Mini-Symposium

Mechanisms of Neuronal Alternative Splicing and Strategies for Therapeutic Interventions

Eduardo Javier Lopez Soto,[1] Michael J. Gandal,[2] Thomas Gonatopoulos-Pournatzis,[3] Elizabeth A. Heller,[4] Diou Luo,[5] and Sika Zheng [6]

[1]Department of Neuroscience, Brown University, Providence, Rhode Island 02912, [2]Department of Psychiatry, Semel Institute, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California 90095, [3]Donnelly Centre, University of Toronto, Toronto, Ontario M5S 3E1, Canada, [4]Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-5158, [5]Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011, and [6]Division of Biomedical Sciences, University of California at Riverside, Riverside, California 92521

Many cellular and physiological processes are coordinated by regulatory networks that produce a remarkable complexity of transcript isoforms. In the mammalian nervous system, alternative pre-mRNA splicing generates functionally distinct isoforms that play key roles in normal physiology, supporting development, plasticity, complex behaviors, and cognition. Neuronal splicing programs controlled by RNA-binding proteins, are influenced by chromatin modifications and can exhibit neuronal subtype specificity. As highlighted in recent publications, aberrant alternative splicing is a major contributor to disease phenotypes. Therefore, understanding the underlying mechanisms of alternative splicing regulation and identifying functional splicing isoforms with critical phenotypic roles are expected to provide a comprehensive resource for therapeutic development, as illuminated by recent successful interventions of spinal muscular atrophy. Here, we discuss the latest progress in the study of the emerging complexity of alternative splicing mechanisms in neurons, and how these findings inform new therapies to correct and control splicing defects.

Key words: **alternative splicing; CACNA1B; spinal muscular atrophy (SMA); autism spectrum disorder (ASD); axonogenesis; chromatin**

Introduction

Alternative pre-mRNA splicing expands the mammalian transcriptome in the nervous system, paving the way for neuronal differentiation and plasticity. The transcriptome and proteome are customized by alternative splicing based on neuronal subtype, and developmental and physiological states. Recent publications show that disruption of alternatively spliced isoform levels, rather than disruption of gene expression levels, is a major source of pathological effects in psychiatric and neurological diseases (Gandal et al., 2018); splicing-correcting therapies are being successfully applied to alleviate disease symptoms in both animal models and in humans (Hua et al., 2011; Finkel et al., 2016; Sinha et al., 2018).

Received June 24, 2019; revised Aug. 9, 2019; accepted Aug. 10, 2019.

Author contributions: E.J.L.S. defined the scope of the review, edited the style, wrote abstract, introduction, a specific section, final remarks, and made the schematic Figures; M.J.G., T.G.-P., E.A.H., D.L., and S.Z. (in alphabetical order) wrote the specific sections, and revised and approved the final manuscript.

This work was supported by Warren Alpert Fellowship Award to E.J.L.S., Canadian Institutes of Health Research (CIHR) Postdoctoral Fellowship to T.G.-P., European Molecular Biology Organization Postdoctoral Fellowship to T.G.-P., Ontario Institute for Regenerative Medicine (OIRM) Postdoctoral Fellowship to T.G.-P., Charles E. Kaufman Foundation Young Investigator Award to E.A.H., Whitehall Foundation Grant to E.A.H., National Institutes of Health-National Institute on Drug Abuse Avenir Director's Pioneer Award to E.A.H., National Institutes of Health Grants NS055925 and R21NS101312 to Ravindra Singh (Diou Luo is a PhD student in Singh Laboratory), National Institutes of Health Grants R01MH116220 and R01NS104041 to S.Z., and SFARI Bridge to Independence Award to M.J.G. We thank Drs. Benjamin J. Blencowe, Diane Lipscombe, Eric W. Ottesen, Joonbae Seo, Natalia N. Singh, Ravindra N. Singh, and Kristin Webster for providing critical input on the review.

The authors declare no competing financial interests.

