domain at the distal end of the C-ter interacts with the calcium-sensing apparatus, consisting of the EF-hand and IQ motif, to profoundly blunt CDI (8-11). From the profiles of the exemplar traces in Figure 3C and 4A, it was clear that the 43* variant still displayed robust CDI. To quantify the degree of CDI exhibited by the channels, the fraction of current that remained at a given time point after depolarization (residual current; i.e. \( r_{30} \) and \( r_{300} \) for 30 and 300 ms, respectively; Figures 6A-D) to different voltages were determined. The difference between the residual current of Ba\textsuperscript{2+} and Ca\textsuperscript{2+} is a measure of CDI strength (\( f \)-value). Here we calculated the \( f \)-value obtained at 10 mV (\( f_{10} \); Figures 6E and 6F). In addition, because the channels exhibit a fair amount of calcium-independent inactivation (VDI), we also used the ratiometric approach to calculate CDI. Here, “netCDI” is obtained by taking the ratio of the remaining current of Ca\textsuperscript{2+} to Ba\textsuperscript{2+} mV (\( \text{netCDI}_{10} \); Figures 4I and 4J). This method compliments the classical \( f \)-value measurements by avoiding the underestimation of CDI strength in the presence of strong VDI (31-33).

For the Ch-WT channel, the \( r_{30} \) for Ca\textsuperscript{2+} was slightly lower than for Ba\textsuperscript{2+} (Figure 6A). This became more evident with residual currents measured at a longer depolarization time of 300 ms (Figure 6B), indicating a small degree of inactivation by Ca\textsuperscript{2+} (\( f_{10} \), 30 ms, 0.05 ± 0.03; 300 ms, 0.18 ± 0.06. netCDI\textsubscript{10}, 30 ms, 0.06 ± 0.03; 300 ms, 0.33 ± 0.14 ). In the native WT channel, however, this is less pronounced (Figure 6E; \( f_{10} \), 30 ms, 0.02 ± 0.02; netCDI\textsubscript{10}, 0.02 ± 0.02) and even barely detectable at 300 ms (Figure 6F; \( f_{10} \), 0.00 ± 0.03; netCDI\textsubscript{10}, 0.00 ± 0.03).

In contrast, 43* led to a gross increase in CDI in both the chimera and native channels at 300 ms (Figures 6E and 6F; \( f_{10} \), 0.63 ± 0.03 and 0.46 ± 0.03, respectively; netCDI\textsubscript{10}, 0.79 ± 0.11 and 0.48 ± 0.03, respectively). Even as early as 30 ms, CDI was already very robust with an increase of at least 8-fold in Ch-43* (\( f_{10} \), 0.47 ± 0.03; netCDI\textsubscript{10}, 0.48 ± 0.07) and >13-fold in the native spliced variant (\( f_{10} \), 0.27 ± 0.02; netCDI\textsubscript{10}, 0.28 ± 0.02). The residual current of 43* channels in Ca\textsuperscript{2+} exhibits a U-shaped dependence on voltage (Figures 6C and 6D) that is distinctive of CDI.

**Voltage-dependent inactivation (VDI) is suppressed by 43* splicing** – An appreciable amount of slow inactivation in the absence of Ca\textsuperscript{2+} is apparent in the current trace profiles of Ch-WT (Figure 3C) and, to a lesser degree, Ca\textsubscript{v}1.4 WT (Figure 4). We next queried whether voltage-dependent inactivation (VDI) may be modulated by alternative splicing in the C-ter. Figure 6G shows the percentage inactivation of peak \( I_{\text{Ba}} \), evoked at \( V_{\text{max}} \), as it decays over various time points. Inactivation of \( I_{\text{Ba}} \) through Ch-WT increased steadily over time; displaying 66.0 ± 5.1
% inactivation at 300 ms after depolarization and reaching $85.3 \pm 3.6\%$ by the end of 1-s. Truncation of the distal portions of the C-ter by 43* splicing resulted in a channel that inactivated in Ba$^{2+}$ more slowly compared to Ch-WT. At 300 ms, $I_{Ba}$ inactivation for Ch-43* ($21.9 \pm 3.2\%$) was about three-fold slower than Ch-WT ($p < 0.0001$, unpaired $t$-test), and at 1-s the inactivation of Ch-43* was half that of WT (Ch-WT, $85.3 \pm 3.6\%$; Ch-43* $45.7 \pm 4.3\%$; $p < 0.0001$, unpaired $t$-test). The native Ca$\nu$1.4 WT displayed a much lower VDI compared to the chimera, exhibiting only $14.3 \pm 2.5\%$ inactivation at 1-s. The presence of exon 43*, however, still reduced VDI of the native channel by half ($7.1 \pm 1.6\%$).

