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Review

Alternative Splicing in the Mammalian Nervous System: Recent Insights into Mechanisms and Functional Roles

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High-throughput transcriptomic profiling approaches have revealed that alternative splicing (AS) of precursor mRNAs, a fundamental process by which cells expand their transcriptomic diversity, is particularly widespread in the nervous system. AS events detected in the brain are more highly conserved than those detected in other tissues, suggesting that they more often provide conserved functions. Our understanding of the mechanisms and functions of neural AS events has significantly advanced with the coupling of various computational and experimental approaches. These studies indicate that dynamic regulation of AS in the nervous system is critical for modulating protein-protein interactions, transcription networks, and multiple aspects of neuronal development. Furthermore, several underappreciated classes of AS with the aforementioned roles in neuronal cells have emerged from unbiased, global approaches. Collectively, these findings emphasize the importance of characterizing neural AS in order to gain new insight into pathways that may be altered in neurological diseases and disorders.

Introduction

Neurogenesis is characterized by global changes in the transcriptomes and proteomes of differentiating cells. Many of these changes are critical for the transition from neural stem or precursor cells to neurons. Subsequent steps of neuronal development, including neuronal migration, axonal and dendritic outgrowth, establishment of synaptic connections, and neuronal plasticity, are further refined by coordinated, spatio-temporal crosstalk among various gene regulatory pathways. In recent years, fundamental roles for alternative splicing (AS) in neural development and in the establishment and function of neuronal networks have become increasingly evident.

AS is one of many processes that mediate gene regulation in metazoans. During AS of precursor mRNA (pre-mRNA), different combinations of 5′ and 3′ splice site pairs are selected, resulting in the generation of diverse mRNA and protein variants. Advances in high-throughput, genome-wide technologies along with the development of computational tools have spurred the identification of novel AS events to unprecedented levels. These data indicate that AS regulation is highly variable among species and that the frequency of AS increases with species complexity (Barbosa-Morais et al., 2012; Merkin et al., 2012), with transcripts from ~95% of human multi-exon genes undergoing AS (Pan et al., 2008; Wang et al., 2008).

Despite the overall extensive species specificity of AS, many of the identified AS events are conserved and are subject to cell-, tissue-, or developmental-specific regulation (Calarco et al., 2011; Kalsotra and Cooper, 2011). Remarkably, AS is especially prevalent and more highly conserved in nervous system tissues of vertebrates (Barbosa-Morais et al., 2012; Jelen et al., 2007; Merkin et al., 2012). It is thought that such high AS frequencies may contribute to the functional complexity of the nervous system. Mutations in neural RNA-binding proteins (RBPs) involved in AS regulation and aberrations in neural AS patterns have been linked to neurological disorders and disease (Licatalosi and Darnell, 2006).

Fundamental goals in the field are to elucidate the functions of the AS events detected in transcriptome profiling studies and to determine their mechanisms of regulation. Understanding how AS impacts the activities of proteins is central to addressing how global impairments in AS networks may result in cellular dysfunction. This review discusses recent studies incorporating high-throughput technologies, computational methods, and focused approaches to provide timely insights into emerging principles of AS in the nervous system. We direct the reader to several reviews that describe related topics and past discoveries (Braunschweig et al., 2013; Calarco et al., 2011; Darnell, 2013; Irimia and Blencowe, 2012; Li et al., 2007; Licatalosi and Darnell, 2010; Norris and Calarco, 2012; Zheng and Black, 2013).

An Overview of the *cis*- and *trans*-Acting Factors that Regulate Alternative Splicing

A conserved set of *cis*-acting elements known as the core splicing signals (5′ and 3′ splice sites, branch site, and polypyrimidine tract) guides the interactions between spliceosomal components and pre-mRNA. However, these core signals are not sufficient to ensure correct splice site selection and to regulate AS. These decisions are further regulated by the combinatorial control of short, degenerate RNA motifs known as exonic/intronic splicing enhancers or silencers, which are bound by *trans*-acting splicing factors. Notably, analyses of the arrangement of clusters of such motifs led to the development of RNA splicing maps, which correlate the binding position of an RBP

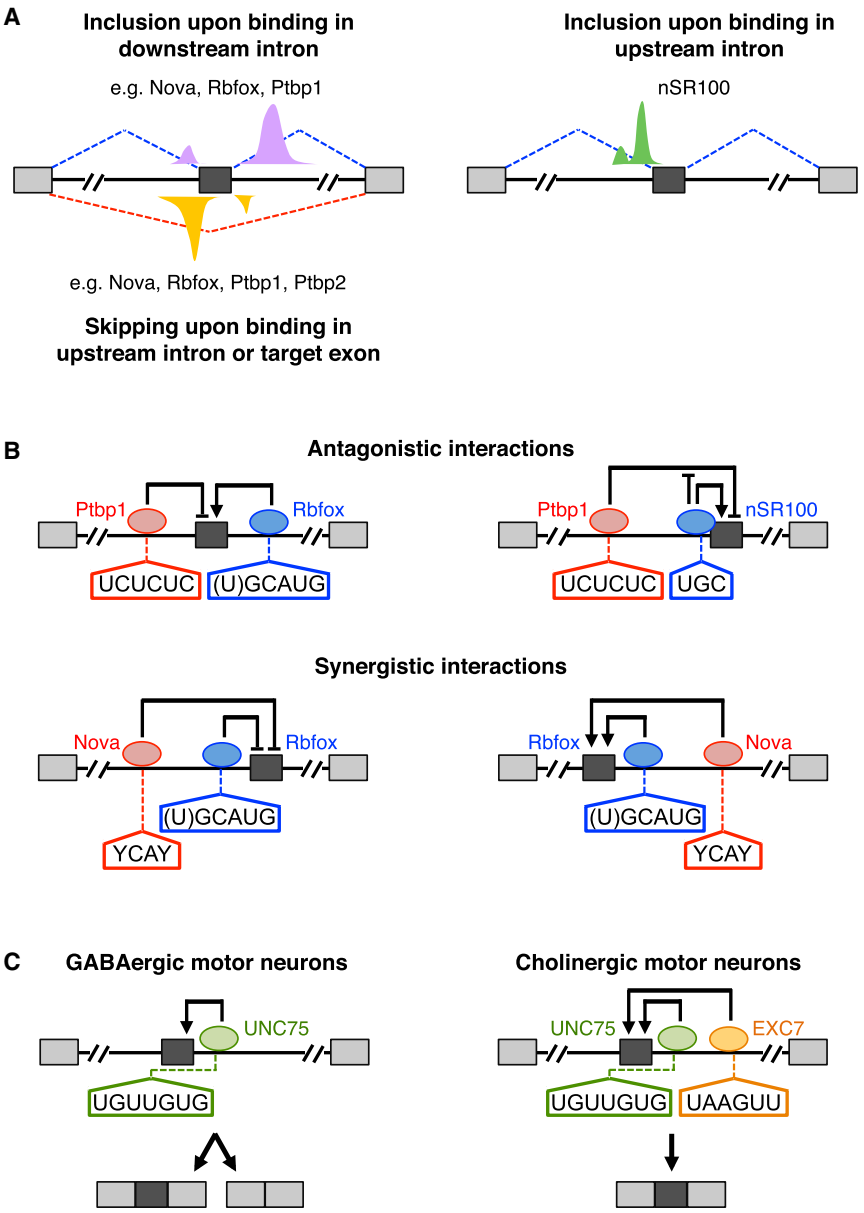

Figure 1. Position-Dependent Activities of Splicing Regulators

(A) Representative RNA splicing maps of tissue-enriched AS factors. The position of CLIP-Seq tag enrichment (purple, yellow, or green curves) around the regulated alternative exon (dark gray box) typically correlates with enhancing (blue lines) or repressive (red lines) activities of most tissue-enriched splicing factors characterized so far (left panel). Binding of the factor downstream of the target exon is usually associated with exon inclusion, while binding within or upstream of the exon is associated with exon skipping. However, nSR100 does not display position-dependent asymmetric regulation and instead binds to the upstream intron to promote exon inclusion (right panel).