Correspondence should be addressed to Eduardo Javier Lopez Soto at Eduardo_lopez_soto@brown.edu.

https://doi.org/10.1523/JNEUROSCI.1149-19.2019

Alternative splicing dictates exon composition of mRNAs that will be translated into the final protein. This mechanism generates numerous exon combinations and, consequently, functionally different isoforms from a single multiexon gene. The best-studied mechanisms for determining alternative exon usage involve recognition of *cis*-regulatory elements on pre-mRNAs by RNA-binding proteins (for reviews, see Fu and Ares, 2014 and Vuong et al., 2016). RNA-binding proteins act as trans-acting splicing factors that inhibit or promote exon usage during alternative splicing. But epigenetic factors also act as molecular regulators of splicing (for review, see Luco et al., 2011) (Fig. 1). The RNA polymerase II (Pol II) can interact with DNA-binding proteins and histone post-translational modifications, altering transcription elongation rate or recruiting RNA splicing factors, to promote or inhibit alternative exon usage (Luco et al., 2010; Shukla et al., 2011; Ding et al., 2017; Maslon et al., 2019). The fundamentally different nature of these mechanisms calls for different design principles for therapeutics intended to alter splicing.

The advances in transcriptome and epigenome profiling technologies, together with sophisticated bioinformatic analyses, have vastly improved the detection and quantification of mRNA isoforms in the nervous system. Assigning functional roles for these splicing isoforms is more challenging, but isoforms of some proteins, including REST, PSD-95, and NRXN1, have been thoroughly characterized, and the progress on this front is accelerating.

Rest encodes the RE1-silencing transcription factor, a master repressor of neuronal-specific genes during cell differentiation. During development, the activation of neuronal genes depends on the release of REST binding from neuronal gene loci (Ballas et al., 2005). This can be achieved by REST post-translational degradation, transcriptional repression, or inactivation through alternative splicing (Ballas et al., 2005; Westbrook et al., 2008; Raj et al., 2011; Nakano et al., 2012). The RNA-binding protein SRRM4 promotes *Rest* exon 4 inclusion, triggering a frameshift in *Rest* mRNA that truncates REST, impairing its gene-silencing function and thus derepressing many neuronal genes (Raj et al., 2011; Nakano et al., 2012). Splicing-inactivated REST in the mechanosensory hair cells is required for normal hearing in humans and mice. Mutations causing either loss of function of SRRM4 in mice or inhibition of the frameshifting splicing event in humans are associated with dominantly inherited deafness (Nakano et al., 2012, 2018).

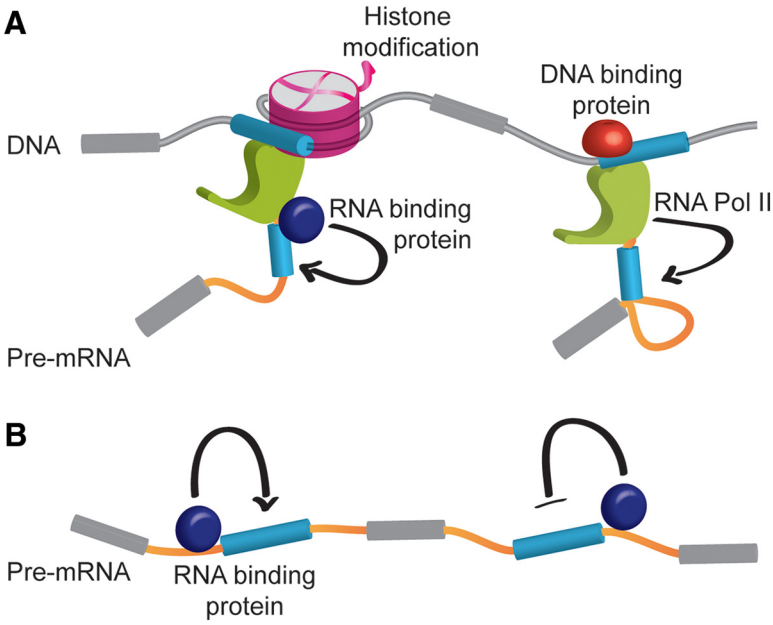

Figure 1. Key mechanisms of alternative pre-mRNA splicing. ***A***, Histone modifications and DNA-binding proteins associated with alternative exons (light blue rectangles) can interact with the RNA Pol II and regulate exon usage by recruiting RNA-binding proteins that recognize pre-mRNA-binding motifs or by slowing down RNA Pol II elongation rate and favoring the inclusion of alternative exons. ***B***, RNA-binding proteins regulate alternative splicing by binding pre-mRNA and driving inclusion or exclusion of alternative exons (light blue rectangles). Gray rectangles represent constitutive exons.