43* splicing mediated an increase in current density – Previous reports of LTCC with C-ter deletions had described gross increase in current density (Ca$\nu$1.1: 34, Ca$\nu$1.2: 35, Ca$\nu$1.2: 36). We therefore examined the currents mediated by the 43* channels. Appending exon 43* to either the chimera or native channel both led to a significant increase in current density (Figures 7A and B). The increase in current density, when evoked at 60 mV, was 4.3 times in the chimera (Ch-WT: $35.6 \pm 6.7$ pA/pF, Ch-43*: $153.2 \pm 27.1$ pA/pF; $p < 0.01$, unpaired $t$ test) and 2.1 times larger in the native channel when evoked at 0 mV (Ca$\nu$1.4 WT: $25.5 \pm 4.4$ pA/pF, Ca$\nu$1.4 43*: $53.2 \pm 11.7$ pA/pF; $p < 0.01$, unpaired $t$ test).

Current density is a function of the number of channels expressed on the plasma membrane, the channel’s unitary conductance and open probability. We next measured the gating charge of Ca$\nu$1.4 WT and 43* channels. As gating charge arises from the voltage driven movement of charged residues within the activating channel structure, this gives an index of the quantity of channels expressed on the cell membrane (Figure 7C). Ca$\nu$1.4 43* displayed a gating charge that was more than six-fold smaller than the WT (Figure 7D; WT: $1.28 \pm 0.14$ fC/pF, 43*: $0.19 \pm 0.08$ fC/pF), indicating that the larger current density it exhibits is likely due to a larger single channel conductance or open probability or both. Single channel analyses would be required in order to determine these two properties.

Determinants for activation and inactivation in Ca$\nu$1.4 C-ter – In the 43* splice variant, the distal portion of the C-ter is truncated. This results in the loss of the ICDI, a domain that has been previously demonstrated to be responsible for the suppression of CDI in the Ca$\nu$1.4 channels. Removal of a short length of this ICDI had also been shown to slow VDI (10). It is conceivable that the robust CDI and slow VDI in Ch-43* can be fully attributed to the lack of the ICDI domain. However as the ICDI is composed of only the last 100 amino acids of the C-ter, we do not rule out the possible participation of the segment between exon 43 and ICDI in channel activation and inactivation.

Firstly, we established whether the changes to channel properties by 43* splicing can be fully reproduced by deleting only the ICDI or requires a greater length of the C-ter to be deleted. We next co-expressed a peptide containing the ICDI together with the Ch-43* to determine whether the ICDI itself can reverse the effects of 43* alternative splicing (i.e. suppression of CDI as well as increased VDI). To these ends, another two chimeric channels were constructed. These consisted of the Ca$\nu$1.2 backbone and truncated C-terms of the Ca$\nu$1.4, one terminating after exon 43 and another terminating just before the ICDI (labeled Ch-e43 and Ch-ΔICDI respectively; Figure 7A). The ICDI peptide was also cloned into a mammalian expression vector and fused with an mCherry fluorescent protein (37). These Ca$\nu$1.4 clones were each co-expressed, in HEK-293 cells, with the auxiliary subunits and with either the mCherry-fused ICDI peptide (ICDI-mCherry) or mCherry alone as a control. Only cells that were found to express both green and red fluorescence were selected for whole cell electrophysiological recordings (Supplementary Figure 6).

Hyperpolarized I-V shift by 43* splicing was due to the loss of ICDI – Analysis of the I-V relationship of Ch-ΔICDI showed that deletion of the ICDI from the full-length Ch-WT enabled a hyperpolarized shift reminiscent of that mediated by the 43* splicing (compare between Figure 8D, grey curve, and Figure 3F). This implies that the I-V shift shown by 43* was primarily due to the loss of the ICDI domain. Co-expression of Ch-ΔICDI
with ICDI-mCherry reversed the shift with a 16.3 mV and 25.4 mV more positive $V_{1/2,act}$ in Ba$^{2+}$ and Ca$^{2+}$ respectively (Supplementary Tables 4 and 5, $p < 0.001$, unpaired $t$ test). The slope of activation, $k_{act}$, was also decreased by 3.3 and 3.7 in Ba$^{2+}$ and Ca$^{2+}$ respectively (Supplementary Tables 4 and 5, $p < 0.001$, unpaired $t$ test). In the control experiments, co-expression of mCherry with the Ca$_{	ext{v}}$-a and auxiliary subunits failed to mediate the I-V shifts. Hence, these data demonstrated that the CDI domain modulated the activation kinetics of the channel.

