Programmable macromolecule-based RNA-targeting therapies to treat human neurological disorders

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ABSTRACT

Disruptions in RNA processing play critical roles in the pathogenesis of neurological diseases. In this Perspective, we discuss recent progress in the development of RNA-targeting therapeutic modalities. We focus on progress, limitations, and opportunities in a new generation of therapies engineered from RNA binding proteins and other endogenous RNA regulatory macromolecules to treat human neurological disorders.

Keywords: RNA-targeting therapeutics; gene therapy; neurological disorders; programmable macromolecules

INTRODUCTION

Human nervous system function and stability are complex processes that largely depend on the meticulous regulation of gene expression. Spatial and temporal expression of the intricate protein networks that regulate the nervous system's diverse cellular architecture is, in turn, reliant on posttranscriptional control of RNA.

Particularly in neurons, messenger RNA (mRNA) molecules undergo extensive processing, including splicing, polyadenylation, editing, transport, translation, and turnover (Li et al. 2007; Dillman et al. 2013; Nussbacher et al. 2019). Indeed, multiple genome-wide analyses have revealed that neurons, of all cell types, make the most complex use of alternative splicing to express a vast number of gene isoforms required for every stage of their life cycle and in support of synaptic plasticity (Iijima et al. 2011; Li et al. 2014; Traunmuller et al. 2016; Vuong et al. 2016; Zhang et al. 2016; Baralle and Giudice 2017). RNA editing, too, is a critical processing event, as it regulates synaptic transmission in the editing and splicing of glutamate receptor pre-mRNA (Bratt and Ohman 2003; Rosenthal 2015). As a result, perturbations in RNA metabolism often lead to serious diseases, with disruptions in all forms of RNA processing together defined as a unifying contributing factor in the pathogenesis of many neurodegenerative disorders (Liu et al. 2017). These include Alzheimer's disease (AD)—the most common manifestation of neurodegeneration—as well as Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Dawson and Dawson 2003; Vance et al. 2009; Barmada et al. 2010; Belzil et al. 2013; Nussbacher et al. 2015; Johnson et al. 2018; Hsieh et al. 2019).

It is estimated that up to 1 billion people worldwide suffer from a neurological disorder, yet there are currently few therapeutic options (GBD 2016 Neurology Collaborators 2019). Most have no cures. For many, however, the causative inherited mutation and its underlying mechanism are known—whether by toxic gain-of-function, haploinsufficiency, or the loss-of-function of critical RNA regulatory elements or RNA molecules themselves. As a result, the development of systems for efficient recognition and modulation of RNA in living cells has been a major focus of biotechnology for decades. A great deal of pioneering work has produced several categories of RNA-targeting therapeutics that are capable of modulating pre-mRNA splicing, altering target gene expression, and editing RNA to counteract many disease-causing mutations.

The first clinical applications of RNA-targeting therapeutics were synthetic RNA-targeting oligonucleotides, most commonly single-stranded antisense oligonucleotides (ASOs), designed specifically to hybridize to target RNA via Watson–Crick base-pairing. ASOs are short synthetic strands of chemically modified deoxynucleotides or deoxyribonucleotides (Dhuri et al. 2020). ASOs can alter pre-mRNA splicing by sterically blocking splicing factors

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(splice-switching ASOs or ssASOs), block mRNA translation by preventing ribosome recruitment, or degrade RNA by recruiting RNase H to cleave the targeted transcript (gapmer ASOs) (Bennett and Swayze 2010).

To date, several ssASOs have been clinically approved by the FDA to treat multiple neurodegenerative disorders, including nusinersen for spinal muscular atrophy (SMA), four different ASOs approved for the treatment of Duchenne muscular dystrophy (DMD), and milasen, a personalized ASO designed to treat an individual with Batten disease (Cirak et al. 2011; Mercuri et al. 2018; Kim et al. 2019). In each case, the ssASO treats disease by modulating pre-mRNA splicing of each disease locus, in order to restore the functional protein. Two gapmer ASOs have also been clinically approved (inotersen and mipomersen), both used to treat peripheral nerve damage associated with hereditary transthyretin-mediated amyloidosis (Wong and Goldberg 2014; Benson et al. 2018).