Alternative splicing of *Psd-95* exemplifies the power of splicing control in shaping neuronal differentiation. PSD-95 is an excitatory postsynaptic protein (Cho et al., 1992); and although it is widely considered to be a neural-specific protein, its mRNA is readily detectable outside the nervous system (Zheng, 2016). The underlying mechanism for neural-specific expression of PSD-95 is attributed to different isoforms expressed in neurons versus non-neuronal cells. In neurons, *Psd-95* exon 18 is specifically included to enable PSD-95 translation; whereas in non-neuronal cells, exon 18 is excluded, leading to a frameshift and nonsense-mediated mRNA decay without productive translation (Zheng et al., 2012). During development, the splicing switch occurs before synaptogenesis and is regulated by polypyrimidine tract binding proteins 1 and 2 (PTBP1 and PTBP2), RBM10, and Puf60 (Zheng et al., 2012, 2013). Therefore, neuronal-specific alternative splicing of *Psd-95* enforces neuronal-restricted expression of PSD-95 and ensures the timing of synapse formation.

Alternative splicing of the *Nrxn1* gene generates thousands of neurexin isoforms (Südhof, 2017). Neurexins are presynaptic cell-adhesion molecules essential for synapse formation and synaptic transmission. Learning-induced changes in *Nrxn1*'s exon usage determine binding affinity of neurexins to postsynaptic partners (e.g., Neuroligin 1), impacting synaptic strength (Aoto et al., 2013; Traunmüller et al., 2016; Ding et al., 2017; Dai et al., 2019). A well-studied splicing event is the inclusion of the *Nrxn1* alternative exon 22. In hippocampal neurons, neuronal activation drives the inclusion of *Nrxn1* exon 22; the resulting neurexin isoform is essential for memory consolidation (Ding et al., 2017). In hippocampus, a specific form of histone 3 modification (trimethylation at lysine 9; H3K9me3) cotranscriptionally regulates exon 22 inclusion by slowing the elongation rate of RNA Pol II (Ding et al., 2017); whereas in cerebellum, the RNA-binding protein SAM68 guides the activity-dependent exclusion of exon 22 after *Nrxn1* transcription (Iijima et al., 2011). These observations suggest that alternative splicing of *Nrxn1* is temporally and spatially controlled by different mechanisms in the brain to establish precise synaptic connections.

The aforementioned examples illustrate how neuronal-specific and dynamically regulated splicing events underlie neuronal differentiation and plasticity. Despite the recent progress in the characterization of individual alternative exons, our understanding of the functional roles for the vast majority of spliced isoforms that have been detected in the last years by transcriptome profiling studies, remains limited. A major future goal is to characterize all splicing events with important phenotypic roles, and especially those associated with pathologies. In this review, we summarize recently identified neuronal-specific splicing programs, focusing on individual splicing events and their implications in neurophysiology. We also discuss how disruption of these programs contributes to disease pathology and how understanding these mechanisms can facilitate the development of therapeutic tools to correct mis-splicing events. This review presents recent advances on these fronts as presented at the Mini-Symposium Novel Mechanisms of Neuronal Alternative Splicing and Strategies to Correct Aberrant-Splicing during the Neuroscience 49th Annual Meeting.