Whole-cell electrophysiological recordings of Ch-e43 showed that it displayed an I-V relationship similar to Ch-43* or Ch-ΔICDI (Figures 8B-8G). Similarly, co-expression with ICDI-mCherry led to depolarized shifts in $V_{1/2,act}$ of 15.4 mV and 17.1 mV in Ba$^{2+}$ and Ca$^{2+}$ respectively, as well as a decrease in $k_{act}$ by 4.0 and 3.0 in Ba$^{2+}$ and Ca$^{2+}$ respectively (Supplementary Tables 4 and 5).

Co-expression of Ch-43* with ICDI-mCherry also triggered a right shift in the I-V curve (Figures 8B and 8E). The presence of mCherry did not affect the channel properties as the I-V curve for the control was similar to that of Ch-43* in the previous experiments (Figures 3F and 3J). The depolarized shift in $V_{1/2,act}$ effected by ICDI-mCherry was 16.8 mV in Ba$^{2+}$ and 19.3 mV in Ca$^{2+}$ (Supplementary Tables 4 and 5; $p < 0.001$, unpaired $t$ test). The magnitude of $V_{1/2,act}$ shift in Ca$^{2+}$ reflected a complete reversal of the hyperpolarized shift caused by the 43* splicing. Similarly, co-expression of Ch-43* with ICDI-mCherry restored $k_{act}$ to values similar to Ch-WT (Supplementary Tables 1 and 2).

**Co-expression with ICDI-mCherry suppressed CDI and increased VDI in Ch-43** – The representative traces shown in Figures 9A-F reflect the current profiles obtained when cells in the co-expression studies were subjected to the square-pulse protocol illustrated in Figure 3B. For Ch-43*, Ch-e43 and Ch-ΔICDI co-expressed with the mCherry control the current profile matched that of Ch-43*, whereby $I_{Ca}$ rapidly decayed and $I_{Ba}$ displayed only modest inactivation (Figures 9A, 9C, and 9E). However when co-expressed with ICDI-mCherry the fast inactivation of $I_{Ca}$ was much retarded and in the case of Ch-e43 and Ch-ΔICDI, $I_{Ca}$ decayed as slowly as $I_{Ba}$ (Figures 9B, 9D and 9F).

Ch-43*, Ch-e43 and Ch-ΔICDI when co-expressed with mCherry control, exhibited a residual $I_{Ca}$ with a U-shaped dependence on voltage (Figures 9G, 9I and 9K) that is indicative of CDI. This U-shape residual $I_{Ca}$ was lost when the channels were co-expressed with ICDI-mCherry and the residual $I_{Ca}$ attained profiles similar to residual $I_{Ba}$ when plotted against voltage (Figures 9H, 9J and 9L).

In Figure 9M, the calculated $f_{10}$ as well as netCDI showed that ICDI-mCherry completely eliminated CDI at 30 ms. At 300 ms (Figure 9N), co-expression with ICDI-mCherry suppressed $f_{10}$ from 0.54 ± 0.12 to 0.05 ± 0.15 in Ch-43*, from 0.62 ± 0.08 to 0.04 ± 0.19 in Ch-e43 and from 0.57 ± 0.13 to 0.08 ± 0.16 in Ch-ΔICDI. Calculated values of netCDI also indicated similar degrees of CDI abolishment. Here, ICDI-mCherry suppressed CDI to levels lower than that measured in Ch-WT. This is perhaps due to the greater abundance of the over expressed ICDI-mCherry as well as the increased mobility that ICDI has when expressed as a separate peptide.

Co-expression with ICDI-mCherry enhanced VDI in Ch-43*, Ch-e43 and Ch-ΔICDI. In the absence of ICDI-mCherry, Ch-43* exhibited slow $I_{Ba}$ inactivation with 1.5 ± 0.5 % at 30 ms after activation and only 17.3 ± 3.3 % by 300 ms (Figure 9O). Ch-e43 and Ch-ΔICDI also displayed similarly slow VDI (300 ms, Ch-e43, 18.0 ± 2.6 %; Ch-ΔICDI, 22.4 ± 5.6 %; $p > 0.1$, unpaired $t$ test). When co-expressed with ICDI-mCherry, the inactivation of $I_{Ba}$ through Ch-43* was increased to 7.9 ± 1.5 % at 30 ms and 47.3 ± 3.0 % at 300 ms ($p < 0.001$, unpaired $t$ test). Similarly, for Ch-e43 and Ch-ΔICDI, the presence of ICDI-mCherry increased $I_{Ba}$ inactivation at 300 ms to 37.3 ± 3.9 % and 37.4 ± 3.4 % ($p < 0.001$, unpaired $t$ test), respectively. However, for Ch-ΔICDI, the increase in VDI due to the presence of ICDI-mCherry was only significant at 300 ms, although an average increase was already apparent at 200 ms. Unlike Ch-e43, significant VDI increase due to ICDI-mCherry could be detected from 50 ms onwards. This ‘delayed’ response displayed by Ch-ΔICDI may be attributed to the additional sequences of the C-ter that Ch-ΔICDI has compared to Ch-e43 and Ch-43*.
Taken together the data, so far, demonstrated that the hyperpolarized shift in I-V relationship, the restoration of CDI as well as slow VDI conferred by 43* splicing was predominantly due to the absence of the ICDI domain rather than other portions of the C-ter. This is because the construct with only the ICDI deleted, Ch-ΔICDI, displayed similar activation and inactivation properties as Ch-43*. Besides, further deletion of the remaining length of C-ter between the ICDI and exon 43 did not significantly alter these properties. Moreover, co-expression with a peptide containing the ICDI was able to restore Ch-WT-like characteristics to all three constructs; although VDI in Ch-ΔICDI required longer depolarization to respond. Therefore it seems that the ICDI is a multi-function domain capable for modulating activation and inactivation properties of the channel.

