The neuronal splicing factor Nova controls alternative splicing in N-type and P-type Ca\textsubscript{2} channels

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Many cellular processes are involved in optimizing protein function for specific neuronal tasks; here we focus on alternative pre-mRNA splicing. Alternative pre-mRNA splicing gives cells the capacity to modify and selectively re-balance their existing pool of transcripts in a coordinated way across multiple mRNAs, thereby effecting relatively rapid and relatively stable changes in protein activity. Here we report on and discuss the coordinated regulation of two sites of alternative splicing, e24a and e31a, in P-type Ca\textsubscript{2}.1 and N-type Ca\textsubscript{2}.2 channels. These two exons encode 4 and 2 amino acids, respectively, in the extracellular linker regions between transmembrane spanning segments S3 and S4 in domains III and IV of each Ca\textsubscript{2} subunit. Recent genome-wide screens of splicing factor-RNA binding events by Darnell and colleagues show that Nova-2 promotes inclusion of e24a in Ca\textsubscript{2}.2 mRNAs in brain. We review these studies and show that a homologous e24a is present in the Ca\textsubscript{2}.1 gene, Cacna1a, and that it is expressed in different regions of the nervous system. Nova-2 enhances inclusion of e24a but represses e31a inclusion in Ca\textsubscript{2}.1 and Ca\textsubscript{2}.2 mRNAs in brain. It is likely that coordinated alternative pre-mRNA splicing across related Ca\textsubscript{2} genes by common splicing factors allows neurons to orchestrate changes in synaptic protein function while maintaining a balanced and functioning system.

Introduction

Genes that encode the Ca\textsubscript{2}\textalpha\textsubscript{1} pore-forming subunits of voltage-gated calcium channels are large. Each Ca\textsubscript{2}\textalpha\textsubscript{1} gene includes 40–45 constitutive exons that are invariable in processed mRNA, as well as several alternative exons that are optional and cell-specific. Alternatively spliced (AS) exons serve two major functions. First, inclusion or exclusion of certain AS exons can shift the reading frame of mRNAs and result in early termination of protein translation, which may result in mRNA degradation by nonsense mediated decay.\textsuperscript{1,2} Cells use these exons to regulate protein expression levels downstream of transcription.\textsuperscript{3,4} Second, AS exons can modify protein sequence and thereby modify protein function. This is achieved by the inclusion, exclusion or substitution of alternatively spliced exons that encode as few as one and up to ~100 amino acids in key domains of the channel protein.\textsuperscript{5}

In this paper we focus on the regulation of alternatively spliced exons that give rise to protein splice isoforms of Ca\textsubscript{2} channels with unique expression patterns and functions.

The decision to include, skip or substitute a given AS exon usually occurs in the cell’s nucleus (although see ref. 7) and depends on interactions among the spliceosome, splicing factor proteins and the transcribed pre-mRNA. The spliceosome, a highly specialized macromolecular complex, is composed of five small nuclear RNA proteins (snRNPs) and hundreds of associated proteins and is common to all cell’s nucleus (reviewed in ref. 8). Components of the spliceosome bind at intron-exon boundaries and catalyze intron removal and exon joining. Additionally, cis elements in pre-mRNAs and the cell-specific splicing factors that bind to them direct cell-specific AS. Collectively they define the cell’s unique mRNA isoform profile.

Cell-specific splicing factors enhance or repress AS exons, and their expression patterns are tissue-, activity- and cell-specific.\textsuperscript{9} Our discussion here focuses on existing and new evidence that Nova, one of the earliest discovered tissue-specific splicing factors, regulates the pattern of AS exons in Ca\textsubscript{2} channels.\textsuperscript{10} Our emphasis on Nova is driven by two important facts. First, Nova is the first—and to date the only—splicing factor validated as a regulator of AS splicing of Ca\textsubscript{2} pre-mRNAs.\textsuperscript{11} Second, Nova-2 knockout mice exist, providing a way to test directly the consequences of Nova-2 loss on AS exon composition in Ca\textsubscript{2} mRNAs.\textsuperscript{12}