(B) Examples of combinatorial activities of AS regulators. Top left: Ptbp1 and Rbfox proteins bind on opposite sides of the regulated exon and exert opposing effects. Top right: Ptbp1 and nSR100 proteins both bind upstream of the regulated exon and nSR100 can counteract the negative activity of Ptbp1. Bottom: Nova and Rbfox proteins act synergistically to repress (left) or enhance (right) inclusion of the regulated exon. The binding motif sequences of the factors are indicated.

(C) Cell-type-specific expression and combinatorial effects of UNC75 and EXC7 result in distinct neural AS patterns in different neuron subtypes in *C. elegans*. The binding motif sequences of the factors are indicated.

with its effect on AS regulation (Figure 1A) (Darnell, 2010; Witten and Ule, 2011). This was first demonstrated for the neural splicing regulator Nova (see below); clusters of YCAY Nova binding motifs positioned downstream of regulated exons are associated with exon inclusion, whereas YCAY clusters located within or upstream of target exons are associated with exon skipping (Ule et al., 2006). This asymmetric position-dependent activity was subsequently found to apply to several other tissue-enriched splicing modulators (Licatalosi et al., 2012; Llorian et al., 2010; Tollervey et al., 2011; Weyn-Vanhentenryck et al., 2014; Xue et al., 2009), although an exception to this pattern has been reported (Raj et al., 2014).

The amalgamation of these and other *cis*-features into an integrated "splicing code" (Wang and Burge, 2008) resulted in a breakthrough in understanding tissue-dependent AS patterns. Machine-learning algorithms have been developed that reliably predict AS regulation in mouse and human tissues from input genomic sequence (Barash et al., 2010; Xiong et al., 2015; Zhang et al., 2010). Most of the *cis*-regulatory features that are predictive of tissue-dependent splicing patterns are located within ~300 nucleotides of the splice sites. These regions are highly enriched in binding sites of RBPs known to be important for AS regulation and, in general, are more conserved than distal intronic sequences (Barash et al., 2010; Xiong et al., 2015). However, changes in the location or presence of a largely conserved set of *cis*-features surrounding exons have resulted in the rapid evolution of species-dependent AS regulation (Barbosa-Morais et al., 2012; Brooks et al., 2011; Jelen et al., 2007; Merkin et al., 2012, 2015). Differences in key *trans*-regulatory proteins, many of which are themselves alternatively spliced and display species-/lineage-specific AS, likely also contributed to the remarkable divergence in AS patterns between species (Barbosa-Morais et al., 2012; Merkin et al., 2012). It will be of interest to determine the functions and regulatory targets of species- and lineage-specific isoforms of splicing factors.

The major classes of splicing factors that regulate constitutive and alternative splicing comprise the heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR)

proteins, the majority of which are widely expressed across tissues. In addition, several splicing regulators have been identified that display cell- or tissue-specific or enriched expression (Calarco et al., 2011; Kalsotra and Cooper, 2011; Li et al., 2007). Notably, most of these proteins are enriched in neural and/or muscle tissues, which harbor among the most extensive yet conserved repertoires of AS events in vertebrates (Barbosa-Morais et al., 2012; Braunschweig et al., 2013; Merkin et al., 2012).

Splicing-sensitive microarrays and high-throughput RNA sequencing (RNA-seq) have greatly facilitated the discovery of AS events across tissues and during development, with RNA-seq representing the current method of choice (Calarco et al., 2011; Irimia and Blencowe, 2012; Licatalosi and Darnell, 2010). RNA-seq datasets have provided reliable quantitative measurements of the dynamics of AS patterns during neuronal differentiation (Braunschweig et al., 2014; Hubbard et al., 2013; Irimia et al., 2014; Raj et al., 2014) and have been further utilized to investigate the contribution of AS to transcriptome complexity in brain tissues (Barbosa-Morais et al., 2012; Merkin et al., 2012). Furthermore, multiple variations of in vivo UV-induced crosslinking immunoprecipitation coupled to high-throughput sequencing methods (herein referred to as CLIP-Seq) have revolutionized transcriptome-wide studies of RNA-protein interactions (Darnell, 2010; König et al., 2011). These assays determine the direct in vivo targets of an RBP and provide further insight into the molecular mechanisms of RNA-protein interactions. Data from CLIP-Seq studies can also be integrated with transcriptome-wide AS regulatory data from RNA-seq and/or microarrays to derive RNA splicing maps. This approach was first demonstrated using Nova CLIP-Seq data (Licatalosi et al., 2008), and the results were consistent with the previous RNA splicing map obtained by analyzing Nova binding motifs surrounding target exons (Ule et al., 2006). It is worth noting that analyses of CLIP-Seq data are subject to technical challenges associated with nucleotide crosslinking biases, unique mapping of short reads, and the abundance of target transcripts. Establishing RNA splicing maps using computational approaches is therefore a valuable complementary approach because it can also be used to analyze sets of splicing events associated with specific motifs or transcripts (i.e., those of low abundance) that are not amenable to detection by CLIP-Seq procedures (Barash et al., 2010; Cereda et al., 2014; Raj et al., 2014; Xiong et al., 2015; Zhang et al., 2013).

Neural Splicing Regulatory Networks

Nova Proteins

Tissue-specific AS is often organized at the level of "splicing regulatory networks," which consist of specific subsets of co-regulated exons within functionally related genes. These exon networks are likely coordinated to ensure proper development and function. This type of regulatory control was initially described for Nova1 and Nova2 (Ule et al., 2005). Nova proteins were identified as neuronal antigens targeted by autoimmune responses in patients with paraneoplastic opsoclonus myoclonus ataxia (POMA), a neurological disorder characterized by dysfunction of the motor nervous system (Buckanovich et al., 1993; Yang et al., 1998). *Nova1* knockout mice display extensive apoptosis in brain stem and spinal cord motor neurons resulting in gross motor failure and eventual postnatal death (Jensen et al., 2000). On the other hand, *Nova2* null mice exhibit misregulation of the activity-dependent long-term potentiation of the slow inhibitory postsynaptic current (Huang et al., 2005). *Nova* double knockout mice are paralyzed and die immediately after birth (Ruggiu et al., 2009). Using a combination of *Nova2*$^{-/-}$ mice and splicing microarray analysis, Darnell and colleagues established a Nova regulatory network consisting of neural alternative exons in genes associated with synaptic and axon guidance roles (Ule et al., 2005) and subsequently expanded this network using CLIP-Seq data and computational approaches (Licatalosi et al., 2008; Ule et al., 2006; Zhang et al., 2010).

Rbfox Proteins

Similar approaches have since been used to investigate the roles of additional neural-enriched splicing factors. The Rbfox family consists of three paralogs: Rbfox1 (also known as A2bp1), Rbfox2 (also known as Rbm9), and Rbfox3 (also known as NeuN). Using CLIP-Seq, Rbfox-RNA interactions were recently mapped in the mouse brain (Lovci et al., 2013; Weyn-Vanhentenryck et al., 2014). The RNA-binding profiles of the three Rbfox proteins were found to be comparable, in line with the strong sequence similarities between their RNA-binding domains and the RNA sequence motifs that they recognize (Weyn-Vanhentenryck et al., 2014). A Bayesian network was used to model the Rbfox splicing network by merging computationally predicted binding sites, CLIP-Seq data, Rbfox-dependent exons, and several evolutionary characteristics. A significant fraction of Rbfox-regulated AS events that exhibit dynamic changes in inclusion levels during brain development were also identified (Weyn-Vanhentenryck et al., 2014). These studies complement and extend previous investigations of the networks of exons regulated by Rbfox proteins (Jangi et al., 2014; Yeo et al., 2009; Zhang et al., 2008).