DISCUSSION
Voltage-gated calcium channels are extensively alternatively spliced and such post-transcriptional modifications diversify the biophysical properties of the channel. In our work, we identified 19 alternative splice variants of CaV1.4 in the human retina, 16 of which are completely novel. Importantly, we showed that alternative splicing can restore CDI to the CaV1.4 channel that was previously reported to be CDI-insensitive (4,6). Also, the splice variant 43* exhibited a substantial hyperpolarize shift in activation as well as an increase in current density. We had also characterized each C-ter splice variant’s recovery from inactivation in the chimeric channels; and showed that 43* dramatically slowed recovery while 45a- slowed recovery only at the early phase. The involvement of proline residues within proline-rich exon 45 was also implicated. However, because voltage inactivation was much reduced in the native channel we were unable to recapitulate these effects here. Nonetheless, the figures for the recovery experiments are given in the supplementary section for reference (Supplementary Figures 8 and 9).

While in previous studies artificially engineered truncations of the C-ter of CaV1.4 have determined that removal of ICDI resulted in disinhibition of CDI (9-10), we now showed that this process may be natively regulated by alternative splicing. This is found in the 43* variant that introduces a termination signal into the C-ter, 137 residues from the IQ motif. The placement of the stop codon by the novel exon cassette is strategic because while maintaining the integrity of the calcium-sensing apparatus, it also generates a channel with a twice larger current density than the original. This increase in current density was not seen in another C-ter-truncated CaV1.4 (L1591X mutant; 10), whereby the entire length of C-ter immediately after the IQ was remove. This data suggested the importance of the additional sequences retained by the 43* splicing. In support of this, an appreciable degree of conservation across the LTCC isoforms is found in the proximal part of this region (Supplementary Figure 3). In CaV1.1 and 1.2, truncation of the C-ter after this region also gave rise to increased current density (Cav1.1: 34, Cav1.2: 35, Cav1.2: 36). In addition, a segment of this region (PCRD, Supplementary Figure 3) was also defined here to be the binding target of a distal C-ter autoinhibitory domain (DCRD) that is responsible for repressing current density. Sequences homologous to the DCRD may also be found within the ICDI of CaV1.4. This raises an important question of whether the PCRD and DCRD homologues of CaV1.4 interact. This is especially so in the context of abolishment of CDI by ICDI because the requirement for the “A” region, encompassing the PCRD, for ICDI efficacy have been reflected in several previous works (9-11). Investigating this interaction may mould our understanding on the two mechanisms that have been described for ICDI (8,11).

The increased current density in CaV1.4 43* may be due to one or a combination of three causes: an increase in expression of the channel on the plasma membrane, an increase in unitary conductance, and an increase in open probability. We have shown that this was not due to an increased membrane expression. On the contrary, CaV1.4 43* membrane expression was six-times lower than WT channels. In the CaV1.2 truncated channel, there was neither an increase in channel expression, as shown by gating charge analyses, nor an increase in single-channel conductance (35). In CaV1.3, there was no increase in expression density detected either (38). For
CDI, activation and current density: $\text{Ca}_v1.4$ splice variants

$\text{Ca}_v1.4$, it remains to be seen, with single-channel analyses, if $43^*$ splicing could alter unitary conductance and open probability.

The $43^*$ splicing also resulted in a hyperpolarize shift in activation and this may appear to conflict with some of the previous reports on other C-ter-truncated LTCCs. However, such left shifts were observed in C-ter-truncated LTCCs co-expressed either with $\beta_{2a}$ or $\beta_3$ ($\text{CaV}1.2, [36], \text{CaV}1.3, [38], \text{CaV}1.1, [39]$) and not seen when co-expressed with $\beta_{1b}$ ($\text{CaV}1.3, [40], \text{CaV}1.2, [41]$).

