Published in final edited form as:

Curr Opin Neurobiol. 2019 August ; 57: 26–31. doi:10.1016/j.conb.2018.12.013.

Alternative Splicing of Neuronal Genes: New mechanisms and New Therapies

Diane Lipscombe, PhD and

Robert J and Nancy D Carney Institute for Brain Science, Department of Neuroscience, Brown University. Providence, RI 02912, USA. diane_lipscombe@brown.edu

Eduardo Javier Lopez Soto, PhD

Robert J and Nancy D Carney Institute for Brain Science, Department of Neuroscience, Brown University. Providence, RI 02912, USA. ejlopezsoto@gmail.com, eduardo_lopez_soto@brown.edu

Abstract

Dynamic changes in alternative splicing during the life cycle of neurons supports development, plasticity, and is implicated in disease pathology. Cell-specific alternative splicing programs coordinate exon selection across networks of functionally connected genes. In this opinion piece we highlight recent publications that identify some of the molecular mechanisms – RNA and DNA binding proteins and epigenetic modifications – which direct cell specific exon selection during pre-mRNA splicing. Aberrant splicing patterns are signature features of a growing number of diseases of the nervous system. Recent publications demonstrate the value of delineating basic mechanisms that dictate exon choice to inform the development of new therapeutic strategies that correct or compensate for damaging deficits in alternative splicing.

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Alternative splicing – a universal mechanism

Alternative splicing is a form of RNA processing that is critical for virtually every stage in the life cycle of a neuron – starting from early neuronal differentiation, to axonal guidance and synapse formation, to supporting cell signaling and plasticity, and for programmed cell death [1–6]. The capacity of multi-exon genes to generate hundreds to thousands of splice variants is on full display in the nervous systems of animals (e.g. neurexin gene; see Fig. 1) [7]. There are technical challenges associated with identifying alternative mRNA splice isoforms across cell-types of multi-cellular organisms. However, transcriptome analyses of

Corresponding author: Diane Lipscombe PhD, diane_lipscombe@brown.edu, Robert J and Nancy D Carney Institute for Brain Science, Department of Neuroscience, Brown University. Providence, RI 02912, USA.

Declarations of interest: none.

tissues and single cells are revealing the rich palettes of alternative splice isoforms across cell-types, development, adaptation, and in disease [1,4,6,8,9]. In the nervous system, alternative splicing of pre-mRNA is a central mechanism underlying many neuronal functions including adaptation in response to the ever-changing external environment. Cell-specific changes in the patterns of alternative splicing of pre-mRNA, and the ensuing modifications in protein activity, adjust cell function and network dynamics on timescales that are faster; more subtle; and through processes that are potentially less energetically demanding, as compared to those associated with changes in gene expression.

AS is the rule rather than the exception—Next-generation sequencing studies suggest that more than 90% of human multi-exon genes undergo alternative splicing and improved methods for single molecule sequencing are revealing the degree of coordinated splicing in individual RNAs as well as across genes, according to cell state [6,7,9]. Recent discoveries provide exciting insights into the neuronal-specific mechanisms and factors that regulate exon selection and have motivated research on *identifying the regulators that control the action of splicing factors*. In this opinion piece, we focus on recently published data that identify cellular mechanisms that regulate alternative splicing in neurons and, for a couple of example, how these findings informed promising new therapies.

Cellular factors that control alternative splicing

Central nervous systems are hotbeds of alternative splicing of pre-mRNAs and some splice sites are evolutionarily conserved, consistent with a central role in encoding properties essential for neuronal function. We now know many of the *trans*-acting nuclear splicing factor proteins that bind *cis*-elements in pre-mRNAs to either promote, or hinder recruitment of the spliceosome at intron/exon boundaries (see Fig. 1). Splicing factors bind to single or clusters of RNA motifs that are typically located in introns, to enhance or inhibit target exon inclusion. A subset of known *RNA binding protein families* includes polypyrimidine track binding proteins (PTB), RBFOX, NOVA, SR-rich (serine/arginine-rich), STAR, and heterogeneous nuclear ribonucleoproteins (hnRNPs). Genome-wide splicing factor binding maps at different developmental time points, in different tissues, and in disease form the initial framework of a code to eventually predict coordinated state- and cell-specific splicing patterns for networks of genes. However, as highlighted in a recent analysis of the binding specificities of >70 recombinant human RNA binding proteins, as assessed *in vitro* using an array of oligonucleotides, several factors including RNA secondary structure and neighboring nucleotide sequence, have the potential to influence RNA binding protein interactions with target RNAs [10].