Central-nervous-system-specific deletion of *Rbfox1* results in mice with increased susceptibility to spontaneous and induced seizures and heightened excitability of neurons in the dentate gyrus (Gehman et al., 2011). In contrast, mice with central-nervous-system-specific deletion of *Rbfox2* are not prone to seizures but rather exhibit abnormal cerebellar development, including reduced size, improper migration and dendritic arborization of Purkinje cells, and increased cell death (Gehman et al., 2012). Furthermore, Rbfox1 and Rbfox2 proteins display spatially and temporally distinct expression patterns in subregions of the developing and adult cerebellum (Gehman et al., 2012). Collectively, these observations suggest that Rbfox proteins have overlapping yet distinct activities during brain development and neuronal function, similar to the observations made with Nova and other splicing regulators (see below).

Ptbp1 and Ptbp2 Proteins

Ptbp1 and its paralog Ptbp2 are known to function predominantly as repressors of neural AS, although a small but significant subset of Ptbp1/Ptbp2-enhanced AS events has also been identified (Boutz et al., 2007; Llorian et al., 2010; Xue et al., 2009). These proteins have largely mutually exclusive expression patterns in the nervous system as Ptbp1 promotes skipping of a neural-specific exon (exon 10) in Ptbp2 transcripts. The consequent introduction of a premature termination codon in Ptbp2

transcripts results in their turnover by nonsense-mediated decay (NMD) (Boutz et al., 2007; Spellman et al., 2007). Ptbp1 is expressed in most non-neural tissues and neural precursor cells but is silenced in developing neurons by the microRNA miR-124 (Makeyev et al., 2007). This mechanism for suppressing Ptpb1 function contributes to the inclusion of the neural-specific Ptbp2 exon, thus facilitating Ptbp2 expression at a later stage during neural development (Boutz et al., 2007; Spellman et al., 2007). Ptbp2 is also subject to autoregulation and cross-regulation by Rbfox2 via AS coupled to NMD (Jangi et al., 2014).

To investigate the role of Ptbp2 during neurogenesis, knockout mice have been recently characterized. *Ptbp2* null mice die shortly after birth and exhibit misregulation of AS in genes involved in cytoskeletal remodeling and cell proliferation (Licatalosi et al., 2012) as well as in neurite growth and synaptic transmission (Li et al., 2014). Furthermore, *Ptbp2* knockout brains display reduced neural progenitor pools and premature neurogenesis (Licatalosi et al., 2012). Conditional knockout of *Ptbp2* in neurons of the higher forebrain resulted in widespread cortical neuronal death and degeneration in vivo and in vitro (Li et al., 2014). Ptbp2 also plays an important role in embryonic-specific repression of alternative exons. As Ptbp2 expression decreases from its highest levels in the embryonic brain to moderate levels during postnatal development, a cohort of exons switch from being skipped in embryos to displaying enhanced inclusion in adults (Li et al., 2014; Licatalosi et al., 2012; Zheng et al., 2012). Thus, sequential downregulation of Ptbp1 followed by Ptbp2 contribute to the activation of neural exon networks at the appropriate stages of development.

nSR100/Srrm4

The neural-specific SR-related protein of 100 kDa (nSR100/Srrm4) is expressed specifically in neurons of multiple brain subregions and sensory organs (Calarco et al., 2009; Irimia et al., 2014; Nakano et al., 2012; Quesnel-Vallières et al., 2015). nSR100 is highly conserved across vertebrates but is not found in invertebrates, suggesting that its emergence was associated with the increased regulatory and functional complexity of the vertebrate nervous system (Calarco et al., 2009). nSR100 regulates a conserved network of human- and mouse-brain-enriched AS events in genes involved in neural functions, including cytoskeletal organization, guanosine triphosphate hydrolase (GTPase) signaling, and synaptic membrane dynamics (Calarco et al., 2009; Quesnel-Vallières et al., 2015; Raj et al., 2014). Notably, nSR100 promotes expression of Ptbp2 by activating the inclusion of Ptbp2 exon 10, which prevents turnover of Ptbp2 transcripts by NMD (Calarco et al., 2009). Furthermore, nSR100 promotes expression of a subset of neural genes by activating the inclusion of a neural-specific exon in the transcription factor REST/NRSF (see below) (Raj et al., 2011).

Knockdown of nSR100 impairs neurite outgrowth and leads to neurodevelopmental defects in zebrafish and mice, consistent with nSR100's neuronal expression profile and its modulation of neural gene activities (Calarco et al., 2009; Quesnel-Vallières et al., 2015; Raj et al., 2011). Widespread loss of nSR100 in a conditional *nSR100/Srrm4* knockout mouse results in extensive (>85%) neonatal death, in part due to a respiratory defect (Quesnel-Vallières et al., 2015). Further examination revealed abnormalities in the branching of motor neurons innervating the diaphragm, altered formation of cortical layers of the forebrain, and axonal midline crossing defects in the corpus callosum. A mutation in the Bronx waltzer (bv) mouse, which causes deafness, balance defects, and aberrant AS patterns in genes expressed in the inner ear, has been mapped to the *nSR100/Srrm4* locus (Nakano et al., 2012). Collectively, these phenotypes reveal critical in vivo roles of nSR100 during nervous system development.

Additional Neural-Enriched Alternative Splicing Factors

The regulatory targets and mechanisms of additional RBPs with enriched expression in neural tissues and implicated in AS regulation (e.g., Mbnl, TDP-43, Hu/Elav) have also been investigated using similar global profiling techniques as those described above (Charizanis et al., 2012; Ince-Dunn et al., 2012; Modic et al., 2013; Tollervey et al., 2011). It is worth noting that intricate neural splicing regulatory networks are not restricted to the vertebrate nervous system. RNA-seq profiling of two splicing factors in *C. elegans* (UNC-75, related to CELF family, and EXC-7, related to Hu/ELAV family) revealed that they also control AS of distinct groups of alternative exons in genes with neuronal functions (Norris et al., 2014). Similarly, RNA-seq analysis upon knockdown of the splicing factor Pasilla (ortholog of Nova1 and Nova2) in *D. melanogaster* resulted in changes in AS of genes enriched in neuronal activities, cytoskeletal dynamics, and sexual reproduction (Brooks et al., 2011). Thus, genome-wide studies have provided insight into the conserved organizational features of regulated AS imparted by multiple neural-enriched *trans*-factors across different organisms.

Combinatorial Action of trans*-Factors Regulates Neural Splicing Networks*

Tissue-specific AS patterns result from the cumulative effects of the binding and activities of several splicing factors. Pioneering work demonstrated this principle in the context of the neuronal-specific N1 exon of the SRC tyrosine kinase, which is dependent on the combinatorial activities of Ptbp1, Ptbp2, Rbfox, and additional hnRNP and SR proteins (Li et al., 2007).

Complementing the use of advanced computational methods for inferring combinations of *cis*-regulatory elements that control neural-specific AS (Barash et al., 2010; Zhang et al., 2010), RNA-seq and CLIP-Seq datasets can be used for global investigation of the combinatorial activities of multiple AS factors. For example, it was discovered that ~30% of nSR100-activated AS events are repressed by Ptbp1 (Raj et al., 2014). Moreover, nSR100 and Ptbp1 show maximal co-expression in neural precursor cells and immature neurons, where inclusion of target exons was detected (Raj et al., 2014). These and additional observations provided evidence that nSR100 acts during neurogenesis to overcome the negative activity of Ptbp1 and promote its program of exon inclusion (Figure 1B). Systematic investigation of how this exon network is activated during neurogenesis revealed a unique organization of *cis*-regulatory elements, including distally located branch sites and polypyrimidine sequences, as well as the presence of UGC intronic *cis*-elements upstream of target exons that all contribute to suboptimal 3′ splice sites and consequent exon skipping in the absence of nSR100 in non-neural cells (Raj et al., 2014). In contrast, in neural cells, nSR100 binds the UGC motif and interacts with multiple

early spliceosomal components to enhance 3′ splice site recognition. Ptbp1 occupies binding sites located upstream of the nSR100 recognition motif. The separation of the antagonistic *cis*-elements enables nSR100 binding to overcome Ptbp1-mediated repression, facilitating rapid switch-like changes in inclusion levels of target exons. Comparison of RNA splicing maps revealed similar opposing regulatory relationships between Ptbp1 and Nova proteins (Cereda et al., 2014) and between Ptbp1 and Rbfox proteins (Figure 1B) (Li et al., 2015). In contrast to the aforementioned antagonistic activities, a subset of Nova-dependent exons is also synergistically regulated by Rbfox (Figure 1B) (Zhang et al., 2010). Similar approaches can be used to systematically determine regulatory overlap between combinations of other neural splicing factors.