Alternative splicing of *Cacna1b* pre-mRNA modifies $Ca_V2.2$ channel properties across different neurons

Transcriptome profiling across neuronal populations reveals substantial differences in the pattern of alternative splicing of ion channels. The vast majority of large multiexon ion channel genes contain alternatively spliced exons that are expressed in neuronal subtype-specific patterns and that modify protein function (for review, see Lipscombe et al., 2013; Allen et al., 2017; Szczot et al., 2017; Thalhammer et al., 2017; Regan et al., 2018; Bunda et al., 2019). A well-studied example of this is *Cacna1b*, which encodes the functional core of $Ca_V2.2$ voltage-gated calcium channels, and controls presynaptic calcium entry that triggers exocytosis. The Lipscombe laboratory has shown that exon usage during

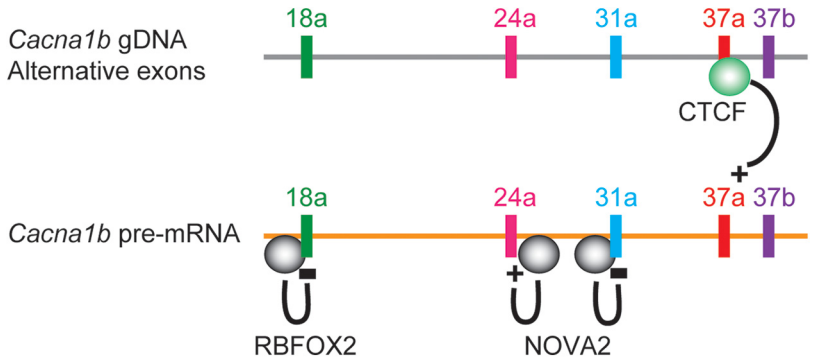

Figure 2. Different mechanisms of *Cacna1b* alternative splicing. The DNA-binding protein CTCF, when bound to genomic DNA locus 37a, promotes exon 37a inclusion during alternative splicing. The RNA-binding proteins RBFOX2 and NOVA2, when bound to *Cacna1b* pre-mRNA, inhibit inclusion of exons 18a and 31a, respectively, but NOVA2 promotes inclusion of exon 24a.

Cacna1b pre-mRNA splicing varies across different neurons to generate distinct patterns of $Ca_V2.2$ isoforms with unique properties and functions (Bell et al., 2004; Lipscombe and Andrade, 2015; Allen et al., 2017). Subsequent research in the laboratory has focused on identifying the mechanisms that regulate alternative splicing of *Cacna1b*. For example, the neuronal splicing-factor families RBFOX and NOVA bind *Cacna1b* pre-mRNA to regulate the usage of alternative exons 18a, 24a, and 31a (Allen et al., 2010, 2017) (Fig. 2). In superior cervical ganglia, RBFOX2 binding upstream of *Cacna1b* exon 18a suppresses its inclusion early in development, but in adults this suppression is reduced and exon 18a is included (Allen et al., 2017). As a result, $Ca_V2.2$ channel trafficking to plasma membrane is promoted in adult neurons because of sequences encoded by exon 18a.

The best studied example of cell-specific alternative splicing of *Cacna1b* involves a pair of mutually exclusive exons (37a and 37b) that encode a 32 amino acid segment in the proximal region of the $Ca_V2.2$ C-terminus. $Ca_V2.2$ channels are located at nociceptor terminals in the dorsal horn of the spinal cord, and this is the site of action of drugs and neurotransmitters that activate G-protein-coupled receptors to downregulate nociception. The neuronal-specific inclusion of exon 37a replaces the more common exon 37b in a subset of nociceptors, resulting in more efficient $Ca_V2.2$ channel membrane trafficking and enhanced G-protein inhibition of $Ca_V2.2$ by μ-opioid receptors (Bell et al., 2004; Castiglioni et al., 2006; Raingo et al., 2007; Macabuag and Dolphin, 2015). This splicing event enhances intrathecal morphine analgesia in nonpathological pain conditions, but it is disrupted following peripheral nerve injury that results in neuropathic pain (Altier et al., 2007; Andrade et al., 2010; Jiang et al., 2013).