Epigenetic modifications have also been shown to influence alternative splicing by mechanisms that are different from those governing the actions of RNA binding proteins. Transcription and pre-mRNA processing are coupled events; transcription rates influence the pattern of alternative splicing and alternative splicing influences transcription [11,12]. *DNA-binding proteins*, including histones, influence RNA Polymerase II (Pol II) kinetics and can alter exon choice during pre-mRNA splicing (see Fig. 1) [11,12]. DNA-binding proteins physically tether Pol II and spliceosome components, to slow Pol II elongation and impact splicing. Slow transcription elongation rates tend to favor alternative exon inclusion by

promoting recruitment of splicing factors and spliceosome to intron-exon boundaries, whereas faster rates favor alternative exon skipping [13–20]. DNA binding proteins that have been implicated in alternative splicing include CTCF binding to *Cd45* gene [16], HP1γ binding to *Cd44* gene [21] and a recent publication, discussed below, shows that *histone H3K9me3 modification* controls cell-specific splicing of a number of genes, including *Nrxn1* gene, during memory consolidation in mice [1] (see Fig. 1). Recent genome-wide analyses of epigenetic markers shows how their occupancy correlates with alternative splicing outcome (exon inclusion or repression) [22–24].

Factors that modify splicing factor action

Many cellular factors regulate the action of *RNA binding protein* splicing factors to influence their cell-specific actions, these factors affect splicing factor expression levels, high order assembly of protein-RNA complexes, posttranslational modification, autoregulation, and alternative splicing [10,25–27]. A recent study by Black's group illustrates the divergent actions of two splice isoforms of the splicing factor RBFOX1 in hippocampal neurons: one isoform localizes to, and acts in the nucleus to affect splicing of target pre-mRNAs; while the other localizes to, and acts in the cytoplasm to affect target mRNA stability. Cytoplasmic acting RNA binding proteins including RBFOX have been shown to bind 30UTRs of several mRNAs to influence mRNA stability [26]. Vamp1 mRNA is a target of RBFOX1 and encodes for VAMP1/synaptobrevin, a v-SNARE that is involved in synaptic vesicle priming and fusion at the neuromuscular junction and in a subpopulation of hippocampal inhibitory neurons (Gtexportal). Relative to VAMP2, VAMP1 is expressed at overall lower levels in brain, but it sits at the center of a protein phosphorylation network suggesting that phosphoregulation of VAMP1, and its associated proteins, is important for regulating neuronal function [57]. Cytoplasmic RBFOX1 stabilizes Vamp1 mRNA by preventing the binding of a microRNA (miR-9) to the 30UTR of Vamp1 mRNA. In the absence of RBFOX1 binding, miR-9 promotes Vamp1 mRNA degradation [26]. This cytoplasmic action of RBFOX1 is cell-specific and by stabilizing Vamp1 mRNA in inhibitory hippocampal neurons, RBFOX1 upregulates inhibitory output and influences the balance of excitation/ inhibitory signaling in hippocampal circuits [26]. This study emphasizes the dual actions of RBFOX1—as a splicing factor or as a regulator of RNA stability—depending on as yet unidentified factors that influence exon choice during alternative splicing of Rbfox1 pre-mRNA.