A key area of future investigation is to understand the mechanisms by which splicing regulators act together. For example, it is currently not known how nSR100 acts in a dominant positive manner over Ptbp1, nor how Rbfox and Nova proteins impart synergistic activities on regulated target exons. Changes in RNA structure and/or the composition of proximal splicing complexes potentially underlie such effects.

Combinatorial activities of multiple splicing factors may also contribute to AS differences between neuronal subtypes. In a recent study, two-color splicing reporters corresponding to genes known to be involved in neural functions were developed to monitor exon inclusion at single-neuron resolution in *C. elegans* (Norris et al., 2014). Reproducible patterns of differential exon inclusion in several reporters were observed between various neuronal subtypes. For example, the exon-16-included isoform of *unc-16* was detected in both GABAergic and cholinergic neurons, while the skipped isoform was only observed in GABAergic neurons. A forward genetics-based screen identified UNC-75 and EXC-7 as activators of *unc-16* exon 16 AS. Notably, lack of EXC-7 expression in GABAergic neurons resulted in partial exon 16 inclusion by UNC-75, while co-expression of the proteins in cholinergic motor neurons stimulated maximal inclusion levels (Figure 1C). Furthermore, RNA-seq data identified a significant subset of co-regulated exons, indicating widespread combinatorial activities of the two proteins.

Investigating Biological Functions of Neural Alternative Splicing Events

The impact of specific neural splice variants on neuronal differentiation, morphology, maturation, and/or activity is generally not understood. Nevertheless, multiple examples demonstrating critical roles of neural isoforms have been reported. For example, expression of the neural isoform of *Disabled-1*, which is involved in Reelin signaling, rescued neuronal migration defects observed in cortices of *Nova2* null mice (Yano et al., 2010). Expression of an isoform including a 6-nucleotide (nt) neuronal "microexon" (see below) in Unc13b, a protein that is important for neuritogenesis, significantly stimulated neurite outgrowth and rescued a neurite extension defect in *nSR100* mutant hippocampal neurons, whereas expression of an isoform lacking this exon did not rescue these phenotypic defects (Quesnel-Vallières et al., 2015). The extent to which other neural AS events are functional remains an important outstanding question.

Alternative Splicing Remodels Protein-Protein Interaction Networks

Recent analyses of the impact of tissue-specific AS networks at the protein level have revealed several important insights. Tissue-specific alternative exons are more often frame preserving than other classes of alternative and constitutive exons (Fagnani et al., 2007; Sugnet et al., 2006; Xing and Lee, 2005) and are enriched in predicted post-translational modification sites (Buljan et al., 2012) such as phosphorylation sites (Merkin et al., 2012; Zhang et al., 2010). Furthermore, tissue-regulated exons, especially those spliced specifically in brain and muscle tissues, frequently overlap unstructured or highly disordered regions that embed conserved linear motifs, which are often the sites of ligand and protein-protein interactions (PPIs) (Buljan et al., 2012; Ellis et al., 2012; van der Lee et al., 2014). Consistent with these observations, proteins encoded by tissue-specific exons tend to have a higher number of interaction partners and form hubs within interaction networks (Buljan et al., 2012; Ellis et al., 2012).

To test whether differential inclusion of brain-enriched nSR100-dependent exons modulate PPIs, an automated co-immunoprecipitation assay using luminescence-based mammalian interactome mapping (LUMIER) was employed (Barrios-Rodiles et al., 2005; Ellis et al., 2012). In this assay, bait cDNA variants with and without a target neural exon are generated for each gene of interest and are fused to Renilla luciferase. The bait isoforms are co-expressed with known or putative Flag-tagged interaction partners (prey). Co-immunoprecipitation of a bait isoform with a prey protein is semiquantitatively measured using a normalized readout from the Renilla luciferase and is used to assess whether inclusion of an alternative exon alters one or more PPIs. It was observed that approximately one-third of analyzed nSR100-dependent exons promoted and/or repressed various PPIs (Ellis et al., 2012). This indicated that an important function of nSR100 is to promote the inclusion of exons that remodel PPI networks during neurogenesis. Further studies are required to determine the precise mechanisms by which AS modulates PPIs. For example, it is not well understood how disordered regions overlapping neural exons contribute to specific partner interactions nor to what extent post-translational modifications of these regions contribute to interaction affinities and/or binding specificities.

Identification and Characterization of a Highly Conserved Program of Neuronal Microexons

Recently, a new RNA-seq pipeline was developed to systematically identify and analyze all major classes of AS events, including cassette-type exons, alternative 5′/3′ sites, retained introns, and "microexons," from a panel of ~50 diverse cell and tissue types from mouse and human (Irimia et al., 2014). Of the ~2,500 AS events with neural-differential regulation, a group of ~300 alternative microexons, 3 to 27 nt in length, display strong enrichment for inclusion in neurons versus all other cell types analyzed (Irimia et al., 2014). Remarkably, there is a strong inverse relationship between the length of an alternative exon and its tendency to be included in neural cells and tissues, with 3- to 15-nt microexons displaying the strongest degree of neural inclusion. Similar results were observed in a parallel study

by Ponting and colleagues, who utilized a broader length definition for microexons (≤51 nt) (Li et al., 2015).

Examples of alternative microexons have been previously reported (e.g., SRC N1 exon) (Black, 1991), but the high degree of neuronal-specific splicing and additional global features of this class of exons had not been previously appreciated. In the recent studies, neuronal microexons were found to be significantly more often conserved and frame preserving than longer neural alternative exons (Irimia et al., 2014; Li et al., 2015). Moreover, sequences of the upstream and downstream flanking intronic regions of microexons were found to be more highly conserved than sequences surrounding longer neural alternative exons, suggesting the presence of important *cis*-acting elements that are critical for their regulation (Irimia et al., 2014; Li et al., 2015).

To search for *trans*-regulators of microexons, Irimia et al. analyzed RNA-seq datasets monitoring AS upon knockdown or overexpression of known neural AS regulators, including Ptbp1, Ptbp2, Rbfox1, Mbnl, and nSR100. Among these, nSR100 had the most pronounced and widespread effects; it was observed to promote the inclusion of more than half of all detected neuronal-specific microexons. Furthermore, nSR100 CLIP-Seq reads overlapping UGC motifs are enriched adjacent to 3′ splice sites flanking microexons, confirming direct regulation by the protein. On the other hand, Li et al. identified Rbfox and Ptbp1 proteins as regulators by searching for conserved hexamer motifs that are enriched proximal to alternatively spliced microexons. CLIP-Seq tags from the three Rbfox proteins are enriched in intronic regions downstream of neural-regulated microexons, suggesting that Rbfox proteins enhance microexon inclusion. In contrast, Ptbp1-RNA crosslink tags display a high density upstream of the microexons, indicating that Ptbp1 represses microexon inclusion. nSR100-regulated events were not analyzed in the latter study, although differences between the results of the two studies can also be attributed to the different length definitions assigned to microexons.

Remarkably, microexons are frequently misregulated in the brains of subjects with autism spectrum disorder (ASD), and these changes significantly correlate with reduced levels of nSR100 (Irimia et al., 2014). Previously, downregulation of RBFOX expression and misregulation of the RBFOX splicing regulatory network—comprising mostly longer alternative exons—were also observed in ASD patients (Voineagu et al., 2011; Weyn-Vanhentenryck et al., 2014). In addition, a recent study demonstrated that nSR100- and Nova1-mediated control of the inclusion of a 12-nt microexon in LSD1 (also known as KDM1A), a histone H3K4 demethylase, contributes to neuronal excitability in mice (Rusconi et al., 2014). This regulation was proposed to be important for susceptibility to seizure and could represent a molecular event that underlies epilepsy.