Double-stranded small interfering RNA (siRNA) and microRNA—engineered RNAs which also target RNA transcripts by Watson–Crick base-pairing—are designed to degrade toxic RNA molecules through RNA interference. Excitingly, five RNAi-based therapies, patisiran, givosiran, lumasiran, inclisiran, and vutrisiran, received clinical approval for the treatment of several rare metabolic disorders including polyneuropathy associated with hereditary transthyretin-mediated amyloidosis (Wood 2018; Gangopadhyay and Gore 2022).

Despite validated therapeutic results, oligonucleotide-based RNA-targeting modalities face many challenges and limitations that prevent their extensive use in treating neurological disorders. For example, even though ASOs have shown great promise in eliminating pathogenic transcripts or modulating RNA splicing in preclinical studies, they are synthetic in construction and thus cannot be encoded within DNA. This prevents ASO delivery by FDA-approved adeno-associated viral delivery vehicles (AAVs) that can continuously express a DNA-encoded gene therapy for long periods of time in vivo. ASOs have to be regularly administered throughout the lifetime of the patient. Furthermore, since current versions of ASOs cannot cross the blood-brain barrier (BBB), delivery methods to the central nervous system (CNS) are invasive and include multiple intrathecal injections. However, future strategies that utilize antisense vectorization may mitigate these concerns (Imbert et al. 2017). Yet other potential challenges reported for ASOs in both clinical and preclinical studies include inadequate target engagement and off-target toxic effects with high doses (Lindow et al. 2012; Dobrovolskaia and McNeil 2015; Burel et al. 2016; Imbert et al. 2017; Yoshida et al. 2019; Kingwell 2021). Such challenges have been highlighted by the multiple gapmer ASOs developed to treat HD. The most promising of these include Roche's phase III ASO candidate tominersen and Wave Therapeutics' phase I/II ASO candidates WVE-120101 and WVE-120102, each failing to show adequate efficacy and safety in their respective clinical trials. In fact, preliminary results from clinical trial NCT03761849 indicated that two independent dosing regimens of tominersen in patients performed slightly worse than placebo on the primary outcome measures of the composite Unified Huntington's Disease Rating Scale (cUHDRS) and total functional capacity (Kingwell 2021). These data suggest that this ASO may have toxic effects in doses required for a therapeutic effect for this neurodegenerative disorder. Revised clinical trials will be conducted to determine if these outcomes resulted from either the repeated high dose used or interference with wild-type HTT protein levels (Kingwell 2021; Rook and Southwell 2022). Unfortunately, Wave's ASO candidates targeting patient-specific single nucleotide polymorphisms on mutant huntingtin (HTT) pre-mRNA showed limited target specificity, which also led to low efficacy and ultimately paused its clinical development (Kingwell 2021).

Whereas RNAi-based therapies can be delivered via AAV, clinical translation has been hindered by immunogenicity caused by double-stranded RNA therapy-triggered Toll-like receptor mediated innate immune responses (Pecot et al. 2011). Delivery of RNAi-based therapies with lipid nanoparticles may elude this immune response (Paunovska et al. 2022). Although, this strategy increases the complexity of clinical development and delivery as lipid nanoparticles cannot cross the BBB. Furthermore, considering that microRNA can bind to target RNA via incomplete base-pairing, off-target silencing of multiple targets is also an issue (Jackson and Linsley 2010; Pecot et al. 2011). Artificial siRNAs and microRNAs also utilize endogenous cellular machinery for controlling normal microRNA biogenesis, causing saturation in high doses that leads to undesired global alterations in gene expression (Boudreau et al. 2009; Khan et al. 2009).

