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Neuronal proteins custom designed by alternative splicing

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The evolution of alternative splicing in eukaryotes greatly expanded the number of functionally distinct proteins that could be produced from a finite gene pool. Extensive in the brains of higher organisms, alternative splicing might be the primary mechanism for generating the spectrum of protein activities that support complex brain functions. Alternative splicing is controlled at the level of individual neurons to custom design proteins for optimal performance. The expression profiles of splice isoforms are modified during development and as neuronal activity changes. Alternative splicing can lead to incremental, long lasting changes in ion channel and receptor activities, independent of changes in gene transcription. Recent studies of tissue-specific splicing factors are revealing how coordinated alterations in alternative splicing of RNA transcripts control synaptic function.

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Introduction: custom tuned proteins for fine control

Recent analyses suggest that at least 75% of multi-exon genes (see glossary) in the human genome are subject to alternative splicing (see glossary) [1]. This form of pre-mRNA post-transcriptional modification has the potential to expand the proteome exponentially, generating a spectrum of activities (Figure 1). Some genes generate tens of thousands of functionally distinct proteins [2]. Exon choice can vary with cell-type, stage of development, and activity. Alternatively spliced exons that encode as few as one or two amino acids can modify protein function. The inclusion or exclusion of alternative cassette exons at protein interaction sites (Figure 2) might act as molecular switches, permitting or preventing association with target proteins. Conversely, the selective inclusion of one of a series of cassette exons (Figure 2) could support graded changes in protein function that underlie modifications in neuronal excitability. This review emphasizes recent studies that support the theory that alternative splicing underlies the complex processing and adaptive properties that define nervous systems of higher organisms.

Molecular diversity on a large scale

Drosophila exhibit one of the most extreme examples of protein diversity to arise from alternative splicing. The *DSCAM* gene encodes a cell adhesion molecule (Down syndrome cell adhesion molecule) essential for defining axonal guidance in both embryonic and adult flies. The highly variable domains among DSCAM isoforms originate from the combinatorial arrangements of alternatively spliced exons selected primarily from three loci. Theoretically, alternative splicing can generate 38 016 distinct DSCAM proteins. This is double the predicted number of genes in the *Drosophila* genome. Half of these splice isoforms possess distinct extracellular immunoglobulin (Ig)-like binding motifs, suggesting a role in cell–cell recognition [2]. However, are all of these isoforms functional and distinct? A series of recent publications tackle this important question [3].

In an impressive study published last year, Zipursky and co-workers [4••] showed that DSCAM isoforms with different Ig-like domains exhibited distinct binding properties. By overexpressing a single DSCAM isoform in two discrete neuronal populations, these authors showed that DSCAM can mediate cell–cell interactions in *Drosophila*, *in vivo*. Specifically, the normal trajectory of interneuron axons across the midline was disrupted when axons of neurons overexpressing DSCAM encountered midline cells that also overexpressed the same DSCAM isoform type. Their data suggested that the extracellular domains of DSCAM mediate cell–cell interactions. Subsequent analyses of 11 DSCAM isoforms *in vitro* provided evidence for homophilic associations, binding between like Ig domains, but not heterophilic associations. Although only a limited number of DSCAM isoforms were tested, the work of Zipursky and co-workers provides an attractive model to explain how alternative splicing might regulate cell–cell interactions during neuronal development. The mammalian homolog of DSCAM is not subject to similar levels of alternative splicing, but other mammalian genes implicated in cell surface recognition, such as the neurexins, can generate thousands of isoforms [5]. Furthermore, different isoforms of Neuregulin-1 act as short-range and long-range attractants to direct the migration of interneurons toward the cortex [6].

Glossary
Alternative splicing: Most eukaryotic genes consist of several discontinuous coding sequences (exons) separated by non-coding sequences (introns). The initial step in transcription involves production of a pre-messenger RNA that represents a copy of the gene and contains exons and introns. This pre-messenger RNA undergoes several forms of processing that include a step called splicing. Introns are removed and exons are spliced together to form messenger RNA. Certain exons are present in all messenger RNAs transcribed from a specific gene (constitutive or invariant exons). Other exons are only present in a subset of messenger RNAs transcribed from a specific gene (alternative exons) (see Figure 2). Alternative splicing defines the process that determines if a specific exon is included or excluded. Alternative splicing is regulated by a number of factors that set the precise exon composition of messenger RNAs depending on cell type, stage of development and cell activity.
Multi-exon: Eukaryotic gene sequences are frequently interrupted by non-coding, intragenic sequences called introns. The majority of human genes therefore contain more than one exon.