Nova Proteins Regulate Alternative Splicing of Synaptic Proteins

Nova proteins, originally discovered as the target antigens in the autoimmune neurological disorder paraneoplastic opsoclonus myoclonus ataxia (POMA), were the first mammalian neuron-specific splicing factors identified.\textsuperscript{10,13,16} Two genes encode Nova proteins: Nova1 and Nova2, although there is some evidence that other members of the Nova family await discovery.\textsuperscript{19} Nova-1 and Nova-2 proteins bind similar motifs, but their expression patterns differ. Nova-1 proteins are limited to subcortical CNS structures
in developing and adult mice. Nova-2 proteins, by contrast, are expressed throughout the CNS and particularly in cortex, olfactory bulb, thalamus, inferior colliculus, inferior olive, and the internal and external granule cell layers of the cerebellum.\textsuperscript{10} Like other splicing factors, Nova-1 and Nova-2 bind to consensus sequences close to target AS exons on pre-mRNAs. The three KH domains mediate Nova binding to clusters of YCAY motifs on target pre-mRNAs.\textsuperscript{10,15}

Nova-1 and Nova-2 knockout mice die 2–3 weeks after birth, but tissue from early postnatal animals shows that Nova is especially important for correct alternative splicing of two inhibitory receptor mRNAs in brain: glycine alpha 2 and GABA\textsubscript{A}.\textsuperscript{16,47} Nova-1\textsuperscript{-/-} mice and POMA patients share a phenotypic similarity—weakness and tremulousness due to motor neuron atrophy—that led Darnell and colleagues to hypothesize a role for disrupted AS of inhibitory synaptic proteins in POMA disease pathology.\textsuperscript{16} Evidence is accumulating that misregulation of alternative splicing of synaptic proteins, including voltage-gated calcium channels, can result in disease.\textsuperscript{18-24}

\textbf{CaV\textsubscript{2.2} is a Nova Target}

Darnell and colleagues have transformed our understanding of the extent, mechanisms, and potential functional importance of Nova-regulation of AS exons in the mammalian brain. By combining protein-RNA crosslinking and Nova-immunoprecipitation, in a technique called CLIP (cross-linking and immunoprecipitation) they were able to identify new Nova RNA targets.\textsuperscript{25,26}

Their studies generated the most comprehensive view to date of the extent and the mechanism of action of a cell-specific splicing factor. Nova binds YCAY clusters in introns generally within 500 nucleotides of target AS exons. When Nova binds clusters upstream of a target AS exon, it generally represses inclusion by blocking U1 snRNP binding; when Nova binds downstream of the target AS exon, it generally enhances inclusion (Fig. 1).\textsuperscript{11}

Subsequent studies have indicated that this appears to be a general feature of alternative splicing regulators, evident for example with not only Nova but Fox2, MBNL, hnRNP L, hnRNP C, YCAY motifs located downstream of e24a in mouse \textit{Cacna1b}.\textsuperscript{11} Splicing regulation of e24a by Nova is conserved in the homologous \textit{Cacna1b} genes of zebrafish and chicken, strongly suggesting that this AS exon plays a critical role in optimizing neuronal function in specific regions of the nervous system.\textsuperscript{28} Additional Nova-2 binding sites have been identified in \textit{Cacna1b}, motivating us to explore whether Nova-2 coordinates inclusion of other AS exons in \textit{CaV\textsubscript{2.2}} and homologous exons in the \textit{CaV\textsubscript{2.1}} paralog.

\textbf{AS Exons in CaV\textsubscript{2} Genes}

Figure 3 highlights some of the known AS exons in three closely related \textit{CaV\textsubscript{2}} genes—\textit{Cacna1a}, \textit{Cacna1b} and \textit{Cacna1e}—that encode \textit{CaV\textsubscript{2.1}}, \textit{CaV\textsubscript{2.2}} and \textit{CaV\textsubscript{2.3}} proteins, respectively. These proteins (in association with accessory subunit proteins) in turn underlie P-type, N-type and R-type currents in neurons. We and others have mapped the tissue distribution and functional consequences of AS exons on channel activity and drug action in mice in vivo.\textsuperscript{20,21,30-49} Several AS exons are conserved among the three \textit{CaV\textsubscript{2}} genes, and in some cases, for example the homologous exons e18a of \textit{Cacna1b} and \textit{Cacna1e}, their inclusion appears to be coregulated.\textsuperscript{31} Here we review published data from the Darnell lab and show new evidence that the same splicing factor, Nova-2, regulates the splicing of homologous exons, e24a and e31a in \textit{CaV\textsubscript{2.1}} and \textit{CaV\textsubscript{2.2}} channels.