These limitations create a need to engineer additional RNA-targeting methods that can regulate RNA metabolism in the nervous system and treat neurological disorders. An ideal RNA-targeting system would be non-immunogenic, have limited off-target effects on the human transcriptome, and be encoded in AAV vectors, allowing long-term continuous production of therapeutic materials in situ and taking advantage of the growing variety of tissue-targeted AAV serotypes which can effectively be delivered into the human CNS (Hocquemiller et al. 2016). Here, in this brief Perspective, we review neurological applications for cutting-edge RNA-based therapeutics and survey their potential to change the therapeutic landscape for many neurological disorders.

DEVELOPMENT OF PROGRAMMABLE RNA-TARGETING THERAPIES WITH EXOGENOUS MACROMOLECULES

DNA-targeting CRISPR/Cas9 technology has been used to perform various genomic engineering tasks (O'Connell

et al. 2014). However, current strategies that edit the genome by first creating double-strand breaks in genomic DNA run the risk of off-target toxicity at unintended sites that may cause permanent and inheritable chromosomal insertions or genome alterations. Therefore, it has been of great interest to develop a similar universally programmable therapeutic modality that can alter gene expression by targeting RNA, a more transient intermediate in the gene expression process.

CRISPR/Cas9 relies on a protospacer adjacent motif (PAM) to recognize its DNA target. Based on this mechanism, *Streptococcus pyogenes* (SpCas9) was adapted to target RNA. This CRISPR system includes SpCas9 with a deactivated DNA nuclease, supplied with a short oligonucleotide containing the PAM sequence (a PAMmer) to direct single-stranded RNA binding and catalyze cleavage in vitro (O'Connell et al. 2014; Nelles et al. 2016). Even in the absence of a PAMmer, catalytically dead RNA-targeting SpCas9 can be fused to fluorescent proteins to visually track mRNA, to endonucleases to degrade repetitive RNAs, and to adenosine deaminase domains to edit RNA in mammalian cells (Batra et al. 2017; Marina et al. 2020). It has even been used in vivo to reverse neuromuscular phenotypes in a mouse model of myotonic dystrophy (Batra et al. 2021). Considered a major advancement, many other RNA-targeting Cas9 systems were subsequently developed, including *Campylobacter jejuni* (CjCas9) (Dugar et al. 2018), *Neisseria meningitidis* (NmCas9) (Rousseau et al. 2018), and *Staphylococcus aureus* Cas9 (SaCas9) (Strutt et al. 2018). However, due to their large sizes, RNA-targeting CRISPR/Cas9 systems have issues with AAV packaging for delivery.

Recently, naturally RNA-targeting type VI CRISPR/Cas systems (CRISPR/Cas13) have been identified and are intriguing candidates for RNA-targeted therapeutics (Abudayyeh et al. 2016; Smargon et al. 2017). These type VI systems, divided into at least four subtypes (A–D) based on the phylogeny of effector complexes, possess dual RNase activities that are capable of processing CRISPR arrays and efficiently degrading target RNAs by continuous cleavage. Cas13d was originally shown to possess several advantages over other Cas13 variants, including smaller size, higher potency, and targeting sequence generalizability (Konermann et al. 2018). In cell-based screenings, nuclear localization sequence (NLS)-fused Cas13d showed a strong ability to cleave target RNA with high efficiency relative to short-hairpin RNA, dCas9-mediated CRISPR interference, and other Cas13 effectors with minimal off-target effects in mammalian cells. It was also recently shown to be expressed safely and robustly in vivo, specifically within the mouse CNS when delivered via AAV to target neurodegenerative genetic loci (Powell et al. 2022). These attributes indicate that CRISPR/Cas13d is a promising platform for RNA targeting in clinical applications for neurodegenerative disorders.

Cas13d-based therapeutics demonstrate potential as a possible treatment strategy for neurodegenerative disorders caused by dominantly inherited microsatellite repeat expansions (MREs). These toxic gain-of-function mutations cause aberrant RNA secondary structures that sequester endogenous RNA binding proteins from their cognate RNA targets, and form toxic mutant dipeptide repeat proteins. MREs have been implicated in over 30 neurological disorders, including myotonic dystrophy and HD, highlighting the need to develop an allele-specific therapeutic strategy that can selectively degrade toxic expanded-RNA (Schwartz et al. 2021).