Our data may be used to provide insights on certain discrepancies between the currents obtained when recording from the rod photoreceptors versus the current characteristics of cloned $\text{Ca}_v1.4$ heterologously expressed in cells. Cloned $\text{Ca}_v1.4$ channels characterized in a heterologous expression system are activated by membrane depolarization to potentials that are more positive compared to the rod photoreceptor dark resting membrane potential of $\sim$-40mV (17,42-44). This posed a problem because rod photoreceptors hyperpolarize upon activation by light; apparently implying that in the normal operating range of the rod photoreceptor membrane potential $\text{Ca}_v1.4$ channels are minimally activated or not at all. Also, when native calcium currents in the rod photoreceptor were measured, they could be evoked at $\sim$30mV more hyperpolarized potentials than that obtained from cultured cells expressing cloned full-length $\text{Ca}_v1.4$ channels (12-14). It is possible that the native currents resulted from the presence of other calcium channel isoforms (i.e., T-type). Taken together, these findings seemingly excluded $\text{Ca}_v1.4$ from the role of inducing neurotransmission at the photoreceptor synapse (45). However given its localization together with its association with CSNB2 etiology (1-2,13), $\text{Ca}_v1.4$ activity should play an important role in normal rod photoreceptor function. The possibility of channel modulation by intracellular binding factors was thus explored and it was found (18) that interaction with CaBP4 was able to shift the activation of the $\text{Ca}_v1.4$ channel by $\sim$10 mV, therefore pushing the channel into the operating range of the rod photoreceptor. In this work, we showed that post-transcriptional modification of the $\text{Ca}_v1.4$ channel by alternative splicing provided another means to shift channel activation potential. Alternative splicing generating the novel exon $43^*$ mediated a $\sim$10 mV more hyperpolarized activated $\text{Ca}^{2+}$ current.

Although $43^*$ variants comprised only 13.6% of total $\text{Ca}_v1.4$ transcripts expressed in the human retina, it exhibited a two-fold larger in current density. Therefore the current contributed by $43^*$ variants may make up a larger proportion of total endogenous $\text{Ca}_v1.4$ calcium current in the retina.

Another discrepancy between the characteristics of $\text{Ca}^{2+}$ currents measured in heterologously expressed $\text{Ca}_v1.4$ and the native currents measured rod photoreceptors and retinal bipolar synapses is the presence of CDI in the native currents (15-17). We likewise propose that the CDI observed in the rod photoreceptors and retinal bipolar cells may have been contributed by alternatively spliced variants of $\text{Ca}_v1.4$ like those containing exon $43^*$ that exhibits robust CDI. The main difference between the CDI exhibited by the $43^*$ splicing and the CDI shown in these reports is that the native CDI occurred with slower kinetics.

In addition to differences in experimental setup (i.e. levels of charged carriers used, strength of $\text{Ca}^{2+}$ buffering, etc), the conditions present under the more physiological setting may modulate the rate and degree of CDI. For example, in $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$, the presence of calcium-binding proteins was shown to reduce CDI strength (46). Also, CDI measured from rods and bipolar cells had different inactivation time constants (15), and CDI was stronger and the rate of recovery from inactivation was pronouncedly slower in rods present in retinal slices than in isolated rod cells (16).

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FOOTNOTES
The abbreviations used are: CaM, calmodulin; CDI, calcium-dependent inactivation; CSNB2, congenital stationary night blindness type-2; C-ter, cytosolic/carboxyl-terminus; ICDI, inhibitor of calcium-dependent inactivation; I-V, current-voltage; LTCC, L-type calcium channel; SSI, steady-state inactivation; VDI, voltage-dependent inactivation.

FIGURE LEGEND
FIGURE 1. Schematic illustration of Cav1.4 alternative splicing in channel structure. The four repeat domain structure of the voltage-gated calcium channel is represented here and annotated with the relative exon positions, alternatively spliced loci and important functional domains. Exon boundaries are indicated by thin lines. Exons are numbered in order from 1 to 48. The α interaction domain (AID; binding site for the β-subunit), EF-hand, IQ-motif and the inhibitor of calcium-dependent inactivation domain (ICDI) are denoted using shaded ovals. Alternatively spliced exons are labelled in parentheses the relative positions in the channel schematic darkened. The exact site and mode of alternative splicing is detailed in Supplementary Figure 3 and the nomenclature used to denote the alternatively spliced exons is given in Supplementary Figure 6.
FIGURE 2. Alternative splice exons in Ca\(_{v}1.4\). The left column lists (in bold) the types of alternative splicing found in Ca\(_{v}1.4\) transcripts. The nomenclature used to denote the type of splicing is described in Supplementary Figure 6. The percentage occurrence of each alternative splice form in full length Ca\(_{v}1.4\) transcripts are given in parentheses, together with the number of clones analysed. A description on the mode of splicing follows, with the consequent alterations to the translation reading frame as well as amino acid changes. The splice scheme in the middle column illustrates the mechanism of splicing in terms of the exact splice junction used by the wild type (WT) and alternative modes of splicing. Dotted boxes indicate the extended boundaries of alternative exons and the exon cassettes. Italicised are the canonical gt...ag sites found almost invariably on the intronic side of splice junctions. Underlined are stop codons. The right column lists the channel structures that are affected by the alternative splicing.