Finding functional isoforms

There is an abundance of mRNAs in the mammalian brain that contain discrete regions of sequence variation but not all are produced from *bona fide* splicing and not all are functional. Functionally relevant splice isoforms are expressed at significant levels in a specific tissue, cell-type, or during a particular stage of development. Recent studies have, therefore, focused on the role of specific ion channel splice isoforms in identified neurons with defined functions [7,8].

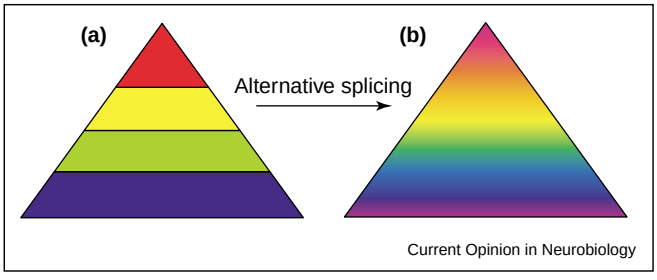
Cell-specific expression of ion channel splice isoforms

Perhaps the best demonstration that alternative splicing of ion channel pre-mRNAs can regulate neuronal function comes from studies of calcium activated-potassium channels (Slo) in chicken cochlea. Here, the pattern of alternative splicing of *Slo* transcripts closely follows the tonotopic map of hair cell tuning along the basilar membrane [9]. This remarkable correlation between the expression of splice isoforms of *Slo* and the tuning frequency in hair cells shows that alternative splicing can optimize neuronal excitability over a range of frequencies.

Certain other neurons in the central nervous system are noted for their ability to sustain high frequency firing. In this case, voltage-gated $K_V3.1$ and $K_V3.2$ potassium channels have been implicated in driving efficient repolarization essential for rapid spike firing. However, not all the features of recombinant $K_V3.1$ and $K_V3.2$ channels, whether expressed separately or in combination, parallel those of native potassium currents. In particular, recombinant K_V3 channels require strong depolarizations to open, whereas K_V channels with more negative activation thresholds are needed to sustain the rapid repolarization of fast spiking neurons. Recently, Surmeier and co-workers [8] used single cell reverse transcriptase–polymerase chain reaction (RT–PCR) combined with electrophysiological recordings of the same cells to profile different K_V mRNAs in fast spiking and regular spiking neurons. They identified a specific potassium channel splice isoform, $K_V3.4a$, that is co-expressed with $K_V3.1$ in parvalbumin-containing, GABAergic globus pallidus projection neurons, parvalbumin-containing GABAergic CA1 hippocampal interneurons, and glutaminergic subthalamic nucleus neurons, all fast-spiking. By contrast, $K_V3.4a$ mRNA was conspicuously absent in regular spiking neurons of hippocampus, striatum and basal forebrain. Importantly, the authors also demonstrated that recombinant $K_V3.4a$ channels co-expressed with $K_V3.1$ could reconstitute potassium currents with low activation thresholds that mimicked the native currents. Other K_V channels are clearly crucial for reconstituting fast spiking properties, but it is remarkable that $K_V3.4a$ appears to be an essential contributor to this process in three distinct neuronal populations in different brain regions.

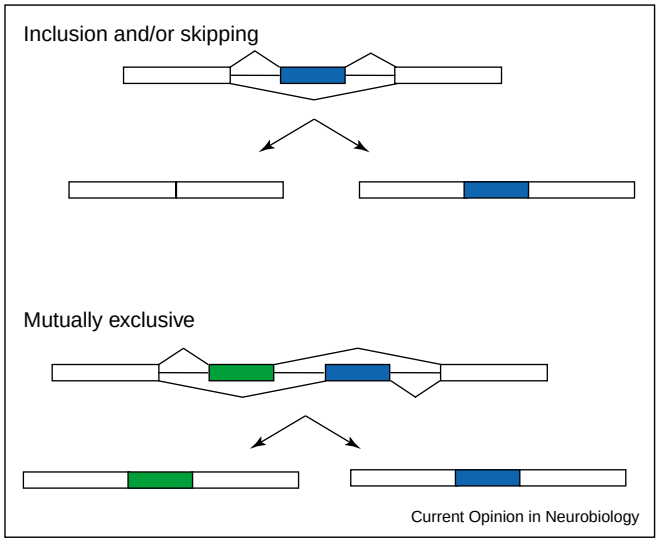
My research group applied similar single cell RT–PCR techniques to reveal cell-specific splicing of N-type calcium channel $Ca_V2.2$ pre-mRNA in sensory neurons of the dorsal root ganglia [7]. N-type calcium channels regulate neurotransmitter release in the dorsal horn of

Figure 1



Alternative splicing generates a spectrum of protein activities. In this illustration, protein activity is represented as color. Alternative splicing substantially increases the range of different activities that can be generated from a limited gene pool (compare a with b). **(a)** The sharp boundaries between activities encoded by different genes are converted to **(b)** gradual transitions in the presence of alternative splicing.

