Molecular Neurobiology

ISSN0893-7648/02/26(1): 21–44/$16.00

Functional Diversity in Neuronal Voltage-Gated Calcium Channels by Alternative Splicing of $Ca_v\alpha_1$

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Abstract

Alternative splicing is a critical mechanism used extensively in the mammalian nervous system to increase the level of diversity that can be achieved by a set of genes. This review focuses on recent studies of voltage-gated calcium (Ca) channel $Ca_v\alpha_1$ subunit splice isoforms in neurons. Voltage-gated Ca channels couple changes in neuronal activity to rapid changes in intracellular Ca levels that in turn regulate an astounding range of cellular processes. Only ten genes have been identified that encode $Ca_v\alpha_1$ subunits, an insufficient number to account for the level of functional diversity among voltage-gated Ca channels. The consequences of regulated alternative splicing among the genes that comprise voltage-gated Ca channels permits specialization of channel function, optimizing Ca signaling in different regions of the brain and in different cellular compartments. Although the full extent of alternative splicing is not yet known for any of the major subtypes of voltage-gated Ca channels, it is already clear that it adds a rich layer of structural and functional diversity".

Index Entries: Alternative splicing, calcium channels, human genome, isoforms, calcium signaling.

Structural Diversity Underlies Functional Specialization

A growing number of congenital, neurological disorders, including familial hemiplegic migraine, spinocerebellar ataxia-6, and X-linked stationary night blindness, link to various voltage-gated Ca channel genes, underscoring their importance in supporting normal cellular function *(1–3).* Voltage-gated Ca channels regulate the rapid entry of Ca ions into excitable cells during membrane depolarization. In turn, Ca entry through these channels controls neurotransmitter release, neurite outgrowth, gene expression, and muscle contraction *(4–6).* Given the wide array of Ca signaling pathways that these channels are linked to, structural

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and functional diversity is required to couple specific voltage-gated Ca channels to specific pathways. Understanding the mechanisms that give rise to structural diversity among voltage-gated Ca channels, not only provides information about Ca signaling in neurons, but also points to structural domains on the Ca channel important for the transduction of intracellular signals.

Within the nervous system, the structural and functional diversity of voltage-gated Ca channels is remarkably well-utilized. Individual neurons select from several voltage-gated Ca channel genes to determine the composition of the final multisubunit Ca channel complex. In addition to the genes that encode $Ca_v\alpha_1$ subunits described below, four $Ca_v\beta$ and three $Ca_v\alpha_2\delta$ auxiliary subunits are expressed in neurons *(7–9).* These genes are also subject to alternative pre-mRNA splicing *(8–11).* A third group of genes encode γ subunits that may also bind to $Ca_v\alpha_1$ *(12).* The γ subunit was first co-purified from skeletal muscle along with $Ca_v1.1\alpha_1$ *(13,14).* Currently, eight genes are known to encode γ subunits, but it is not yet clear how many associate specifically with Ca channel $Ca_v\alpha_1$ subunits. At least one γ subunit (γ_2, *stargazin*) couples to the AMPA receptor, suggesting that this class of auxiliary protein may play a more general role in targeting or modulating membrane receptors and ion channels *(15).*

Of the major subunits thought to comprise voltage-gated Ca channels, the $Ca_v\alpha_1$ subunit constitutes the functional core of each channel and, consequently, dictates its primary functional properties. To date, 10 $Ca_v\alpha_1$ subunit genes have been identified in mammals, nine of which are expressed in the nervous system. They segregate into three major subgroups based on structural, pharmacological, and biophysical differences (*see* Fig. 1): $Ca_v1\alpha_1$ (L-type; dihydrophyridine (DHP)-sensitive), $Ca_v2\alpha_1$ (P/Q-, N-, and R-types; DHP-insensitive), and $Ca_v3\alpha_1$ (T-type; also DHP-insensitive). Traditionally, the $Ca_v3\alpha_1$ T-type family of Ca channels has been referred to as low voltage-activated (LVA) to distinguish them from L-, P/Q-, N-, and R-type channels that are collectively thought to activate at higher voltages (high voltage-activated, HVA). However, $Ca_v1.3\alpha_1$-containing L-type channels have recently been shown to activate in the "low voltage" range *(16–18)* and in this respect are functionally similar to T-type Ca channels. Homologs of the three major $Ca_v\alpha_1$ gene families exist in both the *Drosophila melanogaster* and *Caenorhabditis elegans* genomes indicating evolutionary conservation *(19).* Unfortunately, there is little conservation among the various naming systems used over the years to distinguish voltage-gated Ca channels. The most recently proposed nomenclature is based on sequence homology and is used throughout this review *(20).*

The expression pattern of each $Ca_v\alpha_1$ gene is tightly regulated, depending on cell-specific and developmental factors that, together with characteristic structural features, permit coupling to specific effector proteins and second-messenger systems. For example, certain members of the $Ca_v1\alpha_1$ gene family link excitation to contraction in muscle cells *(21,22),* whereas $Ca_v2\alpha_1$ family members link excitation to neurotransmitter release in neurons *(4),* and $Ca_v3\alpha_1$-channels contribute to rhythmic firing in pacemaker cells of the heart and in thalamic neurons of the brain *(21,23,24).* However, products of a given $Ca_v\alpha_1$ gene may couple to more than one effector system and regulate different functions depending on, among other factors, where in the animal and where in the cell they are expressed. L-type $Ca_v1.3\alpha_1$ subunits are believed to control neurotransmitter release in hair cells of the inner ear *(25),* have been implicated in pacemaking in heart *(17),* and, in neurons, L-type Ca channel genes $Ca_v1.2\alpha_1$ and probably $Ca_v1.3\alpha_1$ play prominent roles in controlling gene expression *(26).* Further, $Ca_v2\alpha_1$ genes, while dominating in the control of transmitter release at presynaptic nerve terminals throughout the mammalian nervous system, can also couple to regulation of gene expression and mRNA stability, presumably in the soma *(27,28).* The function and distribution of Ca channel subtypes may also change during development. For example, before synapses are formed