It is currently unclear to what extent misregulation of neuronal exons contributes to neurological disorders. For example, does disruption of neuronal AS impact neuropathology and behavioral characteristics of patients with ASD? Generating mouse models with altered expression of known splicing regulators of neuronal microexons may provide a valuable means to investigate the role of these short exons in epilepsy and ASD. Another exciting application of the mouse models could be to develop and test potential therapeutics for disorders, for example, agents that act to modulate the expression of critical splicing regulators such as nSR100. In this regard, it is interesting to note that strategies for manipulating the expression of the Survival of motor neuron (SMN) protein, a key factor required for snRNP assembly, have been effective in correcting disease-associated phenotypes in mouse models of spinal muscular atrophy (Foust et al., 2010; Hua et al., 2011).

Functions of Neuronal Microexons

An important question stemming from the aforementioned observations is whether the inclusion of a few amino acids can alter the activity of the encoded protein. Most neuronal microexons exhibit pronounced switch-like regulation—from low to very high inclusion levels—during late stages of neuronal differentiation (Figure 2A), suggesting that they may function in terminal neurogenesis (Irimia et al., 2014). Notably, unlike longer neural exons that overlap disordered regions enriched in linear binding motifs, microexons are highly enriched in modular interaction domains associated with cellular signaling (e.g., PTB, SH2 domains). Thus, inclusion of microexons represents a complementary mechanism for regulating PPI networks and other ligand interactions that are important for neuronal function (Irimia et al., 2014; Li et al., 2015).

Consistent with this view, insertion of a 6-nt microexon in the nuclear adaptor protein Apbb1 enhances its interaction with Kat5/Tip60, a histone deacetylase (Irimia et al., 2014). The Apbb1 microexon adds two charged residues (Arg and Glu) to a phosphotyrosine-binding domain (PTB) domain, which binds Kat5. Alanine substitution experiments and structural modeling suggest that the insertion of these two residues enhances binding of Kat5 by increasing the interaction surface of Apbb1. Similarly, inclusion of a 9-nt microexon in AP1S2, which is a subunit of the adaptor-related protein complex 1 (AP1) complex that functions in the intracellular transport of cargo proteins, strongly promotes an interaction with AP1B1, another AP1 complex subunit (Figure 2B) (Irimia et al., 2014).

Several studies have highlighted an important role for microexons in regulating neurite extension (Figure 2B). For example, it has been shown that a neural-specific 21-nt microexon in *Zfyve27*, which encodes Protrudin, a membrane protein involved in polarized vesicular trafficking in neurons, increases its interaction with the vesicle-associated membrane protein-associated protein (VAP) (Ohnishi et al., 2014). Furthermore, expression of transcripts including the neural microexon in Zfyve27-deficient hippocampal neurons promoted neurite outgrowth more efficiently than expression of transcripts lacking the microexon. In another recent example, inclusion of the aforementioned 12-nt microexon in LSD1 (LSD1+8a isoform) switches the activity of the protein from a co-repressor to a co-activator by promoting an interaction with the supervillin protein (SVIL). Together, these interactions result in demethylation of the repressive H3K9me2 mark to activate the expression of target genes and impact neurite morphogenesis (Laurent et al., 2015).

Microexons also overlap functionally important domains of transcription factors involved in nervous system development. For example, microexons in three members of the Mef2 family of transcriptional activators (Mef2A, Mef2C, and Mef2D) overlap transactivation domains and are highly included in brain

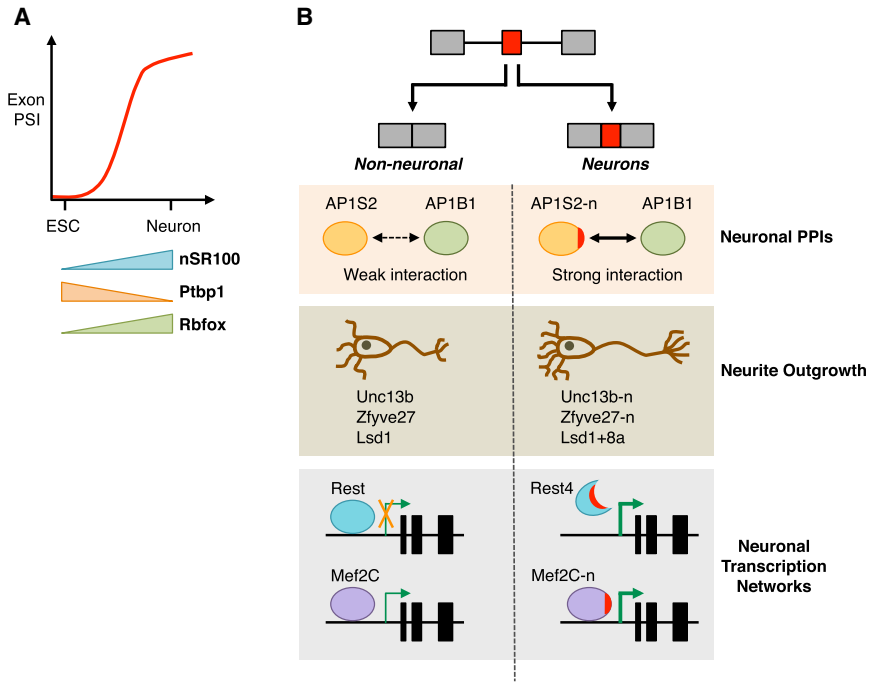

Figure 2. Microexon Regulation and Function

(A) Median percent spliced in (PSI) levels of alternative neural microexons (top panel), and changes in gene expression (bottom panel) of Rbfox (blue), Ptbp1 (orange), and nSR100 (green) proteins during in vitro differentiation of embryonic stem cells (ESCs) to cortical glutamatergic neurons (Hubbard et al., 2013). Opposing activities and expression levels of Rbfox, nSR100, and Ptbp1 regulate microexon inclusion.

(B) AS of microexons (indicated in red) can impact neuronal protein-protein interactions (PPIs), neurite outgrowth, and neuronal transcription networks.

tissues (McDermott et al., 1993; Yu et al., 1992; Zhu et al., 2005). Each of the microexon-containing isoforms has been shown to possess increased transcriptional activator function compared to the isoforms lacking these exons based on reporter assays (Zhu et al., 2005). Furthermore, as embryonic stem (ES) cells differentiate into neurons, these microexons display nSR100-dependent switch-like regulation from complete skipping to >85% inclusion in neurons (Raj et al., 2014). Taken together, these data suggest that inclusion of Mef2 microexons during neurogenesis may convert Mef2 isoforms to more potent activators, thus regulating the expression of a network of neural genes (Figure 2B). However, Mef2 transcriptional targets and the functions of the Mef2 splice variants are not well established in neuronal cells, and therefore require further investigation.

Although generally frame preserving, microexons can also modulate protein activity by altering the reading frame. For example, an nSR100-promoted, 16-nt neuronal microexon in mouse *REST/NRSF* transcripts, a transcriptional repressor that silences a multitude of genes involved in neural functions in non-neuronal cells, introduces a termination codon that produces a truncated isoform known as REST4 (Raj et al., 2011). This variant lacks critical zinc finger DNA binding domains required for transcriptional silencing of target genes, thereby enabling their expression during neural differentiation (Figure 2B). Downregulation of REST is critical for neuronal maturation in in vitro models of neuronal differentiation (Su et al., 2006, 2004; Xue et al., 2013). However, low levels of REST expression are still detected in neurons. A proposed function of neuron-specific REST4 is to act as a dominant-negative modulator by hetero-oligomerization and sequestration of full-length REST (Shimojo et al., 1999). However, it currently remains to be determined to what extent neural-specific AS of REST transcripts contributes to neuronal differentiation and maturation and whether REST4 has additional functions in this process.