Figure 2



Two forms of regulated alternative splicing. Eukaryotic genes consist of several discontinuous coding sequences (exons) that average 50–300 nucleotides in length. Non-coding sequences (introns) lie between exons and are more variable in length, ranging from 200 to several thousand nucleotides. The spliceosome and specific splicing factors mediate intron removal and exon splicing. Dinucleotide sequences, gt and ag, reside at the respective 5' and 3' boundaries of more than 99% of introns. Invariant, constitutively expressed exons (black) are shown flanking alternatively spliced exons (blue/green). Exons might be included or excluded (top) or a series of homologous exons might be mutually exclusive, whereby only one will be selected and included in the final fully spliced mRNA (bottom). Other examples of alternative splicing not depicted here include the use of multiple 5' donor and/or 3' acceptor splice sites, and the use of alternative 5' promoters. Even more subtle variations arise from the direct editing of specific RNA nucleotides.

the spinal cord from nociceptive neurons and are the targets of the analgesic Ziconotide. Consequently, there has been intense interest in establishing if nociceptive neurons express a specific splice isoform of the N-type channel. The human N-type *$Ca_V2.2$* gene contains several sites of alternative splicing that are differentially regulated in the nervous system and that modify channel properties [10]. In the C-terminus coding region of the *$Ca_V2.2$* gene a pair of mutually exclusive exons, 37a and 37b, are present that differ by 14 out of the 32 amino acids that they encode. All $Ca_V2.2$ clones isolated and sequenced before our study contained only one of these exons, exon 37b. We reasoned that exon 37a-containing $Ca_V2.2$ isoforms must be restricted to a subset of neurons not represented in the database. We confirmed this, showing that $Ca_V2.2$-exon 37a was expressed primarily in dorsal root ganglia of rat and at low levels in other parts of the nervous system including brain. Furthermore, we showed exon 37a was preferentially expressed in nociceptive neurons of dorsal root ganglia by separating neurons based on capsaicin-sensitivity and applying single cell RT–PCR in the same cells. Exon 37a inclusion results in larger N-type currents compared with those of the default 37b-containig splice isoform [7]. The functional significance of this large current phenotype to nociception needs to be determined. However, exon 37a-containing splice isoforms of $Ca_V2.2$ can be added to the list of unique genes expressed by nociceptive neurons. Alternative splicing of other ion channel pre-mRNAs in dorsal root ganglia might also be unique [11]. Coordinated alternative splicing events among several types of ion channels in dorsal root ganglia could contribute to tuning the responsiveness of individual neurons to sensory stimuli. Alternative splicing could also bring about the remodeling of sensory neuron excitability that occurs during chronic pain states. Shifts in the pattern of alternative splicing in sensory neurons in neuropathic conditions might suggest new approaches for therapy. For example, the activity of the splicing factor Htra2-β1 can be upregulated by butyrate and other chemicals, to restore significantly the levels of the spinal motor neuron gene product (SMN1) in cell cultures from patients with proximal spinal muscular atrophy [12].

Alternative splicing modifies synaptic strength

An essential feature of adaptive, complex nervous systems is their capacity to modify neuronal output as sensory input changes. Modifications in ion channel and neurotransmitter receptor functions underlie adaptive changes in neuronal excitability and synaptic efficacy. There are numerous, recent examples of changes in alternative splicing of ion channels and synaptic proteins during development: voltage-gated Ca_V2 calcium channels [10,13], synaptosomal protein 25 kDa (SNAP-25) [14], postsynaptic density-95 (PSD-95) [15], alpha-

amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) [16], muscarinic glutamate receptor (mGluR) [17], kainate receptor [18], neuregulin [6], and receptors for gamma amino-butyric acid (GABA) and glycine [19].