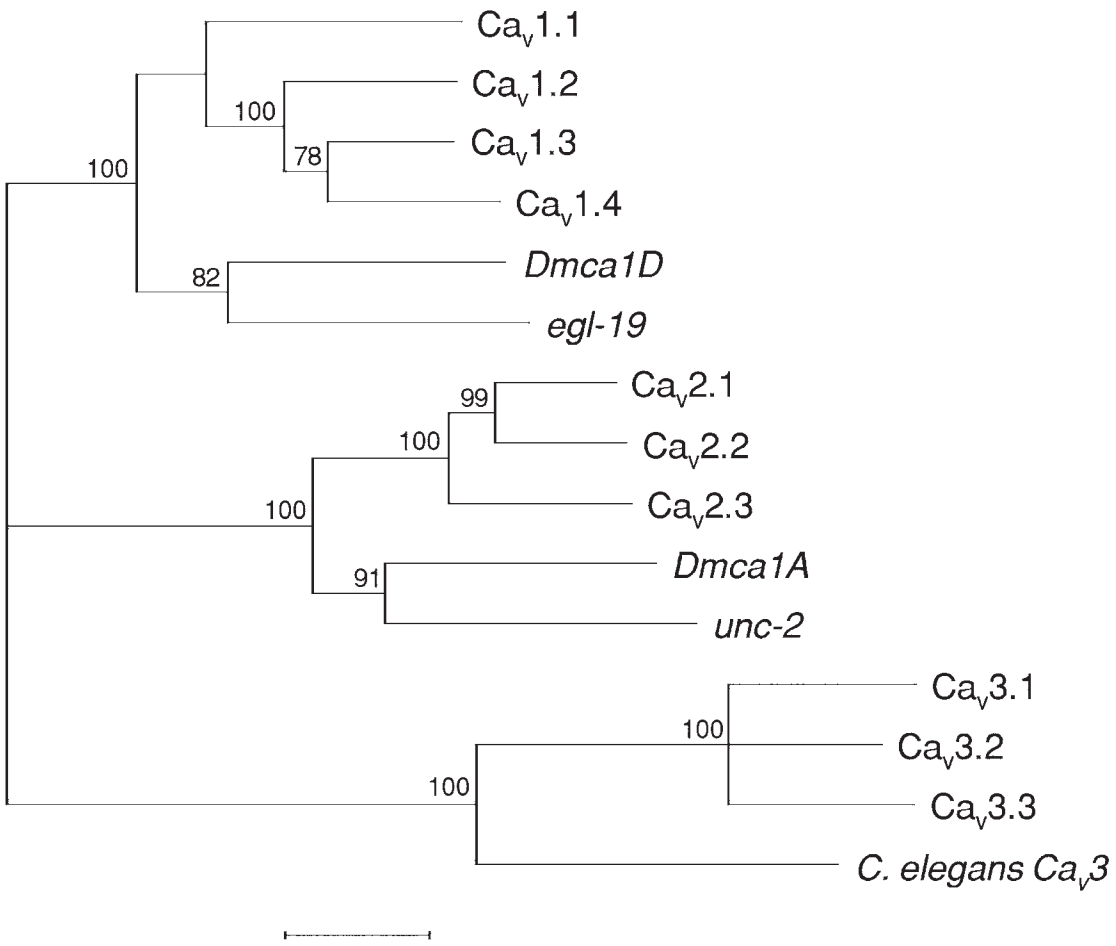

Fig. 1. Calcium channel $Ca_v\alpha_1$ subunit gene tree. The sequences for the human genes $Ca_v1.1$ (GenBank Accession #L33798), $Ca_v1.2$ (AJ224873), $Ca_v1.3$ (M76558), $Ca_v1.4$ (AJ224874), $Ca_v2.1$ (AB035727), $Ca_v2.2$ (M94173), $Ca_v2.3$ (L27745), $Ca_v3.1$ (AF190860), $Ca_v3.2$ (AF05196), and $Ca_v3.3$ (AF211189) were aligned, along with gene sequences of *Drosophila Dmca1A* (U55776) and *Dmca1D* (U00690), and *C. elegans unc-2* (AAB36868), *egl-19* (AAC47755), and Ca_v3 (AAA79201). Alignments were carried out using transmembrane domains and short linkers, but excluding the variable N- and C-termini, and intracellular loops I-II, II-III, and III-IV. A branch-and-bound tree search using maximum parsimony was performed to find the optimal tree (Genetic Computer Group, paupsearch and paupdisplay programs). Confidence values determined by bootstrap analysis are provided for each node. A highly similar tree was also obtained using minimum evolutionary distance as the optimality criterion. The original tree is unrooted. The representation has been rooted using the midpoint method. The scale bar corresponds to 1 substitution per 100 amino acids.

between granule cells and Purkinje cells of the cerebellum, N-type $Ca_v2.2\alpha_1$ Ca channels regulate granule cell migration from the molecular layer to their final destination in the inner granule layer *(29)*, whereas after synapse formation the N-type Ca channel contributes to neurotransmitter release at the granule cell to Purkinje cell synapse *(30)*. The T-type $Ca_v3.3\alpha_1$ gene also undergoes a particularly striking change in its expression during development. $Ca_v3.3\alpha_1$ mRNA is widely expressed in the juvenile rat brain, whereas, in the adult, expression of this gene is restricted to the striatum *(31)*.

The presence of multiple $Ca_v\alpha_1$ genes with cell-specific and developmentally controlled expression patterns is clearly important for generating multiple Ca channels specialized for particular functions. Association of $Ca_v\alpha_1$ subunits with various auxiliary subunits, including $Ca_v\alpha_2\delta$ and $Ca_v\beta$, further augments structural and functional diversity of the final voltage-gated Ca channel complex *(32,33).* Temporal changes in the expression of $Ca_v\beta$ subunits in brain have been documented that are thought to contribute to the maturation of Ca channel currents *(34).* The importance of auxiliary subunit-mediated modulation of Ca channel function is the focus of other reviews *(7,9).*

Alternative Splicing $Ca_v\alpha_1$

Functional diversity within the $Ca_v\alpha_1$ gene family is enhanced several-fold as a result of extensive alternative pre-mRNA splicing. It has been known for some time that pre-mRNAs from the various $Ca_v\alpha_1$ genes undergo alternative splicing *(35–44).* (Also see additional references for recent studies of alternative splicing in the $Ca_v3\alpha_1$ gene family [*31, 45–47*].) While the biological significance and full extent of pre-mRNA splicing is not as yet known for any $Ca_v\alpha_1$ gene, it is likely to be one of the main mechanisms for fine-tuning the properties of voltage-gated Ca channels to achieve a high degree of functional specialization. Consistent with this, alternative splicing of $Ca_v\alpha_1$ pre-mRNAs is differentially regulated among distinct regions of the nervous system *(36,37,43,48–53)* and at different stages during development *(37,50).* Functional studies addressing the impact of alternative splicing on Ca channel behavior, however, have lagged behind molecular and genomic characterization of the various $Ca_v\alpha_1$ genes. Recently, studies have demonstrated that alternative splicing in the $Ca_v\alpha_1$ gene family can influence channel behavior and pharmacology *(48,49,53–68).*

Several biophysical studies of native, neuronal voltage-gated Ca channel currents point to functional diversity within the major subtypes of Ca channels, complementing evidence for structural diversity within a given $Ca_v\alpha_1$ subunit gene. For example, although N-type Ca currents recorded from a variety of neurons are universally inhibited by ω-conotoxin GVIA and activate at relatively depolarized membrane potentials, there is diversity within this class of Ca channel with respect to toxin-sensitivity, the time course and voltage-dependence of inactivation, single channel conductance, gating behavior, and sensitivity to G-protein-mediated modulation *(69–76).* The molecular origin of the functional diversity among native N-type Ca currents has not been fully established, but likely arises, at least in part, from alternative splicing of the pore forming $Ca_v2.2\alpha_1$ subunit. This review will focus on alternative pre-mRNA splicing in the $Ca_v2\alpha_1$ gene family, with particular emphasis on the N-type $Ca_v2.2\alpha_1$ gene.

RNA Processing

The majority of eukaryotic genes are composed of multiple discontinuous coding sequences (exons) that are usually short, averaging between 50–300 nucleotides in length, separated by noncoding sequences (introns) of highly variable lengths, usually 200 to several thousand nucleotides. Messenger RNAs are transcribed as precursors (pre-mRNAs) containing intervening introns. Introns are removed and exons spliced together to form mature mRNAs. This splicing reaction takes place in the spliceosome (mostly U2-type or U12-type; [*77*]), a complex that includes five small nuclear ribonucleoprotein particles, more than 50 distinct proteins, as well as a subset of cell-type specific splicing factors. In the vast majority of genes, dinucleotides, most commonly *gt* and *ag* or, less commonly, *at* and *ac,* are found at the 5′ and 3′ intron boundaries, respectively (e.g., Fig 5B; >99% of introns have *gt-ag* at their 5′ and 3′ ends, respectively; [*78*]. There is also a recognition sequence (the branch point) usually located 10–20 nucleotides upstream of the 3′ splice site that invariably contains an adenosine and to which components of the splicing machinery bind *(77,79,80).* Constitutively expressed exons are invariant

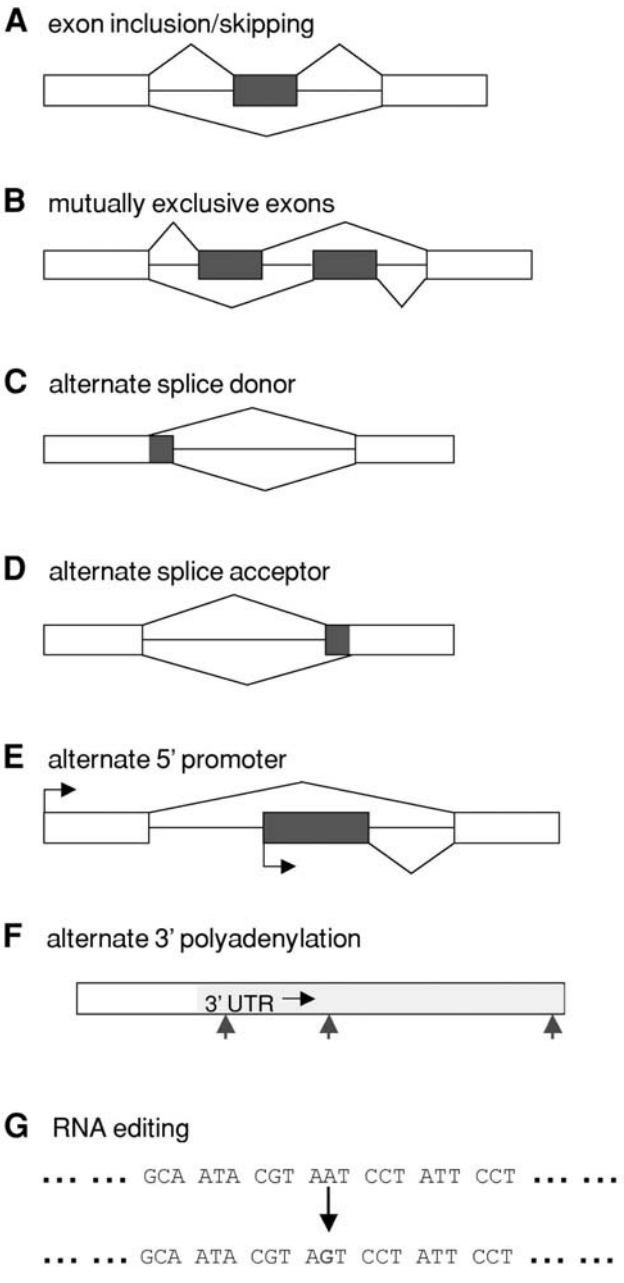

Fig. 2. Different mechanisms generate diversity among mRNAs, including alternative splicing. Various postranscriptional mechanisms for generating diversity among mRNAs are presented in schematic form. Variant regions are shaded. Diversity among $Ca_v2.2\alpha_1$ mRNAs expressed in the rat nervous system arises from at least four forms of RNA processing including exon inclusion/skipping (**A;** exons 18a, 24a, 31a), mutually exclusive exon expression (**B;** exons 37a/37b), the use of alternate splice acceptor sites (**D;** exon 10), and alternate 3′ end polyadenylation (**F;** *see* ref. *28*). In addition, the *Drosophila* Ca channel *Dmca1A* appears to undergo RNA editing (**G;** *155).*

among mRNAs derived from a single gene, while other exons are alternatively expressed in subsets of mRNAs depending on cell-type, developmental stage, and activity *(81–83).* Current estimates from bioinformatic-based analyses suggest that 35–60% of human genes are subject to alternative splicing in at least one site, an estimate that will almost certainly increase as the EST database expands *(78,80).*