Identification of Cryptic Nonsense-Mediated Decay Exons

The coupling of AS with NMD (AS-NMD) plays an important role in modulating the levels of proteins, including numerous splicing regulators and core spliceosomal components (Jangi et al., 2014; Lareau et al., 2007; Saltzman et al., 2008; Yap and Makeyev, 2013). In recent studies, important roles for AS-NMD in the regulation of neuronal gene expression have been uncovered. By profiling mRNA abundance in the brains of *Nova* double knockout mice, over 200 transcripts involved in synaptic functions were found to display reduced mRNA levels (Eom et al., 2013). Unexpectedly, CLIP-Seq experiments revealed that Nova binds to the introns of these regulated transcripts and promotes skipping of a previously unannotated class of neuronal exons. Because the expression of these exons is generally not observed at high levels in brain transcripts from wild-type mice, and their functional significance was unclear, they were described as being "cryptic." The inclusion of cryptic exons in the absence of Nova introduces premature termination codons that initiate NMD of transcripts (Figure 3A). Furthermore, the cryptic exons are subject to activity-dependent regulation; the induction of seizures in mice resulted in changes in AS of these exons, likely as a result of a change in the localization of Nova from the nucleus to the cytoplasm (Figure 3A). Thus, Nova-dependent dynamic regulation of cryptic exons may represent a novel mechanism to modulate the levels of synaptic proteins in neurons after seizure.

Functions of Intron Retention in the Regulation of Neural Gene Expression

Transcript and protein abundance can also be modulated by intron retention, which either prevents the export of transcripts to the cytoplasm, leading to nuclear RNA degradation, or else can result in turnover of the retained intron-containing transcripts in the cytoplasm via NMD (Figure 3B) (Ge and Porse, 2014; Yap and Makeyev, 2013; Yap et al., 2012). It was previously shown that Ptbp1 negatively regulates the expression of several

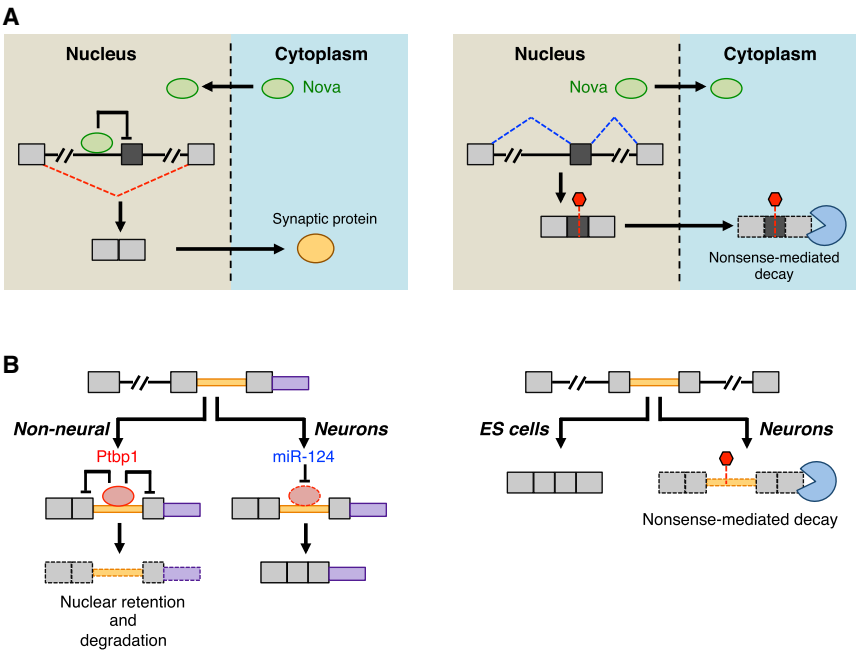

Figure 3. Regulation of Neural Gene Expression via Alternative Splicing

(A) Activity-dependent nucleocytoplasmic shuttling of Nova protein can alter AS of cryptic exons and modulate the levels of synaptic proteins via introduction of a premature termination codon (red pentagon) and nonsense-mediated decay.

(B) Intron retention can modulate gene expression via nuclear sequestration or nonsense-mediated decay. Left: In non-neural cells, Ptbp1 promotes retention of terminal 3′ introns, leading to their nuclear sequestration and degradation, thereby suppressing the expression of several synaptic genes. However, miR-124-mediated silencing of Ptbp1 in neurons enables splicing and expression of these genes. Right: As embryonic stem (ES) cells differentiate into neurons, intron retention is used as a mechanism to downregulate expression of cell division genes via introduction of a premature termination codon (red pentagon) and nonsense-mediated decay. Orange bar, retained intron. Purple bar, 3′ UTR.

proteins with neuronal functions, including Ptbp2 and Gabbr1, via AS-NMD (Makeyev et al., 2007). To test whether Ptbp1 has a more extensive role in neural gene expression, RNA-seq profiling was performed following Ptbp1 knockdown in neuroblastoma cells (Yap et al., 2012). A small yet significant subset of genes—several of which encode pre-synaptic proteins—was upregulated under these conditions. Surprisingly, these transcripts are not subject to NMD. Instead, Ptbp1 inhibits splicing of introns at the 3′ end of these genes, resulting in their nuclear retention and turnover via components of the nuclear RNA surveillance machinery (Figure 3B). As Ptbp1 is downregulated during neuronal differentiation, the target introns are spliced out and the corresponding mature mRNAs are detected in the cytoplasm. These observations suggest that Ptbp1-mediated control of intron retention is an important mechanism governing protein expression during neuronal development.

To investigate whether intron retention is utilized as a widespread gene-regulatory mechanism in mammals, an RNA-seq pipeline was designed to detect intron retention events and applied to more than 40 diverse mouse and human cell and tissue types (Braunschweig et al., 2014). Remarkably, retained introns were detected in transcripts from most genes and displayed higher complexity and regulatory conservation in the nervous system. Furthermore, intron retention was inversely correlated with global cytoplasmic mRNA steady-state levels, consistent with its known functions in nuclear sequestration and NMD. To determine the functional significance of alternative retained introns in neural cells, the expression levels of transcripts with regulated intron retention were monitored during in vitro differentiation of ES cells into cortical glutamatergic neurons. Genes exhibiting higher intron retention in neurons were enriched in functions (e.g., cell cycle) that are not critical or less required for the physiology of differentiated neurons, whereas genes with reduced levels of intron retention in neurons compared to ES cells were enriched in synaptic functions (Figure 3B). These findings support the proposal that cells frequently employ intron retention as an on/off switch to ensure the expression of biologically relevant subsets of genes.

Global Profiling of Alternative Splicing in Neural Cell Adhesion Factors

The mammalian neurexin genes and the *Drosophila Dscam1* gene represent the most extensive cases of AS regulation documented so far. The remarkable diversity of AS patterns in these two neural cell recognition factors plays a key role in the establishment of neuronal circuits. Together with genetic approaches and single-neuron imaging tools, RNA-seq now provides a powerful method to comprehensively probe the AS landscape and dynamics of neurexin and Dscam1 isoforms in neural cells and during development.

Neurexin Proteins

Neurexins and neuroligins are *trans*-synaptic cell adhesion proteins that interact with each other and are located on the presynaptic and post-synaptic membranes, respectively. These proteins play critical roles in synapse organization/assembly, synaptic transmission, and synaptic identity. Each of the three mammalian neurexin genes is transcribed from two independent promoters, resulting in the generation of six principal neurexins (Nrxn1α– Nrxn3α, Nrxn1β–Nrxn3β). Neurexins are subjected to highly regulated and extensive AS at up to six canonical splice sites (SS#1–SS#6), whereas neuroligins possess one canonical splice site (SS#A), with the exception of neuroligin 1, which additionally contains SS#B.