The Lipscombe laboratory has identified the factors that promote inclusion of exon 37a during *Cacna1b* splicing. Unexpectedly, *Cacna1b* exon 37a inclusion is controlled by the DNA-binding protein CTCF: CTCF binding to exon 37a promotes its inclusion in nociceptors. CTCF is a ubiquitously expressed chromatin regulator that has been shown to influence alternative exon usage by pausing RNA Pol II (Shukla et al., 2011). In a subset of nociceptors, epigenetic modulation of CpG methylation in locus 37a controls CTCF binding and thus controls *Cacna1b* exon 37a usage. Peripheral nerve injury alters the epigenetic marks in *Cacna1b* exon 37a, reducing CTCF binding and limiting exon 37a usage, thus contributing to the reduced morphine analgesia in neuropathic pain. This study underscores the importance of identifying the factors that control cell-specific alternative pre-mRNA splicing that impact drug action in disease states, and could inform the development of therapies to increase drug efficiency.

Genomewide interrogation of microexon regulators reveals genetic links to autism spectrum disorder (ASD)

ASD is a neurodevelopmental and neuropsychiatric disorder characterized by impaired social interactions and repetitive patterns of behavior that affects >1% of the population. Technological advances in genomics have revealed that ASD is not a single disease but rather a heterogeneous spectrum of etiologically distinct conditions, which result in common behavioral manifestations. Despite the profound genetic heterogeneity of ASD, this disorder is characterized by the disruption of convergent molecular and cellular mechanisms, such as synaptic function, activity-dependent transcription, and localized translation (de la Torre-Ubieta et al., 2016; Quesnel-Vallières et al., 2019). A major advancement in our understanding of the underlying causes of ASD has emerged through transcriptome profiling studies, which revealed that alternative pre-mRNA splicing is often mis-regulated in ASD patients (Irimia et al., 2014; Parikshak et al., 2016; Gandal et al., 2018; Quesnel-Vallières et al., 2019).

Alternative microexons (3–27 nucleotides) represent the most highly conserved class of splicing events and tend to preserve the open reading frame, often overlapping domains that mediate protein–protein interactions (Black, 1995; Irimia et al., 2014; Li et al., 2015; Ustianenko et al., 2017). The Blencowe laboratory recently discovered that an extensive network of neuronal microexons is frequently disrupted in the brains of ASD patients (Irimia et al., 2014). Approximately 120 microexons, enriched in synaptic genes, display reduced inclusion in the brains of >30% of analyzed individuals with idiopathic autism. Notably, the expression level of neuronal-specific splicing factor SRRM4, which is critical for the splicing of most neuronal microexons, is reduced in the brains of ASD subjects that display attenuated microexon inclusion (Irimia et al., 2014). SRRM4 is a vertebrate-conserved RNA-binding protein critical for nervous system development (Calarco et al., 2009; Raj et al., 2011; Quesnel-Vallières et al., 2015). Importantly, mice haploinsufficient for *Srrm4* recapitulate reduced microexon inclusion and display multiple ASD-like features, including altered social behavior, increased sensitivity to environmental stimuli, and altered synaptic transmission, strongly suggesting that microexon mis-regulation is causally linked to autism (Quesnel-Vallières et al., 2015, 2016).

Despite the importance of microexons in nervous system development and disorders, the molecular mechanisms that regulate microexon usage during splicing are not well understood. This was addressed by developing a CRISPR-based genomewide screening strategy that identified >200 microexon regulators, including chromatin, protein turnover, and RNA processing factors enriched for genetic links to autism (Gonatopoulos-Pournatzis et al., 2018). These results revealed that SRRM4 regulates neuronal microexon usage by forming interactions with the SR-related proteins SRSF11 and RNPS1, as well as an intronic enhancer element, and that these interactions are required for early splicing complex assembly. Furthermore, critical chromatin regulators, including EP300 and CREBBP, impact microexon splicing by controlling SRRM4 expression. Interestingly, muta-

tions and/or alterations in the expression of SRSF11, RNPS1, EP300, and CREBP are linked to neurodevelopmental disorders (Nguyen et al., 2013; Korzus, 2017; Lim et al., 2017; Yuen et al., 2017), providing additional genetic evidence that disruption of microexon splicing underlies nervous system development disorders.