\textbf{Nova-2 Regulates e24a and e31a in CaV\textsubscript{2} Channels}

E24a and e31a encode short sequences in the S3-S4 extracellular linkers of domains III and IV, respectively, of \textit{CaV\textsubscript{2.1}} and \textit{CaV\textsubscript{2.2}}. In separate studies, the Snutch and Williams labs showed that inclusion of e31a in \textit{CaV\textsubscript{2.1}} decreases the affinity of \textit{\omega}-agatoxin IVA for the channel -10-fold, while inclusion of e31a in either channel slows channel activation and deactivation kinetics.\textsuperscript{33,49,50} We analyzed gating currents from \textit{CaV\textsubscript{2.2}} channels containing e31a and showed that it affects the putative S4 voltage sensor.\textsuperscript{41} Most relevant to this discussion, we found e31a is absent (\textit{CaV\textsubscript{2.2}}) or at lower levels (\textit{CaV\textsubscript{2.1}}) in rat brain compared to peripheral ganglia.\textsuperscript{36} E24a of \textit{Cacna1b}...
have shown that e31a is found in CaV2.1 mRNAs in dorsal root ganglia (DRG) and superior cervical ganglia, similar to the distribution of e31a of CaV2.2.50 However, e31a-containing CaV2.1 mRNAs are also expressed in hippocampus and cerebellum albeit at levels lower than in ganglia, an expression pattern that is different from e31a of CaV2.2 which is at very low levels in all regions of the CNS.50

Putative Nova-2 binding sites have been mapped very close to the 31a exons of Cacna1b and Cacna1a genes. As discussed above, exons 31a of both genes are found at very low levels in brain, suggesting that Nova-2 might repress e31a inclusion. Consistent with this, we found an increase in the percentage of CaV2.1[e31a] transcripts (from 30% to 80%) and CaV2.2[e31a] transcripts (from not detectable to ~45%) in RT-PCR amplified products from Nova-2 knockout mouse brain cDNA compared to wild-type brain cDNA (Fig. 4). In cerebellum (CB), the increase in e31a-containing CaV2 sequences in Nova-2-/- was smaller (CaV2.1: from 20% to 50% and CaV2.2: ~10% to 30%) compared to the rest of the brain. But in DRG, the proportion

![Figure 2. Nova binding motifs are located in the intron downstream of e24a of CaV2.2. The genomic sequence in this region of Cacna1b is shown, and the amino acids encoded by e24a indicated (SFMG). The Nova binding motifs (black squares) are shown below the genomic sequence and were mapped to the UCSC genome browser (July 2007 mouse assembly). Nova binding motifs are shown within 70 nucleotides and downstream of e24a in Cacna1b. The Nova binding site track was provided by SFmap. SFmap is a computational tool that uses an algorithm to predict splicing factor binding sites by identifying binding site motif clusters and evaluating the conservation of these clusters across species.](image)

![Figure 3. Alternatively spliced exons in the CaV2 genes. The locations of constitutive exons (black) and conserved alternative exons (colored) in mouse Cacna1a, Cacna1b and Cacna1e genes. Gene diagrams are adapted from the UCSC genome browser (Feb. 2006 mouse assembly). Alternative exons 37a and 37b are found in all three CaV2 channel genes. An alternative exon e18a is present in Cacna1b and Cacna1e, but a homologous exon has not been identified in Cacna1a. Alternative exons e24a and e31a are conserved in Cacna1a and Cacna1b, but equivalent exons have not been identified in Cacna1e.](image)

CaV2.1 and CaV2.2 e31a

Exons 31a in CaV2.1 and CaV2.2 map to the extracellular linker between transmembrane spanning segments 3 and 4 in the IV domain of both channel proteins. An AS exon is located in this region of several CaVα1 subunits and in many species, including human and Drosophila. E31a in both CaV2.1 and CaV2.2 encode two amino acids, NP in CaV2.1 and ET in CaV2.2. We have shown that e31a is found in CaV2.1 mRNAs in dorsal root ganglia (DRG) and superior cervical ganglia, similar to the distribution of e31a of CaV2.2. However, e31a-containing CaV2.1 mRNAs are also expressed in hippocampus and cerebellum albeit at levels lower than in ganglia, an expression pattern that is different from e31a of CaV2.2 which is at very low levels in all regions of the CNS.

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of e31a-containing sequences was not different in wild-type compared to Nova-2−/− mice (~80–85%); this is consistent with the lack of Nova-2 in this tissue.