Different patterns of alternative splicing and other forms of RNA processing have been shown to occur in mammalian mRNAs. These are summarized in Fig. 2. A single alternatively expressed exon residing between constitutive exons can be either expressed or skipped in the mature mRNA (Fig. 2A). Then again constitutive exons may flank multiple alternatively expressed exons, only one of which is included in the final mRNA (mutually exclusive; Fig. 2B). In the *Drosophila* cell adhesion protein, *DSCAM,* exon 6 of the final mRNA transcript can be encoded by any one of 48 alternative, mutually exclusive exons *(84).* Additional sequence variations can arise from the use of alternate 3′ and/or 5′ splice sites (alternate splice donor/acceptor; Fig. 2C,D). Other mechanisms for generating diversity among mRNAs transcribed from the same gene include the use of alternate 5′ promoters and alternate 3′ polyadenylation/cleavage sites (Fig. 2E,F). Alternate 3′ polyadenylation does not modify the coding sequence, but can influence mRNA stability by altering the length of the 3′ untranslated region (*see* ref. *28).* Once again, bioinformatic-based analyses suggest that this form of RNA processing is common; at least 25% of human genes give rise to mRNAs that use alternate 3% polyadenylation sites *(78).* Finally, subtle differences among mRNAs derived from a single gene can arise from RNA editing where, for example, a single adenosine residue

in the pre-mRNA transcript is converted to inosine and consequently interpreted as a guanosine by the translation machinery (Fig. 2G; *85).* Perhaps the most notable example of RNA editing in the nervous system is the AMPA receptor. A single edited site in the AMPA receptor regulates the permeability of its associated ion channel pore to Ca *(86).*

The biological importance of alternative splicing as a critical mechanism for diversifying protein function is probably best exemplified in *Drosophila* where extensive work has shown that differential splicing of a host of genes determines the sex of the fruit fly *(87,88).* Other notable examples include differential splicing of the calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA transcript that generates calcitonin in non-neuronal tissues and CGRP in neuronal tissues *(89),* and alternative splicing of the *Slo* gene-pre-mRNA that encodes calcium-activated potassium channels (BK channels) in hair cells of the inner ear. The pre-mRNA of *Slo* is differentially spliced in a graded pattern along the basilar papilla of the inner ear, correlating closely with the tonotopic map of hair cell tuning frequencies along the basilar membrane. In particular, alternative splicing of *Slo* transcripts affects BK channel kinetics and Ca sensitivity. On the cellular level, alternative splicing of *Slo* alters the repolarization rate of inner hair cells and, therefore, the frequency at which the membrane can depolarize *(83,90,91).* There are, however, few other examples where the biological significance of alternative splicing has been so nicely demonstrated. Likewise, little is known about the factors that control tissue-specific and developmentally regulated alternative splicing in neurons *(82).* One exception is a recently identified neuron-specific alternative splicing factor, Nova-1, that controls expression of glycine and $GABA_A$ receptor splice isoforms *(92).*

Alternative Splicing in $Ca_v2.2\alpha_1$

Our laboratory and others have identified and, to different extents, functionally characterized, alternative splice isoforms of the N-type $Ca_v2.2\alpha_1$ gene (*see* Figs. 3, 4; *32,49,53,57, 67,93–97*), while others have studied splice isoforms of the P/Q-type $Ca_v2.1\alpha_1$, $Ca_v2.3\alpha_1$ (often called R-type), L-type $Ca_v1.2\alpha_1$, and T-type $Ca_v3\alpha_1$ genes *(24,31,41,42,44–48,50,52, 54–56,58–61,98–103).* More recently, we and others have analyzed alternative splicing of the L-type $Ca_v1.3\alpha_1$ gene *(16,104).* The locations of sites of alternative splicing in mammalian $Ca_v2.2\alpha_1$ genes that have been confirmed by genomic analysis are summarized in Figs. 3 and 4. The structure of the human $Ca_v2.2\alpha_1$ gene illustrated in Fig. 3 includes exons 16 through 46 whose presence has been confirmed in human genomic sequence and aligned with available cDNAs. Sequence corresponding to exons 1 through 15 of $Ca_v2.2\alpha_1$ is not yet present in public databases. Their approximate locations are based on the published structure of the closely related $Ca_v2.1\alpha_1$ gene (*see* Fig. 3 legend for details; *105).* Alternatively spliced exons occur in the putative extracellular and intracellular linkers and in the C-terminus of $Ca_v2.2\alpha_1$ subunit. There is no evidence as yet for alternative splicing in the transmembrane domains of the $Ca_v2.2\alpha_1$ gene, although splicing in the transmembrane domains of other $Ca_v\alpha_1$ genes has been reported (e.g., *36).* Certain sites of alternative splicing, such as in the IVS3-IVS4 extracellular linker, are conserved among different mammalian $Ca_v\alpha_1$ genes (*see* Fig. 5A). Further, alternative splicing in domain IVS3-IVS4 is even present in the *Drosophila* homolog of the mammalian $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ genes (*DmcalA;* Fig. 5), but not in *C. elegans* (*unc-2;* Fig. 5A). In *unc-2,* transmembrane domains IVS3 and IVS4 and their linker are encoded by a single exon. The precise composition of the alternatively spliced exons that encode segments of domain IVS3-IVS4, however, differ among the various genes. In regions more variant among $Ca_v\alpha_1$ genes, such as the II–III intracellular loop and the C-terminus, sites of alternative splicing are found in a subset of more closely related genes (e.g., exons homologous to e37a and e37b of $Ca_v2.2\alpha_1$, Fig. 3, are also present in $Ca_v2.1\alpha_1$). In this review, we will discuss