Long-term changes in nervous system function, including memory formation, are strongly correlated with a range of epigenetic modifications [22,28,29]. While most studies typically focus on documenting changes in gene expression levels, there is evidence of substantial alteration in exon choice during memory consolidation in mice [28]. The impact of memory-associated alterations in histone methylation marks was recently demonstrated for the neurexin 1 gene, *Nrxn1*. Histone modification accompanies memory formation, altering alternative splicing of *Nrxn1* specifically in memory-activated neurons in the dentate gyrus of the hippocampus [1].

Activity-dependent Nrxn1 contains exon 22 (Nrxn1+22), an isoform which was found to protect memories from extinction [1]. Ding et al., demonstrated that the histone mark,

H3K9me3, pauses RNA polymerase II, promoting exon 22 inclusion during pre-mRNA processing, and consequently leading to synapse restructuring (Fig. 1). The shift in the pattern of Nrxn1 splicing from Nrxn1 Δ22 to Nrxn1 + 22, and the subsequent impact on behavior, were shown to be long-lasting and dependent on the combined action of several proteins: A zinc-finger domain protein, p66α, recognizes a TGATAA motif in exon 22; p66α is phosphorylated by AMP-dependent protein-activated kinase following neuronal activity; HDAC2 binds activated p66α; and Suv39h1 a histone methyltransferase is recruited to exon 22 by p66α-HDAC2 assembly. This set of molecular interactions stabilizes the interaction of H3K9me3 with exon 22 of *Nrxn1* [1].

While Ding and colleagues focused primarily on *Nrxn1* in their study, they also reported H3K9me3-mediated co-transcriptional changes in alternative splicing of several genes implicated in memory formation including *Nrxn2, Nrxn3, Gephyrin*, and *Scn1a* [1]. Methyl-binding protein 2 (MeCP2) is also a well-known regulator of neuronal gene expression, and it too has been shown to influence alternative splicing of a large number of genes by RNA Pol II pausing [30]. Mutations in MeCP2 are the major causes of neurodevelopmental disorder Rett syndrome.

Cell-specific epigenetic markers reshape gene expression programs but, as highlighted for *Nrxn1* gene, such modifications are also now being recognized as having profound impacts on alternative splicing of neuronal genes.

New therapies informed by alternative splicing

A growing number of diseases are linked to aberrant alternative splicing either caused by damaging mutations that disrupt splicing directly, by interfering with *cis*-acting elements and the action of *trans*-acting protein splicing factors; or indirectly by sequestering nuclear and cytoplasmic proteins that regulate pre-mRNA splicing [31]. Aberrant splicing is linked to a growing number of disease pathologies including Rett Syndrome [32], epilepsy [33], autism spectrum disorders [34,35], schizophrenia, bipolar disorder [8], spinal muscular atrophy (SMA)[36,37], frontotemporal dementia [38], parkinsonism [39,40], myotonic dystrophy [41], chronic pain [42], amyotrophic lateral sclerosis [31], and cancer [43].

In recent years, DNA engineering technologies, such as CRISPR-Cas9, have been employed to correct pathogenic mutations in neurons that interfere with alternative splicing and to induce compensatory shifts in alternative splicing, away from a nonfunctional, toward a functional splice isoform [44–46].

Most CRISPR-Cas9 applications that target alternative splicing mechanisms have so far involved editing genomic DNA. In particular, cytidine deaminase mediated mutagenesis has been applied to edit highly conserved *cis*-elements at intron-exon junctions necessary for exon recognition and splicing [44,46]. This tool was applied recently to restore the reading frame of the Duchenne muscular dystrophy (*DMD*) gene and found to rescue its function in induced pluripotent stem cells (iPSCs) derived from patients carrying the DMD pathogenic mutation [44].