Our data show that Nova-2 is a repressor of e31a in both CaV2.1 and CaV2.2 pre-mRNAs in the mammalian brain (Fig. 1). Based on the very low to undetectable levels of e31a-containing CaV2.2 mRNAs in brain, Nova appears to exert stronger repression of e31a in CaV2.2 than in CaV2.1. Additional studies are needed to explore the precise mechanism of Nova action. For example, Nova repression of e31a in CaV2.2 might be stronger compared to CaV2.1, or the inclusion of e31a in CaV2.1 may also be influenced by other as yet unidentified splicing factors, such as a splicing enhancer that promotes e31a inclusion in specific brain regions.

**CaV2.1 and CaV2.2 e24a**

Several observations led us to hypothesize the presence of an AS exon in *Cacnala* equivalent to e24a of *Cacna1b*. These include the overall high degree of conservation between *Cacnala* and *Cacna1b* genes, their close functional roles, the conservation of AS exons 31a that are located in the homologous region of the channel in domain IV (IVS3-IVS4), and the location of a putative Nova binding site in the intron between exons 24 and 25 in *Cacnala*. We located a region in the intron that is strongly conserved among species (Fig. 5A). We used RT-PCR with primers located in constitutive exons e24 and e25 to amplify CaV2.1 mRNA isolated from different regions of the brains of wild-type mice (Fig. 5B). We observed two differently sized bands when we analyzed PCR cDNA products by gel separation, consistent with the presence of two alternative sequences that differ by only a few nucleotides. We confirmed a 12-nucleotide insert encoding the tetrapeptide sequence SSTR in CaV2.1-derived products by sequencing (Fig. 5A).

We would like to know the function of e24a in CaV2.1 and CaV2.2 channels. In CaV2.2, e24a has a relatively minor influence on channel properties. As described above, we only recently...
Channels

Nova Influences a Subset of Alternatively Spliced Exons in CaV2

Other sites of alternative splicing have been studied in detail by our lab (e18a and e37a/e37b in CaV2.2) and by other labs (e37a/e37b in CaV2.1).30,33-35,38,48,54-58 None of these AS exons appears to be regulated by Nova-2. For example, we find no difference in expression patterns of exon 37a of CaV2.2 in Nova-2 -/- and wild-type mice (data not shown). Exon 37a of CaV2.2 is enriched in nociceptors of DRG but is expressed at very low levels in other parts of the nervous system. Likewise, the expression pattern of e18a in CaV2.2 is similar in wild-type and Nova-2 -/- mice (data not shown). Although we focus on Nova-2 in this report, splicing factors other than Nova must regulate cell-specific inclusion of e18a and e37a/e37b. Furthermore, exon selection in vivo is likely to depend on the concerted actions of splicing enhancers and repressors (Fig. 6).4,58,60 The Darnell group recently concluded that ~15% of Nova-regulated exons might also be regulated by Fox proteins, another family of neuronal splicing factors.62

Summary

The nervous system operates with small error margins and adaptive responses are essential to normal neuronal function. These impact neuronal networks, individual neurons, and individual synapses and involve changes in synapse number, morphology and efficacy. Coordinated and temporal changes...
in protein activity and function underlie all these adaptive responses. Alternative pre-mRNA splicing has an impressive ability to generate a vast array of functionally distinct proteins. Alternative pre-mRNA splicing gives cells the capacity to modify and selectively re-balance their existing pool of transcripts in a coordinated way across multiple mRNAs, thereby effecting relatively rapid and relatively stable changes in protein activity.

Alternative pre-mRNA splicing events are predicted to occur in ~95% of multi-exon human genes. In this short report we review limited published data and in addition show evidence that four alternative exons in two closely related calcium channel CaV2.2 subunit genes are regulated by Nova-2. Thus, the ability of Nova to function both as repressor (e31a) and enhancer (e24a), depending on binding location imprinted in each gene, provides an elegant yet simple mechanism to coordinate splicing of homologous exons in related CaV2.2 genes (Fig. 1). This duality of function, based on RNA binding location relative to the target exon, is a property shared by several splicing factors. Cells control and coordinate patterns of alternative splicing by regulating the expression levels and activity of individual splicing factors. Coordinated alternative pre-mRNA splicing across related CaV2.2 genes allows neurons to orchestrate changes in synaptic protein function while maintaining a balanced and functioning system.

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