The combination of AS and alternative promoter usage has the potential to generate thousands of neurexin isoforms. The in vivo repertoire of neurexin isoforms was recently investigated by sequencing full-length transcripts from the adult mouse cortex (Schreiner et al., 2014; Treutlein et al., 2014). Interestingly, transcripts with a previously uncharacterized 27-nt alternative microexon were detected. This exon (SS#6) is highly conserved and is

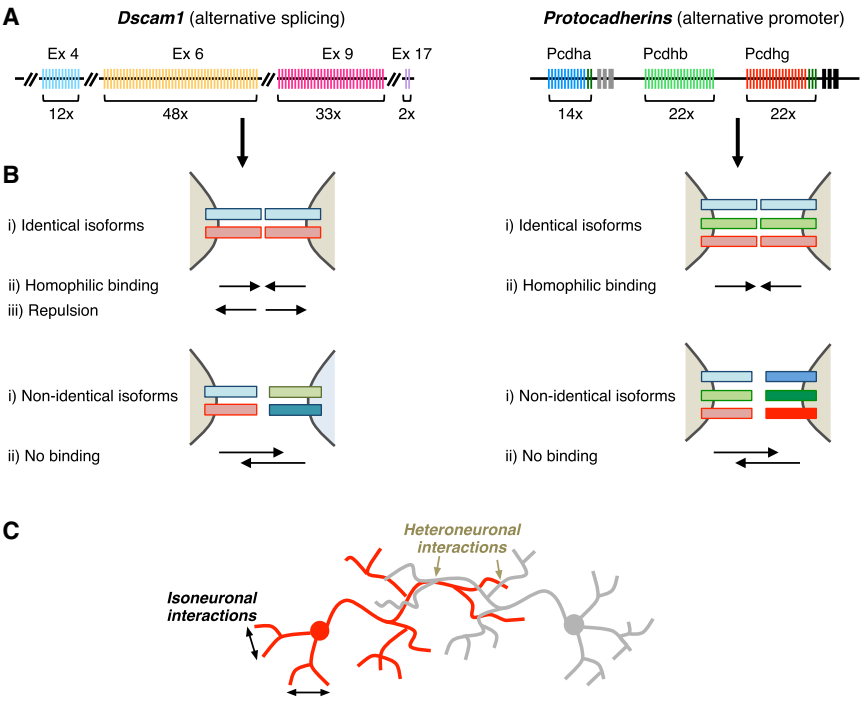

Figure 4. Dscam1 and Protocadherin Isoforms Generate Extensive Diversity to Mediate Neuronal Intercellular Recognition

(A) Schematic representations of the fly *Dscam1* gene (alternative exons only) and mouse *Pcdha*, *Pcdhb*, and *Pcdhg* gene clusters. Variable exons within each of the four Dscam1 exon clusters (Ex 4, Ex 6, Ex 9, and Ex 17) and within each Pcdh subtype are differentially color coded. Constitutive exons are colored gray (Pcdha) and black (Pcdhg).

(B) Expression of identical combinations of Dscam1 or Pcdh isoforms on neighboring cell surfaces triggers homophilic binding. This type of interaction results in contact-dependent repulsion in Dscam1 and Pcdhg isoforms (no data for Pcdha or Pcdhb so far). In contrast, expression of distinct Dscam1 or Pcdh isoform combinations does not initiate binding, allowing neurites to cross each other and/or form synapses.

(C) Homophilic binding and repulsion enable dendritic avoidance between sister neurites of a neuron (isoneuronal) while allowing interactions between neurites of different neurons (heteroneuronal). These interactions play fundamental roles in neural circuit establishment and maintenance.

specific to Nrxn1α and Nrxn3α. Furthermore, it maps to a flexible hinge region that may modulate PPIs. A relatively lower but significant number of neurexin transcripts were identified in which several exons were skipped, resulting in the deletion of multiple domains (Treutlein et al., 2014). Strikingly, all of the novel AS events preserved the reading frame, but the functions of the candidate proteins remain unclear. Collectively, over 2,000 AS variants were discovered, confirming previous predictions regarding the complexity of the neurexin transcript landscape (Schreiner et al., 2014; Treutlein et al., 2014).

Similar to the findings discussed previously, AS of neurexin has been shown to modulate PPIs and ligand interactions. For example, inclusion of the alternative exon at SS#4 (SS4+) decreases the affinity of neurexin for neuroligin 1 SSB+ variants (Chih et al., 2006). Moreover, SS4+ and SS4− isoforms can modulate binding of additional ligands such as CIRL/latrophilin and LRRTMs (Boucard et al., 2012; Ko et al., 2009; Siddiqui et al., 2010). To investigate the physiological relevance of altered interactions upon AS, mice were generated in which SS#4 of *Nrxn3* is mutated so that it is constitutively used but is also flanked by LoxP sites, enabling cre-mediated control of AS (Aoto et al., 2013). Constitutive expression of presynaptic Nrxn3-SS4+ resulted in changes in post-synaptic AMPA receptor abundance and disrupted AMPA receptor trafficking. This phenotype was proposed to result from changes in *trans*-synaptic binding between Nrxn-3 and LRRTMs and/or neuroligins, which interact with AMPA receptors.

Dscam1 and Clustered Protocadherin Proteins

Dscam1 is among the most well studied examples of surface receptors that undergo extensive neural AS—it is predicted to generate approximately 38,016 splice variants (Hattori et al., 2008; Zipursky and Grueber, 2013). The *Dscam1* gene consists of four blocks of alternative exons arranged in tandem, which introduce variability in the transmembrane segment (exon 17) and three extracellular immunoglobulin domains (exons 4, 6, and 9) (Figure 4A). The staggering diversity of Dscam1 is critical for several aspects of neuronal wiring within the fly brain, including regulating growth and targeting of axonal branches (Chen et al., 2006) and for dendritic patterning and self-avoidance (Figures 4B and 4C) (Hattori et al., 2009; Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007).

To globally profile endogenous Dscam1 isoforms with variations in the three ectodomains, a recent study utilized a novel method referred to as CAMSeq (Circularization-Assisted Multi-Segment Sequencing) to analyze the transcriptomes of *Drosophila* brains, developmental stages, and S2 cells (Sun et al., 2013). An important feature of CAMSeq is the use of four sequencing reads from each template to simultaneously determine exon usage in clusters 4, 6 and 9, and as a barcode to identify the sample. Remarkably, 18,496 out of 19,008 theoretically possible isoforms from these three exon clusters were detected. AS in each cluster was found to occur independently of the other clusters, as was previously proposed. Furthermore, Dscam1 isoforms display differences in splicing patterns during development and show a greater variety in brain tissues compared to S2 cells.

Recent evidence corroborates a model for Dscam1 AS in which exons are spliced in a stochastic manner to generate extreme isoform diversity. The in vivo expression of each of the 12 exon 4 variants from the endogenous Dscam1 locus was visualized using distinct splicing reporters (Miura et al., 2013). The choice of an exon 4 variant was found to be different for neurons belonging to the same class and for neurons located in similar positions in individual animals. These observations are consistent with a dynamic, probabilistic mechanism of Dscam1 AS in which a lack of distinctive regulation effectively confers unique

cellular identities that contribute to the avoidance of inappropriate isoneuronal interactions. Random co-expression of multiple Dscam1 isoforms may also serve a cell-intrinsic function. Loss of Dscam1 exon 6 diversity in mechanosensory neurons disrupted growth cone morphologies, while neighboring "wild-type" neurons in contact with the mutants were unaffected (He et al., 2014). Thus, it was proposed that a reduction in the Dscam1 isoform pool likely induces increased same-isoform encounters within a cell, causing more homophilic binding and a gain of Dscam1 function that disrupts axon growth and branching.