In postsynaptic membranes, a splice variant of the synapse-associated protein-97 (SAP-97) recruits AMPA receptors specifically to cortical spines [20•]. The targeting of SAP-97 depends on the presence of a cassette exon that defines the 13 region, located between Src homology 3 (SH3) and guanylate kinase (GK) domains. The 13 region is a site that binds protein 4.1 that, in turn, binds to the cytoskeleton and AMPA receptors. Overexpressing this unique splice isoform of SAP-97 induces spine enlargement and facilitates synaptic transmission in cortical cultures by increasing AMPA receptor number at the synapse. By contrast, similar overexpression studies with isoforms of SAP-97 lacking the 13 domain results in diffuse SAP-97 expression and has no effect on synaptic transmission in cortical cultures. Regulated alternative splicing of SAP-97, therefore, represents a mechanism to up and downregulate the efficacy of glutamatergic synapses. Similarly, in spinal cord, expression of a specific splice isoform of gephyrin, a glycine receptor-tubulin-bridging molecule, sets the level of glycine receptor accumulation at inhibitory synapses [21].

Activity modifies alternative splicing of synaptic mRNAs

Alternative splicing also represents an attractive mechanism for modifying protein function in neurons after changes in activity. Ehlers and co-workers [22••] provided compelling evidence that alternative splicing of the NMDA receptor drives bidirectional modification of synaptic strength, as neuronal activity is turned up or down. These authors initially showed that NMDA receptor accumulation is controlled at the level of its export from the endoplasmic reticulum (ER) to the plasma membrane, and that changes in the electrical activity of cortical cultures affect surface expression of the NMDA receptor. Their most significant finding was that alternative splicing of the C2–C2' domain of the NMDA receptor was the control point connecting these processes. Alternative splicing serves as a gear lever that either holds the receptor in check during periods of increased neuronal activity (C2 variants) or promotes its export from the ER (C2' variants) when activity is inhibited. Bicuculline-induced increases in cortical culture activity were found to shift the pattern of splicing of the NMDA pre-mRNA toward the C2-exon, whereas tetrodotoxin-induced decreases of neuronal excitability promoted C2'-exon inclusion. This new signaling pathway could underlie homeostatic plasticity; the ability of neuronal networks to maintain constant activity in the face of constantly changing input [23]. Certain features of alternative splicing, including the ability to support incremental changes in ion channel and receptor activities, that are relatively long lasting and independent of gene transcription, seem ideally suited to homeostatic plasticity. But what factors link membrane depolarization to pre-mRNA splicing?

Orchestrating splicing in select tissues and cells

Above, I cite examples that illustrate dynamic changes in alternative splicing of individual genes. However, studies of those factors that regulate alternative splicing address how this process underlies coordinated changes in the expression of splice isoforms from multiple genes, which collectively control neuronal function and cell phenotype [24••,25••,26•].

Coordinated alternative splicing in genes that mediate inhibition

Nova proteins were the first mammalian tissue-specific splicing factors to be identified. They are restricted to the nervous system and are mutated in the human disease paraneoplastic opsoclonus myoclonus ataxia (POMA). Building on these observations, Darnell and co-workers [25••] undertook an unbiased screen of RNAs that bound Nova. They used ultraviolet cross-linking and subsequent immuno-precipitation of protein–RNA complexes to identify several Nova interacting RNAs. Significantly, they showed that a disproportionate number (70%) of complexes were formed with RNAs that encode proteins found at the synapse as compared with proteins in other regions of the neuron. An interesting subset of these was involved in inhibition. To hone in on functionally relevant interactions, the authors looked for changes between wild type and Nova$^{-/-}$ mouse brain preparations in splicing patterns at the specific loci identified in their screen. Large deviations were observed in alternative splicing of three genes *JNK2*, *neogenin*, and *gephyrin*; gephyrin is an essential protein for targeting $GABA_A\gamma2$ and GlyR$\alpha2$ receptors to inhibitory synapses. The results of this Nova screen support functional studies discussed earlier that show that the C5-gephyrin splice isoform determines the strength of synaptic inhibition [21].

Clearly many more splicing factors must exist that direct changes in neuronal processes. Synaptic transmission mutants of *Caenorhabditis elegans* were recently used by Hobert and co-workers [26•] to identify two splicing factors: UNC-75 and EXC-7. EXC-7 is a homolog of ELAV (derived from the initial mutant phenotypes: embryonic lethality and abnormal visual systems) a neuronal-specific splicing factor previously identified in *Drosophila*. UNC-75 protein appears to be needed at the mature synapse. Hobert and co-workers compared the severity of deficits in single *unc-75* and *EXC-7* mutants with those in *unc-75* and *EXC-7* double mutants to reveal that these proteins have non-redundant and distinct roles in cholinergic synaptic transmission. Future studies should determine the importance of *unc-75* and its

mammalian homologs for regulating synaptic efficacy at mature synapses.