A major challenge that lies ahead is to determine the functions of individual microexons, especially in relation to autistic phenotypes, and to explore their mechanisms of action. Recent studies have revealed that individual microexons can impact animal behavior (Rusconi et al., 2017; Parras et al., 2018). The Cytoplasmic Polyadenylation Element Binding (CPEB) proteins are RNA-binding proteins that promote cytoplasmic mRNA polyadenylation regulating several biological processes, including learning and memory (Ivshina et al., 2014; Si and Kandel, 2016). Interestingly, inclusion of a neuron-specific microexon in CPEB4 controls the deadenylation and translation of ASD-risk genes, and imbalance in the expression of the *Cpeb4* microexon causes ASD-like phenotypes in mice (Parras et al., 2018). In addition, a microexon in the Lysine Demethylase 1A (KDM1A) controls activity-dependent transcriptional programs and impacts emotional behavior and seizure susceptibility (Rusconi et al., 2015, 2016). Collectively, these studies provide evidence that individual microexons underlie distinct phenotypic outcomes and that disruption of the SRRM4-dependent microexon network contributes to ASD-related phenotypes. The next challenge is to develop high-throughput screening strategies that systematically identify alternative exons, including neuronal microexons, with critical roles in development and whose mis-regulation contributes to disease and disorders. Identification of such splicing events is expected to facilitate the development of therapeutic approaches to revert specific disease phenotypes.

Chromatin-mediated alternative splicing is linked to reward pathophysiology

Chromatin state regulates alternative pre-mRNA splicing, underlying remodeling events by which mature neurons could mount a stable response to environmental stimuli (Ding et al., 2017) (for review, see Luco et al., 2011). The Heller laboratory posits that, in postmitotic neurons, alternative exon usage maintained by stably associated histone modifications underlies chronic disease states, such as addiction. This hypothesis is based on two findings: (1) cocaine exposure changes isoform expression to a far greater extent than it affects total gene expression levels (Feng et al., 2014); and (2) alternative splicing is functionally coupled to specific histone modifications (Auboeuf et al., 2002; Luco et al., 2010; Kim et al., 2011), which are also altered by cocaine exposure (Kumar et al., 2005; Feng et al., 2014). While many neurological diseases, including frontotemporal dementia, Rett syndrome, bipolar disorder, ASD, and schizophrenia, have been linked to large disruptions in isoform expression levels (Licatalosi and Darnell, 2006; Gandal et al., 2018), this mechanism is understudied in the context of drug abuse and addiction. Therefore, the Heller laboratory is currently investigating the hypothesis that cocaine alters alternative splicing in brain reward regions by remodeling chromatin states. To examine this, they analyze the genomewide association between a suite of seven histone modifications and alternative exon usage in nucleus accumbens, a structure central to reward seeking, from mice treated with cocaine or saline. Using novel computational approaches, classical statistical methods combined with machine learning, they found that two histone modifications (trimethylation at lysine 36 of histone 3, [H3K36me3], and methylation of lysine 4 of histone 3 [H3K4me1]) have the strongest association with alternative splicing (Hu et al., 2017).

A second study tested the hypothesis that specific histone modifications could drive development by regulating expression of alternative spliced isoforms during differentiation (Hu et al., 2017). To address this idea, they undertook a comprehensive genomewide analysis that related histone modifications to alternative exon usage during mammalian tissue development. They found that H3K36me3 and H3K4me1 play a dominant role in exon exclusion across all the tissues and developmental time points examined. Specifically, H3K36me3 positioning in alternative exons is the strongest predictor for exon exclusion during alternative splicing in all tissues.