A related strategy, targeting RNA, was recently applied in patient iPSCs to shift the pattern of alternative splicing in the Tau encoding *MAPT* gene, to compensate for pathogenic mutations in *MAPT* that cause frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [45]. Two forms of MAPT are expressed in neurons in humans, 4R and 3R, that contain or lack exon 10 which encodes one of 4 microtubule binding domains in tau. Importantly, higher levels of Tau-4R in human brain is linked to FTDP-17 [38,47]. Damaging mutations in *MAPT* can occur in the intron downstream of exon 10, disrupting an intronic splice silencer and elevating 4R Tau 17 [38,47]. The CRISPR-Cas9 tool dCasRx targets RNA and, when linked to a splice factor, can modify the pattern of alternative splicing [45]. Konermann *et al.* 2018 successfully employed dCasRx fused to hnRNP splicing factors in iPSCs, to shift splicing from Tau-4R toward Tau-3R in cortical neurons. This was achieved by targeting dCasRx-hnRNPs to exon 10 splice acceptors, and two putative exonic splice enhancer sites in *MAPT* gene, thereby promoting exon skipping. It will be exciting to see if this approach has therapeutic benefits.

Finally, one of the most studied examples of alternative splicing linked to a major neurological disorder involves the Survival of Motor Neuron (SMN) protein, an RNA binding protein that is necessary for small ribonucleoproteins (snRNPs) assembly and for RNA splicing. Pathogenic mutations in *SMN1* gene cause spinal muscular atrophy (SMA), a severe hereditary neuromuscular disease linked to high levels of infant mortality [48,49]. All affected individuals carry damaging mutations (often large deletion) of *SMN1*, but copy number of an adjacent, partially functional paralog gene, *SMN2*, scales inversely with phenotypic severity [49,50]. SMN2 fails to compensate for the loss of SMN1 because of protein instability relative to SMN1. *SMN1* and *SMN2* only differ in few nucleotides, but this difference leads to exon 7 skipping in *SMN2*. Critically, the non-truncated SMN2 exon 7-containing protein is fully functional and sufficient to support neuronal survival in both SMN1-null mice and in human iPSCs derived from SMA patients [36,51–53].

These studies motivated therapeutic strategies to promote exon 7 inclusion in SMN2 thereby compensating for SMN1 loss of function in SMA. In December 2016, the US Food and Drug Administration (FDA) approved Nusinersen (Spinraza™, also known as ISIS–SMNRx or ISIS 396443) for use in treating SMA. Nusinersen, an antisense oligonucleotide (ASO) designed to promote SMN2 exon 7 inclusion, was developed by Ionis Pharmaceuticals and taken into clinical trial in partnership with Biogen. Nusinersen is a 2′- O-methoxyethyl phosphorothioate-modified ASO specifically designed to alter splicing of SMN2 and thus increase the amount of functional SMN protein that is deficient in SMA patients. Clinical trials have reported that Nusinersen promotes full-length SMN protein leading to improved motor function in SMA infants compared to untreated children [54,55].

The publications discussed above are powerful examples of the critical importance of basic research informing the design of potentially highly specific, novel therapies for the treatment of severe neurological diseases. Aberrant splicing is linked to a growing number of disease pathologies. As discussed, for technologies to correct and to control alternative splicing defects we need to continue effort to understand the mechanisms that regulate alternative splicing of neural genes. Knowing the cell-specific signals and proteins that control RNA processing will be key to understanding the mechanisms that generate unique cell-specific

patterns of mRNA isoforms and control expression levels of splice isoforms to influence cell function.

Acknowledgements:

Funded by NIH grant NS055251.

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Highlights

- Alternative splicing is dynamic and supports all stages of neuronal function
- Studies reveal molecular mechanisms that direct cell-specific exon selection
- Epigenetic markers regulate exon choice to support development and plasticity
- New therapeutic approaches can correct or compensate for splicing defects