We recently demonstrated the proof-of-principle that a mutant allele-selective RNA-targeting CRISPR–Cas13d system eliminates toxic mutant HTT transcripts in HD patient-derived fibroblasts and iPSC-derived neurons with limited off-target effects on the human transcriptome (Morelli et al. 2023). AAV-mediated delivery of this mutant HTT mRNA targeting Cas13d system to the striatum of heterozygous zQ175 mice, an established mouse model of HD, resulted in allele-selective suppression of mutant HTT mRNA and protein aggregates while maintaining normal HTT mRNA and protein levels, significantly improved motor function, and attenuated striatal atrophy. These phenotypic improvements lasted for at least 8 months without adverse effects, and with minimal off-target transcriptomic effects (Morelli et al. 2023). This strategy also holds promise as a possible therapeutic approach for myotonic dystrophy type 1 (DM1) as we demonstrated Cas13d with CUG-targeting guide RNAs can eliminate toxic RNA foci in a cortical organoid of DM1 (Morelli et al. 2022).

In addition to RNA degradation, nuclease-dead versions of these Cas13 systems have been repurposed for in vivo RNA modifications, including programmable regulation of alternative splicing, A-to-I, C-to-U editing, and m^6A modifications. For example, catalytically inactive dCas13b fused to ADAR2 deaminase domain ($ADAR_{DD}$), an enzyme that deaminates adenosine to inosine in double-stranded RNA, can be directed by guide RNA to a specific RNA sequence of interest (Cox et al. 2017). This programmable RNA editing system, referred to as RNA editing for programmable adenosine to inosine replacement (REPAIR), and other such CRISPR–Cas ADAR fusion systems, can change single RNA nucleotide residues (A to I) within endogenous RNA transcripts in mammalian cells (Marina et al. 2020). Inosine is structurally similar to guanosine and is read as a guanosine by most cellular machinery including during translation, splicing, and reverse transcription, effectively creating an A-to-G edit in RNA.

A-to-I editing sites are not only located in protein-coding regions within RNA, but also within noncoding sequences, including in the 5′ and 3′ untranslated regions (UTRs) and within intronic retrotransposon units such as Alu and long-interspersed elements (LINE). Therefore, future adaptations of ADAR-based editing systems could

be exploited to regulate RNA metabolism by affecting microRNA–RNA interactions, RNA splicing, RNA stability, cellular localization of transcripts, and circRNA formation. The disruption of each is implicated in several neurological disorders.

Excitingly, in addition to A-to-I editing, an engineered $ADAR_{DD}$ evolved to deaminate cytidine enables CRISPR–Cas directed cytidine-to-uridine editing, a system now referred to as RESCUE (RNA editing for specific C-to-U exchange) (Abudayyeh et al. 2019). Together, REPAIR and RESCUE provide a new mechanism capable of reversing disease-causing single-nucleotide G-to-A and T-to-C mutations at the RNA level. This approach could yield a potential treatment strategy for several neurological disorders and beyond, as point mutations are the most common cause of hereditary diseases. Indeed, G-to-A and T-to-C mutations comprise up to 61% of all pathogenic point mutations in humans (Rees and Liu 2018). Its success as an RNA-targeting therapy is unclear in its current form, however, as REPAIR and RESCUE only edit at most ∼30% of target endogenous transcripts in in vitro models. Furthermore, REPAIR and RESCUE have significant off-target effects on the transcriptome, likely in part due to Cas13-$ADAR_{DD}$ fusion associations with endogenous RNAs independent of guide RNA, as recently reported for Cas9 RNA editing in human cells (Smargon et al. 2022). Thus, reengineering of RESCUE may be required to achieve a sufficient editing rate in vivo that can safely mitigate a disease phenotype. Similar strategies that recruit endogenous ADAR to a guide RNA have also been developed for programmed A-to-I editing (Katrekar et al. 2019; Merkle et al. 2019; Qu et al. 2019). These approaches have reduced risks for immunogenicity and off-target effects on the transcriptome compared to strategies using exogenous ADAR.