Given the evidence that H3K36me3 positioning strongly predicts alternative exon usage across various tissues and development, Xu and Heller (2018) next analyzed the role of H3K36me3 in influencing alternative splicing in adult mouse nucleus accumbens. Analysis was performed on ChIP/RNA-Seq datasets from nucleus accumbens following either cocaine (or saline) self-administration or overexpression of SET2, the histone methyltransferase that catalyzes H3K36me3. The authors found an overlapping set of alternative exons that are differentially expressed, are enriched for H3K36me3, and contain the binding motif of the Serine and Arginine Rich Splicing Factor 11 (SRSF11). Unexpectedly, *Srsf11* mRNA is itself differentially spliced and enriched in H3K36me3 in the corresponding alternative exon in cocaine- or SET2-treated tissue relative to controls. Then, they used a targeted epigenetic editing tool to distinguish the direct causal relevance of the H3K36me3 at the alternative exon from pleiotropic, genomewide effects. In this approach, a histone methyltransferase catalytic domain linked to an engineered sequence-specific DNA-binding protein enables methylation of histones located only in the vicinity of the target alternative exon. Interestingly, they found that H3K36me3 enrichment targeted to the *Srsf11* alternative exon promotes exon inclusion during alternative splicing but has no effect on overall *Srsf11* expression.

Together, the combination of machine learning and epigenetic editing tools suggests that H3K36me3 enrichment influences alternative exon usage. The Heller laboratory is currently working to determine the relevance of SRSF11 isoform expression to cocaine-mediated behavior and the precise mechanism by which this histone modification contributes to alternative splicing, either via direct recruitment of RNA-binding proteins or regulation of the kinetics of RNA Pol II.

PTBP2 orchestrates alternative splicing programming required for axon formation

Neurons become polarized in phases: neurites form, then begin to grow before acquiring axonal characteristics (Arimura and Kaibuchi, 2007). Despite significant progress in understanding the cell biology of neuronal polarization, many basic questions remain. For example, although many genes that control axonogenesis, the first step in neuronal polarization, have been identified (Liu and Szaro, 2011; Zollinger et al., 2015; Rao et al., 2017; Tilot et al., 2018), how they are coordinated is unclear. In a quest to identify genetic determinants of cortical neuronal polarization, the Zheng laboratory generated the first transcriptome datasets of cortical neurons before and after axon specification (Zhang et al., 2019). Multipronged analyses revealed a surprisingly strong association between neural-specific splicing events and axonogenesis. The expression levels of previously identified axonogenesis-controlling genes were similar before and after axon extension, but many showed changes in isoform expression. This finding suggests that neuronal polarization is executed in

part by regulating polarity genes through alternative splicing mechanisms. Importantly, the authors found that the alternative exons that are expressed during early axonogenesis are neural-specific, suggesting that they may be part of regulatory mechanisms that distinguish neuronal polarization from planar cell polarity.

The Zheng laboratory found that the RNA-binding protein PTBP2 governs axonogenesis-associated alternative splicing (Zhang et al., 2019). Specifically, *Ptbp2* depletion results in shorter axons of cortical neurons both *in vitro* and *in vivo*. Interestingly, some *Ptbp2* null neurons can produce surplus axons marked by axonal markers Tau1 and AnkG. Therefore, axonal growth is impeded, but axon specification appears to be enhanced in the absence of PTBP2. The unusual phenotype caused by *Ptpb2* knockout likely reflects that the initial period of neurite growth builds the structural prerequisites for axon formation. If the alternative splicing program changes too soon, as happens when *Ptb2* is knocked out, those prerequisites are not met, so the axon cannot be specified. The Zheng laboratory data suggest that the hierarchy of cellular events leading to axon formation is coordinated by PTBP2 regulation of alternative exon usage (Zhang et al., 2019).

Since the polarity genes alter their isoform expression during early axon formation, it is tempting to speculate that alternative splicing gives rise to functionally different isoforms involved in axon formation. Future studies are directed toward identifying the critical PTBP2-regulated events that underlie axonogenesis. Considering that structural and functional impairment of axons is a common manifestation of neurodegenerative, neuroinflammatory, and neurodevelopmental disorders (Medana and Esiri, 2003), this research provides a unique perspective to better understand the genetic, molecular, and cellular control of disease processes.