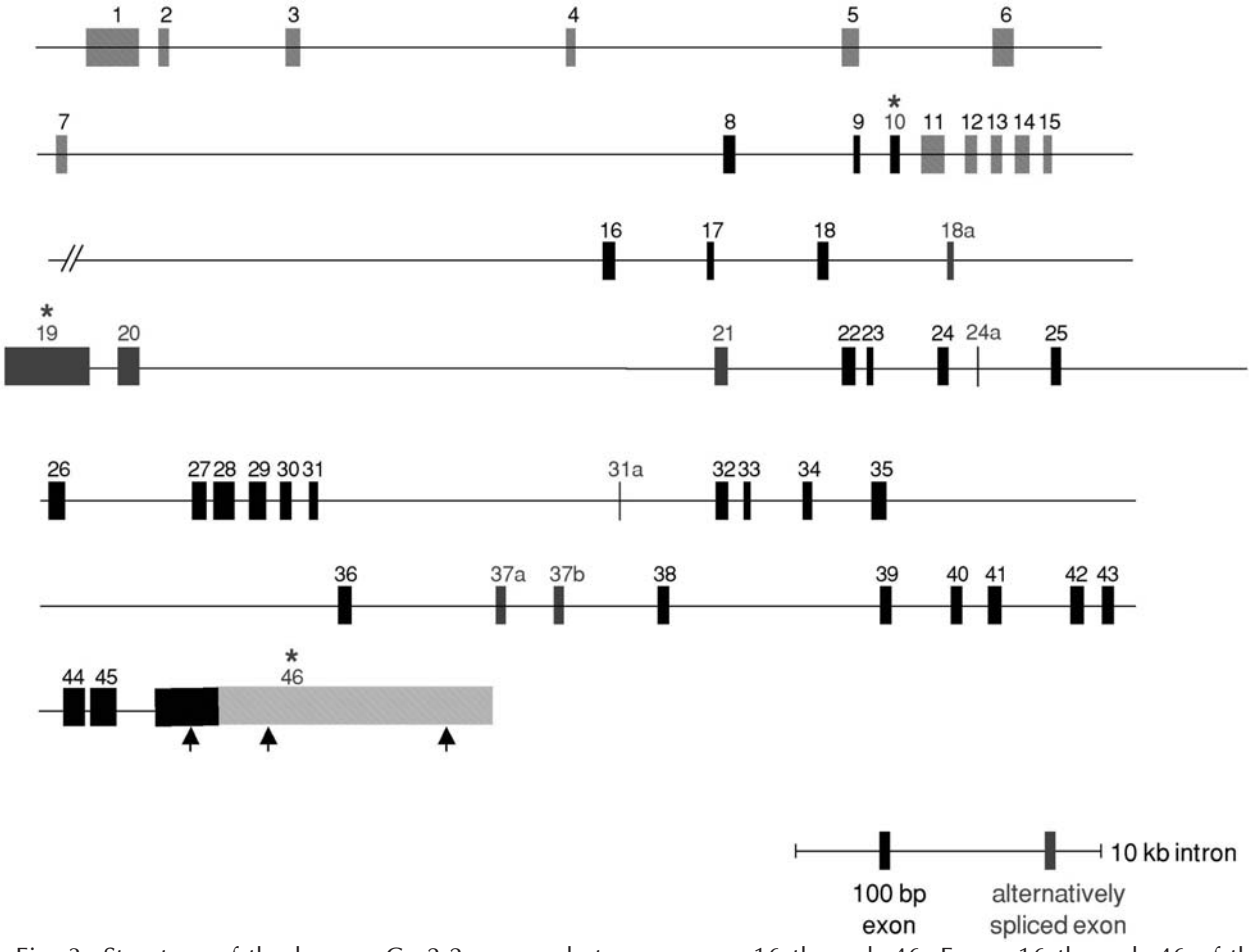

Fig. 3. Structure of the human $Ca_v2.2\alpha_1$ gene between exons 16 through 46. Exons 16 through 46 of the human $Ca_v2.2\alpha_1$ gene were identified in a chromosome 9 cosmid (AL591424) by alignment to the human $Ca_v2.2\alpha_1$cDNA (M94172) and are depicted as solid boxes. They were identified by a BLAST-based sequence similarity search. We manually created sequence alignments from high-scoring hits and defined the exon-intron boundaries based on the consensus splice-junction sequences. We have not found any ambiguity in defining exon-intron boundaries for the exons that have complete genomic sequences. The available sequence of cosmid AL591424 does not contain exons 1 through 15, but they are mapped according to the structure of the closely related $Ca_v2.1\alpha_1$ gene (hashed boxes; *105*), or based on other sequence information. In particular, exons 8 through 10 of $Ca_v2.2\alpha_1$ were identified by Mittman (AF247811 and AF237470). The break mark indicates where the available $Ca_v2.2\alpha_1$ sequence in cosmid AL591424 begins. The human $Ca_v2.2\alpha_1$ gene is located at position 9q34, close to the telomere. It is problematic to obtain sequence from such regions, and this likely explains why the 5′ end of the gene is not yet available. Alternatively spliced exons are light gray and constitutively expressed exons are black. Also, exons where evidence exists that they possess alternate 5′ or 3′ splice sites are noted with asterisks (*), including exons 10, 19, and 46 *(49,95,156).* The alternatively spliced exons 18a, 24a, and 31a were identified in the human genomic sequence based on our analysis of rat $Ca_v2.2\alpha_1$ cDNAs and rat genomic structure, and the identification of dinuceotide consensus-splice junctions and branch points in the human gene *(53,57).* Overall there is significant conservation of gene structure across rat, mouse, and human genes. Exons 19, 20, and 21 also have been shown to be alternatively spliced in human brain *(67).* Exon 37a was identified based on the existence of rat cDNAs containing this sequence (Pan et al., 2001. Society for Neuroscience Abstracts) and earlier predicted on the basis of genomic analysis (Mittman et al., 2000, Society for Neuroscience Abstracts). Arrows indicate the location of three alternative polyadenylation sites AAUAAA in the 3′ untranslated region encoded by exon 46 (*see* also ref. *28*). Exon 46 is hashed part way through because the 3′ end of this exon is based on sequence from rat $Ca_v2.2\alpha_1$ cDNA (AF055477; *28*). Available human $Ca_v2.2\alpha_1$ cDNAs contain only the short form of the 3′untranslated region.

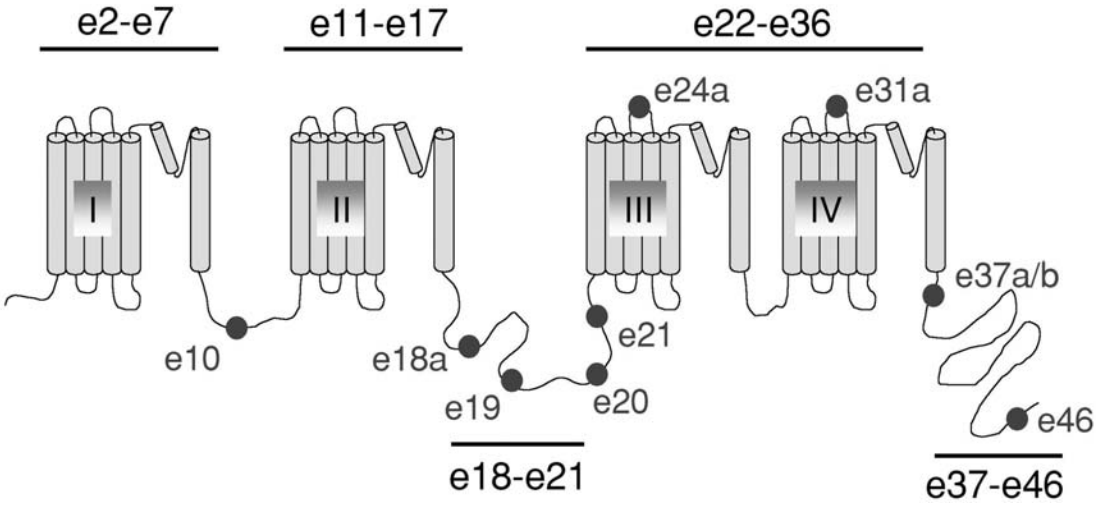

Fig. 4. Putative transmembrane topology of the $Ca_v2.2\alpha_1$ subunit indicating the approximate position of constitutive and alternatively expressed exons. This schematic depicts the classic view of a voltage-gated ion channel comprised of four structurally homologous domains (I, II, III, IV) each containing 6 transmembrane spanning domains (S1-S6) together with a pore region between transmembrane helices S5 and S6. Approximate exon numbers are indicated above or below each domain delineated by horizontal bars. For example, domain I is encoded by exons 2 through 7 (e2-e7), and the intracellular II-III loop is encoded by exons 18 through 21 (e18-e21). The approximate locations of sites of alternative splicing are highlighted by circles (exons 10, 18a, 19, 20, 21, 24a, 3la, 37a/b, and 46).

what is known of alternative splicing in three domains of the $Ca_v2.2\alpha_1$ gene, the IVS3-IVS4 extracellular linker, the II–III intracellular loop, and the C-terminus, as well as their potential importance in Ca channel function. We only have a partial picture of the extent of alternative splicing in the $Ca_v2.2\alpha_1$ gene, and additional sites of alternative splicing will likely be identified in the future. Strategies that will help in identifying these sites are discussed later in this review.

The IVS3-IVS4 Extracellular Linker

The S3-S4 linkers of a number of voltage-gated and structurally related ion channels are important in determining the time course and voltage-dependence of channel activation *(39,49,58,106–108).* In the L-type Ca channel family for example, the domain I S3-S4 linkers of the skeletal $Ca_v1.1\alpha_1$ and cardiac $Ca_v1.2\alpha_1$ genes are critical for determining their very different activation kinetics *(106).* The S3-S4 extracellular linker in the fourth domain (IVS3-IVS4) is subject to alternative splicing in several $Ca_v\alpha_1$ genes, including $Ca_v2.2\alpha_1$ (*see* Fig. 5A). The conservation of alternative splicing in this region is consistent with the idea that the IVS3-IVS4 linker plays a critical role in modulating Ca channel activity *(36,39,49,53,55,56,58,103,109–111).* The IVS3-IVS4 alternatively spliced cassette exons of $Ca_v2.2\alpha_1$ (exon 31a; Figs. 3, 4) and $Ca_v2.1\alpha_1$ are only 6 nucleotides in length (Fig. 5). Exon 31a of $Ca_v2.2\alpha_1$ is present in human, mouse, and rat genes and encodes the dipeptide sequence GluThr (ET; Fig. 5). An equivalent 6 base exon is also present in the *Drosophila* Ca channel gene, *DmcalA (112–114),* encoding the dipeptide sequence HisAsp (HD; Fig. 5). Exon 31a of $Ca_v2.2\alpha_1$ is expressed in a tissue-specific pattern, and its presence alters Ca channel function (*see* below). In rat and human, we have shown that exon 31a is preferentially expressed in neurons of the peripheral nervous system (PNS), but suppressed within the central