FIGURE 3. Current-voltage relationship of Ca\(_{v}1.2\). A, schematic representation of chimera constructs. The channel backbone consists of Ca\(_{v}1.2\) (white box) while the cytosolic tail consists of Ca\(_{v}1.4\) wild type, WT, or alternatively spliced variants, Δ37, 42d+, 43* and 45a- (black). The stop codons for Δ37 and 43* are indicated by white and black filled circles, respectively. Amino acid positions are numbered according to the Ca\(_{v}1.4\) sequence – GenBank Accession Number NP005174. B, voltage protocol for determining current-voltage (I-V) relationship. 400-ms depolarisations to potentials ranging, in 10 mV increments, from -60 to 70 mV (holding potential -90 mV). C, representative \(I_{Ba}\) (grey) and \(I_{Ca}\) (black) traces during a 400-ms depolarisation to 10mV. The \(I_{Ba}\) and \(I_{Ca}\) traces were scaled to enable comparison between the two profiles. Current scale bars were drawn for both \(I_{Ba}\) (grey) and \(I_{Ca}\) (black). The time scales for each \(I_{Ba}\) and \(I_{Ca}\) pair are the same. D, E, F, G, normalised I-V plots for \(I_{Ba}\) of WT, 42d+, 43* and 45a- constructs, respectively. The curves were fitted with the equation described in EXPERIMENTAL PROCEDURES. In parentheses are the number of cells recorded. The WT curve was redrawn in each graph for comparison. H, I, J, K, same as D, E, F, G but for \(I_{Ca}\).

FIGURE 4. Current-voltage relationship of Ca\(_{v}1.4\) wild-type and 43* splice variant. A, representative \(I_{Ba}\) (grey) and \(I_{Ca}\) (black) traces during a 400-ms depolarisation to 10mV. B, normalised I-V plots for \(I_{Ba}\) of Ca\(_{v}1.4\) WT and 43*. The curves were fitted with the equation described in EXPERIMENTAL PROCEDURES. In parentheses are the number of cells recorded.

FIGURE 5. Activation and steady-state inactivation properties of wild-type and 43* splice variant channels. A, voltage protocol used for determining activation properties and an exemplar current trace (WT). Tail currents were measured after 20-ms depolarisation to potentials ranging from -60 to 100mV, in 10mV steps. B, voltage protocol used for determining steady-state inactivation, SSI, properties and an exemplar current trace (WT). Currents evoked at 10mV test potentials were measure before and after 15-s depolarisation to potentials ranging from -120 to 20 mV, in 10 mV steps. A holding potential of -90 mV was maintained for all experiments. C and D, normalised plots for activation (dashed lines) and SSI (solid lines) for chimeric channels Ch-WT and Ch-43*, respectively. For SSI, peak currents obtained after the 15-s inactivating pulse were normalised to that obtained before inactivation and plotted against voltage. The curves are fits with the Boltzmann relationship. For the activation plots, the peak of the tail currents (G) were normalised against the largest peak and plotted against voltage. The curves were fitted using the equation given in EXPERIMENTAL PROCEDURES. The number of cells recorded are given in parentheses; “( )” for SSI and “[ ]” for activation. E and F, same as C and D but for native channels Ca\(_{v}1.4\) WT and Ca\(_{v}1.4\) 43*, respectively.

FIGURE 6. Calcium-dependent inactivation and voltage-dependent inactivation of current through WT and 43* spliced variant channels. A and C, the fraction of peak current, \(I_{peak}\), that remained at 30-ms upon depolarisation to the indicated voltages, \(r_{30}\). The difference between the remaining current for \(I_{Ba}\) and \(I_{Ca}\), \(f\)-value, indicates the strength of calcium-dependent inactivation, CDI. The curves are visual fits.
of the values plotted to facilitate comparison. Number of cells recorded are given in parentheses. B and D, fraction of remaining current at 300-ms of depolarisation, \( r_{300} \), otherwise same as A and C. E and F, \( f_r \) values obtained at 10mV, \( f_{10} \), for \( r_{30} \) and \( r_{300} \) respectively. netCDI is the ratio between the remaining currents for \( I_{Ba} \) and \( I_{Ca} \). G, percentage of \( I_{Ba} \) inactivation during depolarisation to \( V_{max} \) in the time course indicated. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \) (compared to WT; unpaired \( t \)-test).