Common proteins involved in highly specialized tasks

While the search for tissue-specific splicing factors continues, a recent report from Fu and co-workers suggests that splicing factors common to many tissues that control constitutive exon splicing can also control alternative splicing [24••]. Members of serine and arginine (SR)-rich splicing factors were first identified as important for constitutive splicing, and are now implicated in other aspects of RNA processing. A conditional gene targeting strategy was used to investigate the functional importance of one of these proteins, ASF/SF2. Homozygous deletion of ASF/SF2 results in embryonic lethality, but selective deletion of ASF/SF2 in the cardiac lineage at the onset of cardiogenesis produced a novel phenotype. These mice survived to 6–8 weeks after birth and exhibited a selective cardiac defect of hypercontraction. ASF/SF2 regulates alternative splicing of a small subset of genes, all involved in excitation–contraction coupling and differentiation of fetal to adult heart. The authors' most significant finding was that in the absence of ASF/SF2 the CaMKIIδA splice isoform, normally expressed in fetal heart, was retained in the adult. A transgenic mouse that expressed CaMKIIδA from a cardiac promoter mimicked the hypercontractile phenotype of the ASF/SF2 conditional knockout. This elegant study clearly demonstrates that ASF/SF2 is the control point that switches the pattern of CaMKIIδ splicing during normal maturation of the heart [24••].

Splicing and disease severity

There are a growing number of examples of splicing defects underlying human disease. Splicing can also have a role in modifying disease severity. For example, Timothy syndrome is a novel disorder characterized by multi-organ dysfunction including lethal arrhythmias, webbing of fingers and toes, and autism [27••]. This syndrome results from a *de novo* mis-sense mutation G406R in the L-type $Ca_V1.2$ gene. Notably, the mutation is located in only one of a pair of mutually exclusive exons that encode the 6th transmembrane spanning helix of the first domain of the $Ca_V1.2$ subunit. The authors show that the multi-organ involvement is consistent with the expression pattern of this mutation-carrying splice isoform, which represents about 20% of $Ca_V1.2$ mRNAs in the heart. The mutation slows calcium channel inactivation, but the net effect on the L-type current in the heart of affected individuals, assuming the mutant contributes ~10% of the whole cell current in the heterozygote state, is relatively small. Nonetheless, this small change in the whole cell current is estimated to prolong the cardiac action potential and calcium influx by almost 20%. A similar mutation in a region of the $Ca_V1.2$ gene common to all splice isoforms of this crucial ion channel would probably be fatal. Timothy's syndrome illustrates that mutations in alternatively spliced exons of crucial genes might be partially compensated for by the normal expression of splice isoforms that lack the mutation-carrying exon. It follows that a high percentage of hereditary disorders probably arise from mutations that are either located in alternatively spliced exons or that affect regulated splicing events.

Finally, alternative splicing can modify the severity of disease. A four base deletion mutation in the voltage-gated sodium channel gene, *Scn8a* or $Na_V1.6$, causes inherited movement disorders in mice. This mutation in the splice donor site of intron 3 results in $Na_V1.6$ mRNAs encoding truncated, nonfunctional $Na_V1.6$ proteins [28••]. Meisler and co-workers [28••] noticed that the severity of the sodium channel mutation varied greatly in mice dependent on their genetic background. In the extreme, in the C57BL/6J background the chronic movement disorder associated with the *Scn8a* mutation converted to a lethal neurological disease. They pinpointed the cause of the disease severity to a mutation in the sodium channel modifier, *SCNM1* gene that encodes a zinc finger protein and putative splice factor. The SCMN1 mutation impairs splicing of the sodium channel pre-mRNA *in vivo* and is the allele found in C57BL/6J. Impaired splicing in C57BL/6J mice further reduces the level of correctly spliced sodium channel transcripts below the threshold required for survival.

Conclusions

Alternative splicing optimizes the activity of key neuronal proteins. Cell-specific inclusion of exons can introduce new sites of interaction that redirect protein targeting. Subtle modifications in the activity of ion channels and receptors can also support gradual changes in neuronal excitability that, for example, might underlie homeostatic plasticity. Currently, we know only a few of the trans-acting splice factors that must control alternative splicing of synaptic protein pre-mRNAs. Future studies aimed at identifying these factors should reveal new signaling pathways that orchestrate alterations in alternative splicing of multiple pre-mRNAs to control neuronal excitability and synaptic efficacy.

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