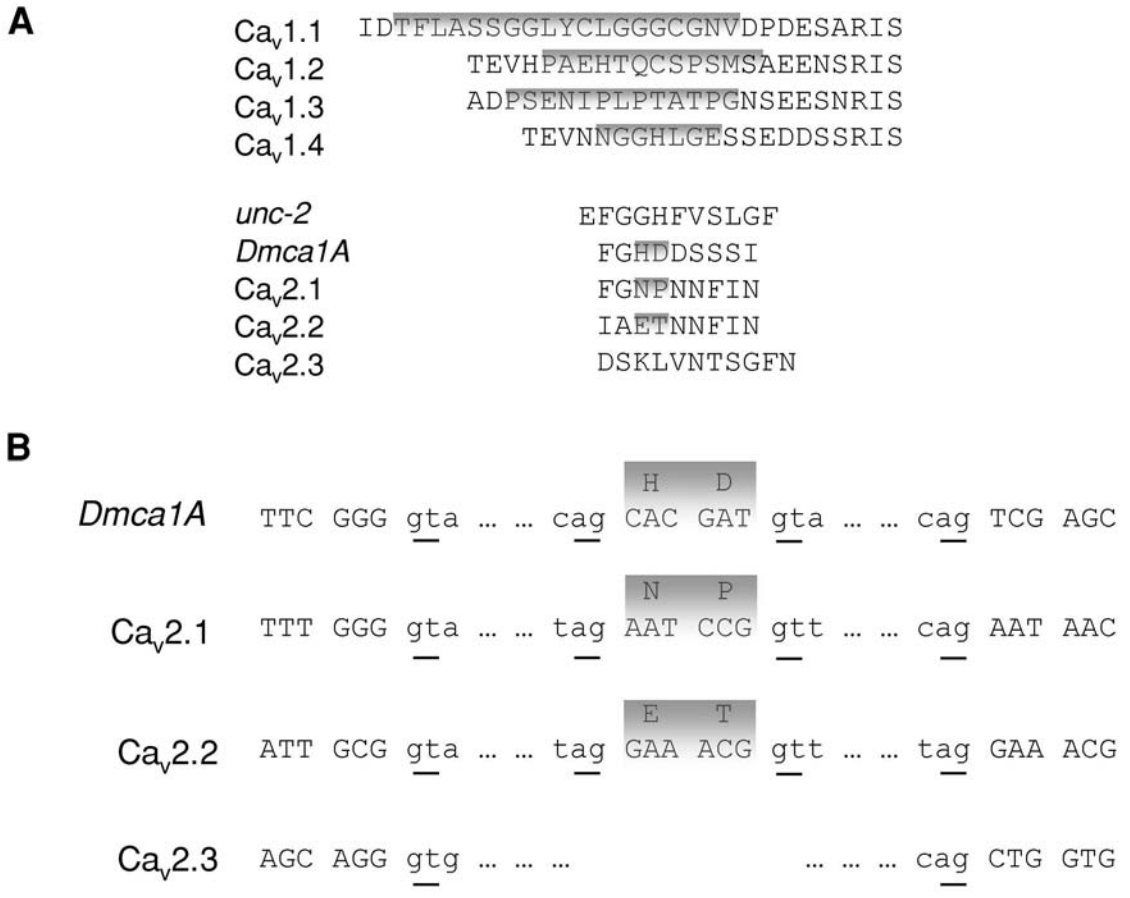

Fig. 5. Alternative splicing in domain IVS3-IVS4 is conserved among several $Ca_v\alpha_1$ genes. **(A)** Amino acid sequences of putative IVS3-IVS4 extracellular linkers of several $Ca_v\alpha_1$ genes are compared. Sequences in these regions that are encoded by alternatively spliced exons are shaded. Evidence for alternative splicing in these genes was obtained from the following sources: $Ca_v1.1\alpha_1$: *(39,110),* $Ca_v1.2\alpha_1$: *(36,39,110),* $Ca_v1.3\alpha_1$: *(16,110,111),* $Ca_v1.4\alpha_1$: *(157),* $Ca_v2.1\alpha_1$: *(55,58,109),* $Ca_v2.2\alpha_1$: *(49,156,158),* $Ca_v2.3\alpha_1$: *(39,159), Dmca1A: (113),* and *unc-2:* Genbank U25119. There is a splice junction between Arg (K) and Lys (L) in the IVS3-IVS4 region of the $Ca_v2.3\alpha_1$ gene, but the intervening intron is short and there is no evidence for the presence of an alternatively spliced exon. **(B)** The splice junctions in the IVS3-IVS4 domain of closely related Ca_v2 genes are conserved in human and *Drosophila.* The intron/exon boundaries in the equivalent domain of the human $Ca_v2.1\alpha_1$, $Ca_v2.2\alpha_1$, and $Ca_v2.3\alpha_1$ genes and *Drosophila Dmca1A* gene are compared. Exon sequence is shown in upper case and intron sequence in lower case. Amino acids are noted above the alternatively spliced exons and the region of alternative splicing is shaded. Dinucleotide *gt-ag* consensus splice junction sequences are underlined. The conserved splice junction in $Ca_v2.3\alpha_1$ is illustrated, for comparison.

nervous system (CNS). Analysis of the $Ca_v2.2\alpha_1$ human genomic structure in this region reveals that exon 3la is flanked by 10kb and 4kb introns, 5′ and 3′, respectively, that contain consensus *ag-gt* splice junctions (Figs. 3, 5B; *49,53).* Similar exon/intron boundaries are present in the equivalent region of the human $Ca_v2.1\alpha_1$ gene (Fig. 5B). The corresponding exon of the human $Ca_v2.1\alpha_1$ gene, encoding the dipeptide AsnPro (NP; Fig. 5), is

located within an approx 9 kb intronic region and its expression is also regulated. However, unlike exon 31a of $Ca_v2.2\alpha_1$, the NP exon of $Ca_v2.1\alpha_1$ is expressed at significant levels in various regions of the CNS *(53,55,56).* How the spliceosome is able to recognize a 6-nucleotide sequence buried within such long introns will be interesting to discover. There is a relatively high degree of conservation of elements in the introns flanking exon 31a in mouse, rat, and human $Ca_v2.2\alpha_1$ genes, suggesting that cross-species sequence analysis might provide insights into key elements regulating expression of this and other alternatively spliced exons.

Functional Consequences of Alternative Splicing in Domain IVS3-IVS4

The presence of exon 31a in domain IVS3-IVS4 of $Ca_v2.2\alpha_1$ shifts the voltage-dependence of N-type Ca channel activation to more depolarized voltages and slows the rate of channel opening *(49,53).* This suggests that N-type Ca channels in brain activate earlier and more rapidly during an action potential, compared to those in peripheral neurons. Indeed, modeling has shown that such differences can impact significantly the magnitude of action potential-evoked Ca entry through N-type Ca channels in neurons *(53).* Alternative splicing of the homologous exon in the closely related $Ca_v2.1\alpha_1$ gene also slows the rate of P/Q-type Ca channel activation *(56,58).* The similar functional consequences of alternative splicing at the IVS3-IVS4 loci of both $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ genes imply a common functional role. $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ subunits are the functional cores of P/Q-type and N-type Ca channels, respectively. Together these two channels couple excitation to transmitter release at the majority of synapses in the mammalian nervous system *(4).* It is therefore highly likely that tissue-specific expression of functionally distinct IVS3-IVS4 splice isoforms of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ plays an important role in tailoring the release of neurotransmitter in different regions of the nervous system.

The mechanism by which modification of the IVS3-IVS4 linker in voltage-gated ion channels affects the kinetics and voltage-dependence of channel opening is not known. Curiously, the IVS3-IVS4 alternative exons of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ encode different dipeptides (ET and NP, respectively), yet both slow Ca channel activation kinetics to a similar degree *(53).* The close proximity of the IVS3-IVS4 extracellular linker to one of four putative voltage-sensors (IVS4) is consistent with the fact that modifications in this region influence channel activation. It is not unreasonable to hypothesize that the length or the secondary structure of the IVS3-IVS4 linker places restraints on the movement of the adjoining S4 helix thereby influencing the kinetics of channel activation. Consistent with this hypothesis we have observed differences in the time course of gating currents, believed to arise from the coordinated movement of the S4 helices, in cells expressing S3-S4 splice isoforms of $Ca_v2.2\alpha_1$ (Y. Lin, S. McDonough, and D.L., unpublished observations). Irrespective of the mechanism by which alterations in the IVS3-IVS4 linkers influence the gating of voltage-gated ion channels, it is clear that this region is an important site for regulating the efficacy of coupling voltage-sensing to channel opening *(106,107).*