Notably, the vertebrate Dscam homologs do not display the extensive levels of AS found in the fly Dscam1. Instead, the mammalian clustered protocadherins (*Pcdha*, *Pcdhb*, and *Pcdhg*), which consist of multiple variable exons organized into discrete clusters and regulated via stochastic alternative promoter usage, are thought to function in an analogous manner (Figure 4) (Zipursky and Sanes, 2010). For example, Pcdhg isoforms mediate homophilic interactions that promote repulsion between sister neurites of retinal starburst amacrine neurons and Purkinje cells, enabling self/non-self-discrimination (Lefebvre et al., 2012). Although Dscam1 isoforms greatly outnumber the Pcdh isoforms (~19,000 Dscam1 isoforms with distinct ectodomains relative to 58 mouse Pcdh isoforms), recent evidence strongly supports the proposal that Pcdhs generate sufficient cell surface diversity to confer single cell identity. It was demonstrated that all three subfamilies of Pcdhs are involved in highly specific and combinatorial homophilic binding (Thu et al., 2014). Pcdhs can also function in multimeric complexes, although the details of these interactions are largely unclear. Together, these two mechanisms likely result in significant cell surface diversity and implicate Pcdhs in heteroneuronal recognition and neuronal circuit assembly (Thu et al., 2014).

Conclusions and Future Directions

In recent years, our understanding of how AS is dynamically regulated in the nervous system and generates isoform diversity with critical functions in neural development has been propelled by technological advances in high-throughput profiling methods and bioinformatics. Together with focused biochemical, molecular, and cell biological methods, we now have a deeper grasp of the mechanisms, functions, and impact of AS on neuronal cell complexity and biology. The widespread detection of previously underappreciated neural AS events such as microexons, cryptic exons, and retained introns and the characterization of the underlying regulatory mechanisms signify a few of the many important strides that have been made. Despite these advances and examples of elucidated functions, the impact of most neural AS events on neuronal functions such as differentiation, morphology, migration, electrophysiological activity, and synapse formation is not known. Furthermore, how such AS changes may affect behavior and other activities is poorly defined. As discussed in the above sections, knockout mouse models of splicing factors and in vitro cell culture systems are useful tools with which to dissect the physiological functions of uncharacterized splice isoforms. Moreover, the advent of CRISPR/Cas9 genome editing (Doudna and Charpentier, 2014; Hsu et al., 2014) has provided a powerful means with which to genetically delete or insert exons of interest in cell culture or whole animal models to systematically examine functions of neural isoforms. Additional key areas of future investigation are discussed below.

Neuronal Cell-Type-Specific Alternative Splicing

A limitation of most current investigations of neural AS regulation is that they do not differentiate between the individual cell types comprising brain tissues, such as neural precursor cells, vascular cells, neurons, and glia. Typically, inferences are made based on AS patterns that represent averages across the whole brain or brain regions. Recently, highly pure populations of several neuronal and non-neuronal cell types from mouse cerebral cortex were isolated and analyzed for differences in AS regulation (Zhang et al., 2014). Notably, thousands of novel cell-type-dependent AS events were identified with neurons possessing the highest degree of specific AS events. These findings suggest that numerous additional targets of neural splicing regulators may have been missed in previous studies of AS regulation using brain samples containing mixed cell types.

Furthermore, the identities of individual neurons are usually not taken into consideration, which masks important AS differences that may exist between neuronal cell types. Gaining deeper insight into AS patterns that are specific to neuronal subtypes and individual neurons represents information that can be valuable for fully understanding how neuronal complexity and wiring arise. The aforementioned study in *C. elegans* revealed multiple examples of neuron subtype-specific AS patterns (Norris et al., 2014). In another study, cell-type-specific AS of Dscam2 was detected in the L1 and L2 visual system neurons in flies (Lah et al., 2014). Dscam2 contains two mutually exclusive exons; the variants Dscam2A and Dscam2B are expressed in L2 and L1 neurons, respectively. Similar to Dscam1, Dscam2 also displays homophilic binding-mediated repulsion. Thus, restricting the expression of distinct Dscam2 isoforms to particular neuron subtypes allows the L1 and L2 neurons to interact without repelling each other. However, loss of cell-type specificity in Dscam2 isoform expression results in multiple aberrations in neuronal wiring, including tiling defects and a reduction in the sizes of synaptic arbors (Lah et al., 2014).

The scarcity of convenient tools to monitor and manipulate isoform expression at the single-cell level has made it difficult to perform large-scale studies of cell-type-specific AS. The advent of single-cell RNA-seq methods (Saliba et al., 2014) could potentially resolve at least part of the challenge and be used to detect additional examples of AS events that are differentially regulated between individual neurons. Furthermore, it may identify cell-specific AS regulators based on differences in expression levels. The translating ribosome affinity purification (TRAP) methodology, in which mice are genetically engineered to produce a fluorescently tagged ribosomal protein in specialized cell types of interest (Doyle et al., 2008; Heiman et al., 2014; 2008), can additionally be used to determine the translation profile of individual cell types and detect cell-specific AS factors and protein isoforms. Moreover, the CRISPR/Cas9 technique can be used to delete neuron subtype-specific exons of interest and examine the functions of corresponding isoforms. These methods in combination with single-neuron imaging techniques

will likely become powerful tools for future investigations into cell-type-specific AS.

Identification of Additional Neural Alternative Splicing Regulators

Currently identified regulators of neural AS events are estimated to account for 40%–50% of AS events differentially regulated between neural and non-neural tissues. Thus, the identification of *trans*-acting factors and mechanisms associated with the control of many additional neural-differential AS events represent important challenges for the future. To address this in a timely and systematic manner, recently developed high-throughput techniques can be utilized to screen for splicing regulators. Fluorescent splicing reporters, which display GFP or RFP expression depending on whether an exon is included or skipped, are a common choice to monitor AS in vivo (Norris et al., 2014; Orengo et al., 2006; Zheng et al., 2013). By screening a cDNA library in a gain-of-function screen or an siRNA library in a loss-of-function screen, changes in the fluorescence signal of the reporter are used as readouts to identify positive or negative regulators of an AS event of interest (Zheng et al., 2013). However, generating splicing reporters that efficiently recapture endogenous splicing patterns is a difficult and laborious process, and the number of events that can be monitored at the same time is limited. Instead, RNAi-based high-throughput screens that incorporate barcoding/multiplexing have been developed to simultaneously profile multiple endogenous AS events in an efficient and cost-effective manner (Papasaikas et al., 2015; Tejedor et al., 2015) and are particularly promising tools for uncovering new regulators of neural splicing.

Long Noncoding RNAs

Furthermore, characterizing interactions between AS factors and long noncoding RNAs (lncRNAs) will be an important avenue of research that may provide additional insights into the dynamics of AS regulation in neurons. For example, the brain-enriched lncRNA Gomafu (also known as Miat) has been suggested to play a role in modulating AS by sequestering the splicing proteins SF1, Quaking-1, and SRSF1 in nuclear compartments in non-activated neurons and releasing them upon neuronal activation (Barry et al., 2014; Tsuiji et al., 2011). In addition, Ptbp1 and hnRNP K were found to be associated with the brain-specific lncRNA TUNA and proposed to be recruited to promoters of genes involved in ES cell pluripotency and neural differentiation (Lin et al., 2014). However, the details of how lncRNAs mediate splicing factor availability and/or activity in neurons are largely unclear, and future work in this relatively new field may result in exciting developments.

Deep Learning

The first iteration of machine-learning-based elucidation of the splicing code demonstrated that it is feasible to systematically decipher complex combinations of *cis*-regulatory elements that underlie regulated splicing events (Barash et al., 2010). Since then, additional parameters have been implemented to enhance prediction accuracy, including the use of expanded training datasets and incorporation of a ''deep learning'' approach that samples thousands of potential models in train-test routines (Xiong et al., 2015). An exciting application of the deep learning (neural network) architecture is to generate (single) cell-type- and tissue-specific predictions of AS regulation, including classes of AS events not captured previously by this approach, such as short-length-range microexons. This strategy should enable the prediction of combinations of splicing regulators that provide critical functions in biologically important AS events, as defined by high-throughput PPI screening and other assays. Moreover, it should facilitate the definition of genomic changes, including normal and disease variants, that contribute to or cause neurological disorders and disease.

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