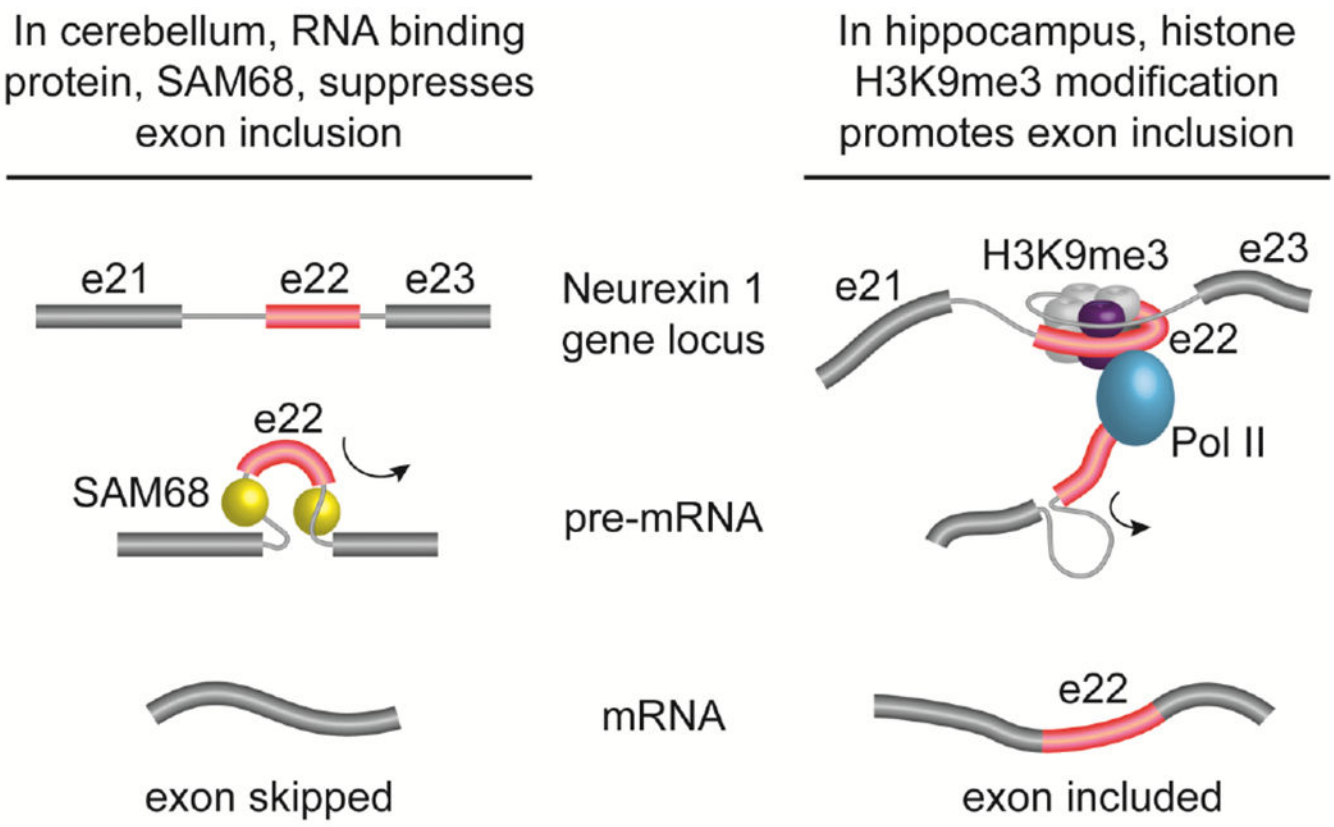

Figure 1. RNA and DNA binding proteins regulate alternative splicing of Neurexin by different mechanisms and in response to different cellular factors.

The *Neurexin 1* gene (*Nrxn1*) generates multiple splice isoforms and contains many alternatively expressed exons. *Nrxn1* exon 22 is alternatively expressed. *Left*: Exon 22 of *Nrxn1* is repressed in both cerebellum and hippocampus by RNA binding protein SAM68 [56]. SAM68 binds *cis* elements in 5' and 3' introns flanking exon 22 (red). *Right*: In response to neuronal activity and memory consolidation, H3K9me3 modification is induced in hippocampus. H3K9me3 modified histone associate with DNA at *Nrxn1* exon 22 locus. Elongation of Pol II is slowed, promoting exon 22 inclusion during pre-mRNA splicing. Activity-induced H3K9me3 underlies a change in the pattern of alternative splicing of *Nrxn1* in hippocampal neurons [1].