Pharmacological Consequences of Alternative Splicing in Domain IVS3-IVS4

The identification of a toxin for the N-type Ca channel that binds preferentially to the S3-S4 linkers of $Ca_v2.2\alpha_1$ would be enormously helpful in discriminating between S3-S4 splice isoforms of this class of channel. The widely used ω-conus toxin, ω-conotoxin GVIA, inhibits IVS3-IVS4 splice isoforms of $Ca_v2.2\alpha_1$ equally well (Z. Lin and D.L., unpublished observations), consistent with structure-function studies that do not place the main site of ω-conotoxin GVIA binding close to the IVS3-IVS4 extracellular linker of $Ca_v2.2\alpha_1$ *(115).* However, there are reasons to hope that in the future other toxins may be identified that bind to and discriminate between S3-S4 splice iso-

forms of the N-type Ca channel. Alternative splicing in the IVS3-IVS4 linker of the P/Q-type $Ca_v2.1\alpha_1$ gene, for example, has been shown to affect ω-agatoxin IVA sensitivity *(55,58).* Based on an observed 10-fold difference in toxin sensitivity between $Ca_v2.1\alpha_1$ IVS3-IVS4 splice isoforms, Snutch and colleagues have proposed that this alternative splicing accounts for the presence of high (P-type) and low (Q-type) affinity ω-aga IVA-sensitive Ca channels in various neurons *(116–118).* Recent evidence, however, suggests that alternative splicing of $Ca_v2.1\alpha_1$ exon 31a is not sufficient to reconstitute the high ω-aga IVA affinity *(51).* Other factors, such as which $Ca_v\beta$ subunit associates with the channel and what cell-specific post-translational modifications the channel undergoes, are also likely to be required in order to generate the complete P- and Q-type Ca channel phenotypes *(33).* Voltage-gated channels other than Ca channels are also inhibited by toxins that target their respective S3-S4 linkers *(55,58,119–121).* In the case of the voltage-gated Na channel α subunit, a single amino acid (Gly) within the IIS3-IIS4 linker plays a major role in β-scorpion toxin binding *(121).*

The Intracellular Linker Connecting Domains II and III

The composition and size of the intracellular linkers connecting domains II and III ($L_{II–III}$) are highly variable among different $Ca_v\alpha_1$ subunits. These cytoplasmic domains play a key role in coupling $Ca_v\alpha_1$ subunits to their respective downstream effector proteins. In skeletal muscle, the $L_{II–III}$ region of $Ca_v1.1\alpha_1$ couples the voltage-gated Ca channel to the ryanodine receptor, creating a physical bridge to the sacroplasmic reticulum *(22).* The $L_{II–III}$ linkers of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ subunits bind the synaptic proteins syntaxin and SNAP-25, consequently $L_{II–III}$ is thought to be critical for coupling P/Q-type and N-type Ca channels to neurotransmitter release in presynaptic nerve terminals *(63,122–125).* The $L_{II–III}$ region of $Ca_v2.2\alpha_1$, encoded by exons 18 through 21, is shown in Fig. 4. The region is dominated by an unusually long exon, e19, that encodes upwards of 200 amino acids and is present in all three $Ca_v2\alpha_1$ genes. Isoforms of all three $Ca_v2\alpha_1$ subunits that differ in the composition of $L_{II–III}$ are expressed in neurons.

$L_{II–III}$ isoforms of P/Q-Type $Ca_v2.1\alpha_1$

$L_{II–III}$ isoforms were first identified for $Ca_v2.1\alpha_1$ using isoform-specific antibodies. Immunolocalization studies suggest that two $L_{II–III}$ isoforms of $Ca_v2.1\alpha_1$ are both present in presynaptic nerve terminals *(64,126,127),* but their precise expression patterns differ *(64).* Interestingly, the two $L_{II–III}$ isoforms interact differentially with SNARE proteins. Whereas a $L_{II–III}$ peptide fragment of the so-called BI isoform of $Ca_v2.1\alpha_1$ binds syntaxin, SNAP-25, and synaptotagmin, a $L_{II–III}$ peptide corresponding to the equivalent region of the rbA isoform of $Ca_v2.1\alpha_1$ does not bind syntaxin, and its interactions with SNAP-25 and synaptotagmin are Ca-dependent *(63,65,128).* These studies imply that the two isoforms of $Ca_v2.1\alpha_1$ might differ in excitation-secretion coupling efficacy at synapses *(125).* Catterall and colleagues have suggested that alternative splicing in $L_{II–III}$ of $Ca_v2.1\alpha_1$ is the mechanism that generates BI and rbA isoforms, however, this awaits confirmation at the RNA and genomic levels.

$L_{II–III}$ isoforms of N-Type $Ca_v2.2\alpha_1$

$L_{II–III}$ isoforms of the N-type $Ca_v2.2\alpha_1$ subunit, identified in mRNA from the mammalian nervous system *(57,67,95,129),* have been shown at the genomic level to arise from cell type-specific alternative splicing of exon 18a *(57),* and, recently, from large deletions of several exons that encode $L_{II–III}$. The $L_{II–III}$ large deletion isoforms identified recently have been found in human brain, and both lack critical elements in this region thought to mediate the binding of synaptic proteins. One of these $Ca_v2.2\alpha_1$ isoforms is reported to be missing exons 19 through 21, resulting in a 382 amino acid deletion from $Ca_v2.2\alpha_1$ *(67).* Another group of $L_{II–III}$ splice isoforms has also been reported to occur in several

$Ca_v\alpha_1$ genes. In this case, alternative splicing causes a frame shift and early truncation of the protein creating a two domain $Ca_v\alpha_1$ subunit *(96,130–133).* The two-domain isoform of $Ca_v2.2\alpha_1$, although not supporting functional channels, acts as a dominant negative that reduces functional expression levels of full-length $Ca_v2.2\alpha_1$ *(96).*

Exon 18a of $Ca_v2.2\alpha_1$

The alternatively spliced exon 18a of $Ca_v2.2\alpha_1$, which encodes a 21 amino acid segment close to the N-terminus of $L_{II–III}$, is of particular interest because its expression appears to be cell-type specific and developmentally regulated. The majority of $Ca_v2.2\alpha_1$ mRNA in adult sympathetic ganglia contains exon 18a (>80%). However, in the adult CNS, exon 18a is suppressed and is lowest in more rostral structures of the brain, such as neocortex (<20% of $Ca_v2.2\alpha_1$ mRNAs contain exon 18a). Evidence from *in situ* hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) analysis suggests that exon 18a is preferentially expressed in regions of the adult rat brain enriched in monoaminergic neurons *(95).* More studies are needed to map the cell-specific splicing patterns of the $L_{II–III}$ region of $Ca_v2.2\alpha_1$ but the close proximity of exon 18a to the putative site of SNARE binding, together with the results of Ghasemzadeh et al. *(95),* raise the possibility that splicing in this region of $Ca_v2.2\alpha_1$ may be important for targeting specific splice isoforms to release sites that contain distinct neurotransmitters. Clearly, it will also be interesting to know whether the presence of exon 18a, which is coincidentally enriched in serine and threonine residues, influences the binding of synaptic proteins.

The presence of the $Ca_v2.2\alpha_1$ alternatively spliced exon 18a in the human brain was recently challenged *(67).* Based on their analyses, Kaneko and colleagues failed to amplify any $Ca_v2.2\alpha_1$ cDNAs that contained exon18a from a fetal human brain cDNA library. We have shown recently that exon 18a is indeed expressed in the adult human CNS, and that its expression in brain is upregulated during development (A.C.G and D.L., unpublished observations).

Functional Consequences of Exon 18a Expression

Although it is still unclear whether inclusion of exon 18a in $Ca_v2.2\alpha_1$ influences the efficacy of neurotransmitter release, its presence in $L_{II–III}$ of $Ca_v2.2\alpha_1$ does have a functional effect on the biophysical properties of the N-type Ca channel. Exon 18a shifts the voltage-dependence of steady-state inactivation of the N-type Ca channel to a more depolarized level (*see* Fig. 6; *57).*

This type of modulation predicts reduced sensitivity of the N-type Ca channel to inactivation by small, prolonged depolarizations of the membrane potential, particularly between –80 mV and –60 mV (shaded region, Fig. 6). Our findings imply that the $L_{II–III}$ of $Ca_v2.2\alpha_1$ is an important site for regulating the voltage-dependence of N-type Ca channel availability. This is consistent with findings that co-expression of SNARE proteins, such as syntaxin or SNAP-25, with P/Q-type or N-type Ca channels shifts their voltage-dependence of inactivation, in this case to more hyperpolarized voltages, presumably via their interaction with the $L_{II–III}$ region *(128,134,135),* and with functional studies of alternative splicing in the $L_{II–III}$ region of the $Ca_v3\alpha_1$ family *(59).* Significantly, the recently identified $Ca_v2.2\alpha_1$ isoforms that are missing large segments of $L_{II–III}$ are also functionally different *(67).* The voltage-dependence of inactivation of both deletion isoforms was shifted toward more depolarized potentials. In addition, the $L_{II–III}$ deletion isoforms recovered more rapidly from inactivation compared to $Ca_v2.2\alpha_1$ channels that contained a complete $L_{II–III}$ *(67).* An interesting observation from this recent study suggests that the $L_{II–III}$ isoform lacking exons 19 though 21 is less sensitive to *Conus* toxins, ω-conotoxin MVIIA and GVIA. The lower sensitivity to ω-conotoxins may arise because both these toxins preferentially inhibit the inactive state of the N-type