FIGURE 7. Current density of \( I_{Ba} \) through WT and 43* spliced variant channels and gating charge. A and B, the peak of tail currents measured at the end of short depolarising pulses evoked at different potentials were normalised against the membrane capacitance (\( C_m \)) of the recorded cell to obtain the current density. The number of cells recorded are shown in parentheses. C, gating charge was calculated by taking the integral of the current transient (grey areas, inset), at the onset, of the depolarizing pulse to the reversal potential of the ionic current. Shown here are representative gating and ionic tail current traces evoked at reversal potential. D, average gating charge for Ca\(\text{V}1.4\) WT (n=14) and Ca\(\text{V}1.4\) 43* (n=21) normalized against membrane capacitance. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \) (compared to WT; unpaired \( t \)-test).

FIGURE 8. Current-voltage relationship of Ch-43* and deletion constructs Ch-e43 and Ch-\(\Delta\)ICDI, co-expressed with ICDI-containing peptide. A, schematic representation of the various constructs. The channel backbone consists of Ca\(\text{V}1.2\) (white box) while the cytosolic tail consists of Ca\(\text{V}1.4\) containing exon 43* (Ch-43*) or deleted at a.a. positions 1718 (Ch-e43) or 1878 (Ch-\(\Delta\)ICDI). A co-expression construct consisting of a 100 a.a. peptide encompassing the ICDI fused to the mCherry fluorescent protein (ICDI-mCherry). The stop codon for 43* is indicated by a black filled circle. Amino acid positions are numbered according to the Ca\(\text{V}1.4\) sequence – GenBank Accession Number NP005174. B-D, normalised I-V plots for \( I_{Ba} \) of Ch-43*, Ch-e43 and Ch-\(\Delta\)ICDI co-expressed with either ICDI-mCherry (black) or mCherry (grey). The curves were fitted with the equation described in EXPERIMENTAL PROCEDURES. In parentheses are the numbers of cells recorded. E-G, same as B-D but for \( I_{Ca} \).

FIGURE 9. Current traces, CDI and VDI of Ch-43* and deletion constructs in co-expression experiments Channels constructs were co-expressed with either ICDI-mCherry or mCherry (labelled on the top) as described earlier. A-F, representative \( I_{Ba} \) (grey) and \( I_{Ca} \) (black) traces during a 400-ms depolarisation to 10 mV. G-L, residual \( I_{Ba} \) (grey) and \( I_{Ca} \) (black) for the corresponding co-expression experiments above, measured at 30-ms and 300-ms as labelled on the axes. M, N, the strength of CDI (\( f_r \)-value and netCDI) calculated at 10 mV for \( r_{30} \) and \( r_{300} \) respectively. O, percentage of \( I_{Ba} \) inactivation during depolarisation to \( V_{max} \) in the time course indicated. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \) (compared to WT; unpaired \( t \)-test).
FIGURE 1
### Alternative exons and changes effected