Novel insights from splicing regulation of the spinal muscular atrophy (SMA) genes

SMA is a devastating autosomal-recessive disorder associated with infant mortality, with an incidence of ~1 in 10,000 live births. SMA is caused by low expression levels of a ubiquitous protein, survival motor neuron (SMN) protein, which plays an essential role in transcription, splicing, translation, RNA trafficking, and cell signaling (Singh et al., 2017b). Homologous deletion or disruption of *SMN1*, the main gene that encodes SMN protein, accounts for >90% of SMA cases (Lefebvre et al., 1995; Wirth, 2000).

Humans have another gene, *SMN2*, which is an almost identical copy of *SMN1*. Due to the predominant skipping of exon 7 during splicing, *SMN2* produces low levels of SMN (for review, see Khoo and Krainer, 2009; Singh and Singh, 2018). Importantly, promoting inclusion of this exon has been exploited for rescuing SMA phenotypes (Hua et al., 2008, 2010, 2011, 2015; Passini et al., 2011; Rigo et al., 2012; Sahashi et al., 2013). Nusinersen (Spinraza), the first drug approved by the FDA for treatment of SMA, is an antisense oligonucleotide that promotes *SMN2* exon 7 inclusion (Singh et al., 2017a). Nusinersen blocks the Intronic Splicing Silencer N1 (ISS-N1) located downstream of *SMN2* exon 7 (Singh et al., 2006; Singh and Singh, 2018). Of note, ISS-N1 has been shown to be a highly inhibitory sequence for *SMN2* exon 7 usage (Singh et al., 2006). In addition to several antisense oligonucleotides that have been successfully used to restore SMN protein, small molecules, and engineered small nuclear ribonucleoproteins have shown promise for correcting *SMN2* exon 7 splicing (Singh et al., 2017a). Recent work by the Singh laboratory has demonstrated the utility of small-nuclear-ribonucleoprotein based approaches in correcting splicing that cannot be corrected by antisense oligonucleotides and small compounds (Singh and Singh, 2019).

A recent report from the Singh laboratory expands the diversity of alternative pre-mRNA splicing events of *SMN* genes. They found that *SMN* genes harbor a surprisingly high content, ~39%, of the most common transposable elements in the human genome, the Alu elements (Ottesen et al., 2017). Multiple intronic repeats of inverted Alu elements in *SMN* genes likely influence the formation of secondary structures in their pre-mRNAs favoring the generation of circular RNAs during alternative splicing (Jeck et al., 2013; Liang and Wilusz, 2014; Wilusz, 2015; Zhang et al., 2016). The Singh laboratory confirmed the formation of a vast repertoire of circular RNAs generated by *SMN* genes during splicing in various human cell lines and tissues (Ottesen et al., 2019). Circular RNAs can affect various aspects of cellular metabolism, including sequestration of RNAs and proteins, and regulation of proteins synthesis and transcription (Barrett and Salzman, 2016). The findings of *SMN* circular RNAs expand the potential role of *SMN* genes in cells. Further studies will explore the function of these newly discovered *SMN* circular RNAs, whether they contribute to SMA pathology, and how they could be exploited for SMA therapeutic and diagnostic applications.

In conclusion, the discovery that alternative splicing is a major contributor to the core phenotypic properties in neurons has inspired investigation of splicing regulators and splicing misregulation in disease. Recent publications highlight the value of comprehensive analysis of alternative splicing in the context of neuronal development, plasticity, and pathology, including neuropathic pain, cognitive dysfunction, addiction, and genetic disorders. The emerging functional and regulatory complexity of alternative splicing generates new questions in the field: What are the main determinants that drive splicing factors to recognize RNA or DNA elements in some neurons, but not in others, even when those splicing factors are ubiquitously expressed? Is alternative exon usage influenced by one factor acting alone or in a coregulated fashion with other factors? What are the functional roles of individual splicing isoforms, and does their disruption underlie specific disease phenotypes? Is targeting these regulators a viable option to restore aberrant alternative splicing programs to baseline as a treatment for splicing-linked diseases? Identifying neuron-specific molecular drivers encompassing alternative pre-mRNA splicing and regulators offers new, attractive drug targets for cognitive disorders, addiction, pathological pain, and genetic disorders that are notoriously challenging pharmaceutical targets.

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