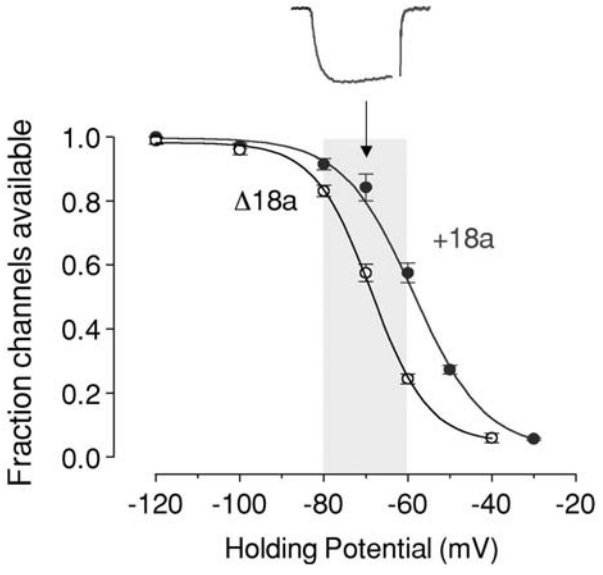

Fig. 6. Alternative splicing in the II-III intracellular loop in $Ca_v2.2\alpha_1$ modifies the voltage-dependence of N-type Ca channel availability. $Ca_v2.2\alpha_1$ splice isoforms differ in the expression of 21 amino acids encoded by exon 18a in the II-III intracellular loop. Normalized, averaged steady-state inactivation curves were calculated from N-type Ca currents induced by expressing $Ca_v2.2\alpha_1$ subunits lacking exon 18a (Δ18a, open circles, $V_{1/2}$ = –69 mV) or containing exon 18a (+18a, solid circles, $V_{1/2}$ = –59 mV). $Ca_v2.2\alpha_1$ splice isoforms were transiently expressed together with the Ca channel β_{1b} subunit in *Xenopus* oocytes. Exemplar currents for each splice isoform, induced by depolarizations to 0 mV from a holding potential of –70 mV, are shown above the graph. Currents were activated by brief depolarizations to 0 mV from various holding potentials between –120 mV and –30 mV. The gray shading highlights the fact that the splice isoforms differ most in their inactivation properties within a physiologically relevant range of holding potentials, between –80 mV and –60 mV. 5 m*M* Ba was the charge carrier. Other experimental details can be found in ref. *(57).*

Ca channel *(136)*, and the deletion isoform of $Ca_v2.2\alpha_1$ is more resistant to voltage-dependent inactivation.

Functional differences among $Ca_v2.2\alpha_1$ splice isoforms may depend on other factors for their expression. For example, the difference in the voltage-dependence of inactivation between $L_{II–III}$ splice isoforms of $Ca_v2.2\alpha_1$ was found to be $Ca_v\beta$ subunit-specific, when studied in *Xenopus* oocytes. Specifically, the N-type Ca channel availability curves of $Ca_v2.2\alpha_1$ splice isoforms differed from each other only when expressed together with either $Ca_v\beta_{1b}$ or $Ca_v\beta_4$ subunits. In contrast, there was no discernable difference between $Ca_v2.2\alpha_1$ splice isoforms when co-expressed with either $Ca_v\beta_{2a}$ or $Ca_v\beta_3$ *(57).* The mechanisms underlying this $Ca_v\beta$ subunit-specificity are not known, but the explanation may be relatively simple. $Ca_v\beta$ subunits have their own effects on the voltage-dependence of N-type Ca channel inactivation. N-type Ca channels containing $Ca_v\beta_{1b}$ and $Ca_v\beta_4$ inactivate at more hyperpolarized voltages ($V_{1/2}$ = –60 mV to –80 mV) compared to N-type Ca channels associated with $Ca_v\beta_{2a}$ and $Ca_v\beta_3$ that inactivate at significantly more depolarized membrane potentials ($V_{1/2}$ = –35 mV to –45 mV). If N-type Ca channel inactivation is most plastic at membrane potentials between –60 mV and –90 mV, differences between splice isoforms would be more apparent in the presence of $Ca_v\beta_{1b}$ or $Ca_v\beta_4$, but less obvious with $Ca_v\beta_{2a}$ or $Ca_v\beta_3$ subunits (*see* Fig: 6). It is nonetheless likely that alternative splicing of $Ca_v2.2\alpha_1$ and the expression of functionally distinct $Ca_v\beta$ subunits *(137)* combine to create a rich array of native N-type Ca channel currents that differ in their sensitivity to inactivation by voltage (*see 21,70,138).* There are other cases where particular Ca channel phenotypes require the presence of specific $Ca_v\beta$ subunits. A particularly interesting example comes from studies of the spinocerebellar ataxia type 6 (SCA6) mutation of the $Ca_v2.1\alpha_1$ gene. SCA6 is a neurodegenerative disorder characterized by selective death of Purkinje cells in the cerebellum. SCA6 is associated with an expansion of a trinucleotide CAG repeat in exon 47 of the $Ca_v2.1\alpha_1$ gene *(102).* The SCA6 polyglutamine expansion in the C-terminus of $Ca_v2.1\alpha_1$ shifts the voltage-dependence of channel activation and the rate of channel inactivation only in the presence of $Ca_v\beta_4$ and not when the channel is co-expressed with $Ca_v\beta_2$ and $Ca_v\beta_3$ *(139).*

Genomic Analysis of $L_{II–III}$

Analysis of the rat and human $Ca_v2.2\alpha_1$ genes reveals that exon 18a is a cassette exon flanked by ~4 and ~4.5 kb introns, 5′ and 3′, respectively, which contain consensus *gt-ag* dinucleotide signature sequences at the splice junctions. Interestingly, the 3′ intron also contains alternative dinucleotide splice-acceptors, *ag*, separated by three nucleotides that encode the amino acid, R758. All $Ca_v2.2\alpha_1$ cDNA rat clones that we have isolated so far contain the R758 codon, while others have reported R758-lacking α_1 clones in mouse neuroblastoma cells *(129)*, bovine chromaffin cells *(32)*, and human fetal brain *(67)*, suggesting that both splice-acceptor sites are utilized *(57)*. The functional significance of R758 is not known, but the presence of alternative 3′ or 5′ splice donor/acceptor sites is not uncommon. Alternative 3′ acceptor sites are also present at the intron/exon junction of $Ca_v2.2\alpha_1$ exon 10 and at the equivalent junction in $Ca_v2.1\alpha_1$. In both genes the splice acceptor sites are also only 3 nucleotides apart and control the presence of an additional amino acid, valine in $Ca_v2.1\alpha_1$ and alanine in $Ca_v2.2\alpha_1$, in the I–II intracellular loop (*see* Figs. 3, 4; *55,58*.) Genomic analysis has also provided insight into the mechanism by which the recently described large deletion $Ca_v2.2\alpha_1$ splice isoforms are generated *(67)*. As mentioned, the shorter $L_{II–III}$ splice isoform is generated by completely skipping exons 19, 20, and 21, and involves the use of consensus *gt-ag* dinucleotide splice junctions. The splicing pattern required to generate the second $Ca_v2.2\alpha_1$ $L_{II–III}$ isoform reported in the Kaneko study is atypical *(67)*.

Putative, alternatively spliced exons are also located in the homologous $L_{II–III}$ regions of the $Ca_v2.1\alpha_1$ and $Ca_v2.3\alpha_1$ genes. In $Ca_v2.1\alpha_1$, a putative 72 nucleotide open reading frame can be identified in the human gene, flanked by consensus *ag-gt* splice junctions. This region may represent a 24 amino acid encoding exon that is alternatively expressed. However, this putative exon cannot account for the presence of $Ca_v2.1\alpha_1$ rbA and BI isoforms that differ in their abilities to bind SNAREs, discussed earlier, because it is located upstream of the variable region that distinguishes them *(63)*. The third member of the $Ca_v2\alpha_1$ gene family, $Ca_v2.3\alpha_1$, contains an isolated exon that encodes 19 amino acids. Though its tissue distribution and functional effects on channel activity have not been fully determined, multiple $Ca_v2.3\alpha_1$ mRNA sequences have been reported, some containing and others lacking the 57 nucelotide sequence (e.g, *41*). RT-PCR analysis also indicates that in $Ca_v2.3\alpha_1$ this exon is differentially expressed in neocortex and cerebellum *(50,52)*.

The C-Terminus

The C-terminus of $Ca_v\alpha_1$ constitutes about one-fourth of the channel protein and has been implicated in regulating various aspects of Ca channel function, including calcium/calmodulin-dependent inactivation, G-protein-mediated modulation, and protein-protein interactions that regulate activity and target the channel to specific cellular compartments *(66,93,101,140–146)*. The C-terminus is also the most divergent domain among different $Ca_v\alpha_1$ genes. Biochemical analyses have provided evidence for the presence of at least two size forms of most $Ca_v\alpha_1$ subunits in the mammalian brain, including $Ca_v2.1\alpha_1$, $Ca_v2.2\alpha_1$, $Ca_v2.3\alpha_1$, $Ca_v1.2\alpha_1$, and $Ca_v1.3\alpha_1$ *(16,42,58,64,147)*. There is now good evidence that alternative splicing in the C-terminus is an important mechanism for generating some of the different size forms of $Ca_v\alpha_1$ subunits *(16,31,38,41,42,50,55,56,93,97,98,102,103,142,148)*. Although the functional consequences of alternative splicing in the C-terminus are not fully determined, effects on inactivation kinetics have been reported for $Ca_v1.2\alpha_1$ and $Ca_v2.1\alpha_1$ *(55,56,139,142,148)*, and on the ability of G-proteins to inhibit the $Ca_v2.2\alpha_1$ N-type Ca channel *(66)*. Further, only certain splice isoforms of the $Ca_v2.1\alpha_1$ gene, those that contain exon 47 in the C-terminus, express the SCA6 polyglutamine expansion that underlies spinocerebellar ataxia type 6 *(102)*.