| Exon | Change | Description |
|------|--------|-------------|
| 2a   | Alternative acceptor site (frame-shift with stop codon) | +VSPYRH+FQGSSQGVRWPNSFFMRPAPSGC** |
| 2x   | (3.4%, n=88) Mutually exclusive exon (in-frame) | DTTPSRPANGAGPGWPLQGECPRWEGSSQGASTLGPATKPRNQHS9HKTGWASQAASRPGILTLNPLRPGSIVYER → AGNLNOALPLPSATRPRVWLGVAEPEANPAKFSHS-KLDFPND |
| Δ4   | (1.2%, n=84) Exon skipping (frame-shift with stop codon in exon 16) | ILG6EDVMYMVG3MAYG0PFPFPGMLVCY1FICFGNY → ECWCASSISSFSSSVATLIPD |
| 16a- | (61.7% alternative splicing in exons 16-18 loci, n=81) | +RRQLQSGPFPACLRCGLP** |
| Δ9   | (9.1%, n=175) Intron retention | (hypothetical) |
| 16a- | (54.9%, n=175) Intron retention (contains stop codon) | +PRLQGSPFLGRCPLPS** |
| Δ16 & 17a+ | Concurrent alternative acceptor sites on two consecutive exons (frame-shift in 16a- restored by frame-shift in 17a+) | ILG6EDVMYMVG3MAYG0PFPFPGMLVCY1FICFGNY → ECWCASSISSFSSSVATLIPD |
| Δ17 & 18a+ | Concurrent exon skipping and alternative acceptor site (in-frame) | ILG6EDVMYMVG3MAYG0PFPFPGMLVCY1FICFGNY → ECWCASSISSFSSSVATLIPD |
| Δ22 | (1.1%, n=89) Exon skipping (in-frame) | ΔLIGYDFATFTSIFTVELLK |
| Δ29 | Exon skipping (in-frame) |ΔROCEVEY1CAGCPFLRYP-KNHGQ1YWRWATNSAFAEFYMLMFL1NTVAL MG |
| Δ32 | (17.9%, n=608) Exon skipping (in-frame) | ΔNGGHLGE |
| Δ32 & 33a | Concurrent exon skipping and alternative acceptor site (frame-shift with stop codon) | ΔNGGHLGE, +LPHFYLLPLPSYAGQAS** |
| Δ32 | (14.1%, n=538) Intron retention (contains stop codon) | +VAHFNNOCSLLLPCPSPYPSLRTLIGPE** |
| Δ36 & 36a+ | (15.2%, n=164) Concurrent novel exon inclusion and alternative acceptor site (stop codon within cassette exon) | +SOCLICUMQ** |
| Δ36a+ | Only (hypothetical) Alternative acceptor site (frame-shift with stop codon) | +MLPCLSDHCNCHW** |
| Δ37 | (0.8%, n=584) Exon skipping (frame-shift with stop codon in exon 40) | ΔLGLFLWAVMNDYDFLRTWSSLPGHHLDE-FKRNWSEYDYGK, +PGHQLLGPGPAEYPAPSGWEAWPTPSQLOETEGHEAP QLFWDDGQHFLCQGPOEPEDQARNPAPGQGAPADAQCHOK DLEaedetae** |
| 42d+ | Alternative donor site (in-frame) | +VGTSFHSFRNLJ |
| Δ39 | (13.6%, n=308) Novel exon (contains stop codon) | +SREDVLCPWPGWFRDPLI** |
| Δ45a | (12.7%, n=716) Alternative acceptor site (in-frame) | ΔSVL3E4DAGTPPCV2SLPPHR |

### Splice scheme

| Exon | Splice site | Description |
|------|-------------|-------------|
| 1    | GTCT…ACA   | TGA |
| 15   | GAC…GTC    | GTG |
| 18   | GAC…ACT    | TCA |
| 20   | GAC…GTC    | GTG |
| 21   | GAC…GTC    | GTG |
| 22   | GAC…GTC    | GTG |
| 27   | GAC…GTC    | GTG |
| 29   | GAC…GTC    | GTG |
| 30   | GAC…GTC    | GTG |
| 31   | GAC…GTC    | GTG |
| 32   | GAC…GTC    | GTG |
| 33   | GAC…GTC    | GTG |
| 34   | GAC…GTC    | GTG |
| 35   | GAC…GTC    | GTG |
| 36   | GAC…GTC    | GTG |
| 37   | GAC…GTC    | GTG |
| 38   | GAC…GTC    | GTG |
| 39   | GAC…GTC    | GTG |
| 40   | GAC…GTC    | GTG |
| 41   | GAC…GTC    | GTG |
| 42   | GAC…GTC    | GTG |
| 43   | GAC…GTC    | GTG |
| 44   | GAC…GTC    | GTG |
| 45   | GAC…GTC    | GTG |

### Structures affected

- **N-ter**:WT Δ 37
- **IS2-IIS3**:WT Δ 17&18a+
- **IS5-S6 linker, IIS6**:WT Δ 22
- **IIS2**:WT Δ 29
- **III-IV loop, IVS1**:WT Δ 32
- **IVS3-S4 linker**:WT Δ 33
- **IVS5-S6 linker**:WT Δ 36
- **IVS6, c-tail, EF-hand**:c-tail

**FIGURE 2**
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6

A

Ch-WT

B

Ch-43*

C

D

E

F

G

% inactivation (1/sec)

% inactivation (1/sec)

0.3 0.6 1.0 1.6 sec

0.3 0.6 1.0 1.6 sec

0.03 0.05 0.3 0.3 1.0 1.0 1.6 1.6 sec

Ca\(_{\text{i}}\) WT (11) Ca\(_{\text{i}}\) 43* (11)
FIGURE 7
**FIGURE 8**

- **Panel A:** Diagram showing the expression of different constructs (Ch-43*, Ch-ΔICDI, Ch-e43) and their respective impact on voltage-gated ion currents (I\text{Ba}, I\text{Ca}).
- **Panel B:** Graphs showing the current-voltage relationship for Ch-43* + mCherry (8) and Ch-43* + ICDI-mCherry (12).
- **Panel C:** Graphs showing the current-voltage relationship for Ch-e43 + mCherry (11) and Ch-e43 + ICDI-mCherry (9).
- **Panel D:** Graphs showing the current-voltage relationship for Ch-ΔICDI + mCherry (7) and Ch-ΔICDI + ICDI-mCherry (13).