In $Ca_v2.2\alpha_1$, the C-terminus is encoded by at least 10 constitutively expressed exons (e37–e46; Figs. 3, 4). The beginning of the C-terminus is encoded by either exon 37a or 37b, which appear to be expressed in a mutually exclusive pattern (Mittman and Agnew, 2000, Society for Neuroscience Abstracts). Functional studies will be required to determine the potential importance of mutually exclusive alternative splicing in the C-terminus of $Ca_v2.2\alpha_1$, however, sequence analysis indicates that exon 37b contains an EF-hand domain that is absent in exon 37a (Pan et al., 2001, Society for Neuroscience Abstracts). Similar mutually exclusive exons are also present in the C-terminus of $Ca_v2.1\alpha_1$ *(55,56).* These exons are differentially expressed in the nervous system *(55),* and recent data suggests that alternative splicing in this region of $Ca_v2.1\alpha_1$ modulates Ca-dependent pre-pulse facilitation of P/Q-type channels (Chaudhuri et al., 2001, Society for Neuroscience Abstracts).

One of the attractions of studying ion channels is the ability to monitor channel behavior with unparalleled precision using electrophysiological recording methods. Consequently, even relatively subtle changes in the biophysical properties of the channel, either gating or ion permeation, can be detected with high resolution. A significant fraction of the $Ca_v\alpha_1$ subunit is, however, devoted to the creation of protein binding sites that may not impact the gate or the ion channel pore directly, but that are essential for signaling. In particular, the C-terminus of the $Ca_v\alpha_1$ subunits is known to be an important region for targeting channels to specific subcellular compartments. Without knowing which proteins bind to the C-terminus of specific $Ca_v\alpha_1$ subunits, the impact of alternative splicing in this region as it relates to targeting has been difficult to address. However, Bezprozvanny and colleagues have demonstrated specific interactions between the C-termini of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ subunits with two modular proteins, Mint1 and CASK, found at presynaptic nerve terminals *(93).* Significantly, this interaction is splice isoform-specific. Only the long C-terminal splice isoforms of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ bind Mint1 and CASK *(67,93).* This implies that alternative splicing in the C-terminus of $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ may be an important mechanism for regulating Ca channel density at presynaptic nerve terminals and consequently the efficacy of synaptic transmission.

How Many Splice Isoforms Can Individual Ca Channel α_1 Genes Generate?

Our discussion thus far has focused on those regions of $Ca_v\alpha_1$ genes, in particular $Ca_v2.2\alpha_1$, where alternatively spliced exons have been identified and, in some cases, their functional effects on the channel characterized. The large size of the $Ca_v\alpha_1$ genes, containing upwards of 50 exons and spanning at least 250 kb in the human genome (Fig. 3), however, suggests an even greater potential for diversity and consequently functional specialization arising from alternative splicing. It is not unreasonable to expect that each $Ca_v\alpha_1$ gene may contain at least 10 sites of alternative splicing. It is possible that certain alternatively spliced exons are always co-expressed in specific tissues such that only a subset of combinations are permitted or preferred *(47,51,139).* However, *if* each site is independently regulated, alternative splicing could give rise to over 1000 distinct mRNAs from each $Ca_v\alpha_1$ gene. This may at first seem unreasonable, but there is precedent for this level of diversity in neuronal genes. For example, as a result of alternative splicing in neurons, three neurexin genes, each containing nearly 20 constitutively expressed exons, can potentially generate more than 1000 isoforms *(149,150).* Similarly, there is evidence for cell-specific expression of several hundred isoforms of the calcium-activated potassium channel *Slo* gene in hair cells of the inner ear *(90,91).* Further, the recently cloned *Drosophila* axon guidance gene, *DSCAM,* is predicted to generate more than 38,000 splice isoforms, a number that exceeds current estimates of the total gene number in *Drosophila (80).*

In order to appreciate the level of structural and functional diversity arising from alternative

splicing, it will be important to identify which exons are alternatively expressed and the alternate splice donor and acceptor sites that regulate their expression. Splice isoforms among the different $Ca_v\alpha_1$ gene families were identified, initially, by comparing cDNAs isolated from a variety of different tissues and different species. More recently, additional sites of alternative splicing in $Ca_v\alpha_1$ genes have been identified from analysis of the human genomic database by comparing RT-PCR-derived cDNA sequences or using specific exon-finding algorithms *(45–47).* If large enough in size, previously unrecognized alternatively spliced exons will be identifiable on the basis of genomic sequence analysis alone. The EST databases, derived from sequencing cDNAs from a variety of normal and diseased tissues, are important adjuncts in identifying alternatively spliced exons, particularly small exons that reside in long intronic stretches. For example, as discussed earlier, the $Ca_v2.1\alpha_1$ and $Ca_v2.2\alpha_1$ genes contain 6 nucleotide, alternatively spliced cassette exons, buried in intronic stretches of >9 kb *(49,53).* These alternatively spliced exons were identified from analysis of cDNAs derived from different regions of the brain and could not have been predicted from the analysis of genomic sequence. Despite their small size, these short cassette exons are functionally significant, influencing Ca channel pharmacology and behavior. However, it is important to point out that analysis of expressed sequences derived *in silico* from the EST database are currently of limited use for analyzing the $Ca_v\alpha_1$ gene family because they are 3' biased, and large genes are not well-represented. RT-PCR has been successfully applied to screen for the presence of splice isoforms in a variety of tissues including different regions of the nervous system. The sensitivity of PCR permits amplification of even rare mRNAs, but even this can be a disadvantage because aberrant or incompletely spliced mRNAs may also be amplified. Aberrant splicing appears to be particularly prevalent in tumor cells *(151)* and cells that express a low level of target mRNAs (W. Xu and D.L., unpublished observations). However, low-abundancy, incorrectly spliced mRNAs can be amplified by RT-PCR from any tissue source. Such products may be readily distinguished from mature mRNAs in cases where relatively large intronic sequences are left unspliced. However, relatively short introns that are in frame may not be readily distinguished from functionally relevant splice sites. In this case, analysis of genomic sequence can help discern aberrant splice products from true splice isoforms. Clearly then the search for additional sites of alternative splicing requires a multi-pronged approach, combining mRNA analysis from a variety of tissues together with genomic and functional analyses. The advent of micro-array technology offers a way to rapidly screen mRNA from a wide variety of tissues, and has the potential to automate and, consequently, improve significantly the task of identifying even very short exons whose expression is tissue-specific. Exon arrays have already been used successfully to map the tissue- and disease-specific expression patterns of genes on chromosome 22 that contains >8,000 exons *(152).*

Given the somewhat overwhelming potential for generating perhaps tens to hundreds of isoforms from alternative splicing in each $Ca_v\alpha_1$ family alone, priority is now being given to characterizing patterns of alternative splicing in $Ca_v\alpha_1$ mRNAs expressed in specific cell-types. For example, single-cell RT-PCR has recently been used to clone the dominant splice isoforms of $Ca_v2.1\alpha_1$ from Purkinje cells *(51)* and more generally single-cell RT-PCR analysis has been used to analyze the pattern of expression of different Ca channel subunits, including $Ca_v\beta$, in identified neurons *(33,153).*

The challenge now is to demonstrate directly how alternative splicing in the $Ca_v\alpha_1$ family impacts signaling in the nervous system. The availability of certain tools, for example isoform-specific toxins and isoform-specific antibodies, will greatly aid in establishing the functional importance and cellular localization of particular splice isoforms. In addition, exon-specific gene targeting of $Ca_v\alpha_1$ family members in mice is an approach that will likely prove useful in the near future.

Diversity in neuronal signaling is a signature feature that separates simple and complex organisms. A surprising finding to emerge from recent genome-sequencing projects is that complexity within an organism does not correlate well with absolute gene number *(154).* Rather, there is a closer correlation between biological complexity and the level of diversity that can be achieved from a set of genes, in large part, due to alternative splicing. Studies of alternative splicing in the $Ca_v\alpha_1$ genes provide important information on the molecular origins of diversity in Ca signaling, but are also likely to contribute toward our general understanding of at least one of the molecular mechanisms that support complex signaling in the mammalian nervous system.

Acknowledgments

We are grateful to Andrew Castiglioni for comments. We also thank the reviewer for helpful suggestions. This work was supported by National Institutes of Health Grants NS29967, NS01927 (D.L.). ACG is a Howard Hughes Medical Institute Predoctoral Fellow.

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