A recently developed C-to-U editor, CURE (C to U RNA editor) may serve as a possible alternative C-to-U editing strategy. This programmable editing system fuses APOBEC3A, a natural cytidine deaminase, to dCas13d with a nuclear localization domain. Preliminary in vitro screenings have demonstrated this system to have a high specificity toward C-to-U editing, with the ability to edit C-to-U up to ∼60% efficiency at endogenous targets. Also, due to its natural affinity for UC dinucleotides, it edits RNA with limited off-target effects on other cytidine residues in the transcriptome compared to RESCUE (Huang et al. 2020).

dCas13d has also been recently adapted to deliver truncated N^6-methyladenosine (m^6A) binding proteins to specific sites on RNA (Wilson et al. 2020). This system can be used as a tool to gain critical insight into neurological function, as m^6A modifications play important roles in neurodevelopment and aging (Widagdo and Anggono 2018; Jiang et al. 2021). It also yields a potential new therapeutic modality for neurodegenerative disorders in which disease progression may involve alterations in m^6A modification, including Alzheimer's disease and Parkinson's disease (Chen et al. 2019; Han et al. 2020).

Despite promising results in vitro and in preclinical models, the bacterial origin of Cas13 may preclude clinical translation. For example, we recently discovered that a fraction of the population already has pre-existing adaptive immunity to Cas13d (Tang et al. 2022). Off-target RNA cleavage by Cas13 orthologs has also been identified in vitro, specifically within human and mouse immortalized cell lines, as well as in vivo (Ai et al. 2022; Kelley et al. 2022; Yunfei Li et al. 2022). Such collateral damage may interfere with its therapeutic use. To counteract these limitations, a synthetic RNA-targeting system made from human-derived proteins that mimics CRISPR/Cas13 technology has been recently developed. This system, referred to as CRISPR–Cas-inspired RNA targeting system (CIRTS), fuses effector proteins including ADAR2 and YTHDF1/2 to a guide RNA hairpin binding protein and a protein designed for ssRNA binding (Rauch et al. 2019). Although in vivo studies on endogenous RNA targets have not been completed yet, like Cas13d, the CIRTS system is encodable by DNA and is less than 2.8 kb, allowing it to be delivered to preclinical models with AAV vectors.

Another potential RNA-targeting therapeutic approach based on eukaryotic RNA binding proteins includes those containing an RNA-binding domain known as the Pumilio homology domain (PumHD) or PUF domain. Each PUF domain contains a tripartite recognition motif (TRM) that recognizes a specific RNA base. Combining multiple TRMs in tandem can specify the RNA sequence motif to which a particular engineered PUF construct binds (Zhou et al. 2021). Like Cas13d and CIRTS, the potential therapeutic applications of PUF constructs seem endless, as such systems are easily programmable and unconstrained by specific sequences in the transcriptome.

Taken together, RNA-targeting constructs engineered from exogenous macromolecules may serve as successful candidates for therapeutic applications, as they are DNA encoded, can be robustly delivered to the CNS, and are programmable, enabling targeting of a wide range of disease-causing RNA sequences (Fig. 1).

CONSTRUCTION OF RNA-TARGETING THERAPEUTICS WITH ENDOGENOUS RNA REGULATORS

Mutations that disrupt existing splice sites and splicing regulatory sequences, or that activate cryptic sites leading to premature terminated or truncated proteins, have been implicated in many neurodegenerative disorders (Tollervey et al. 2011; Vuong et al. 2016). Thus, there is a critical need to engineer RNA-targeting therapies that can regulate pre-mRNA splicing with limited off-target effects on the human transcriptome. U-rich small nuclear ribonucleoprotein particles (U snRNPs), complexes composed of

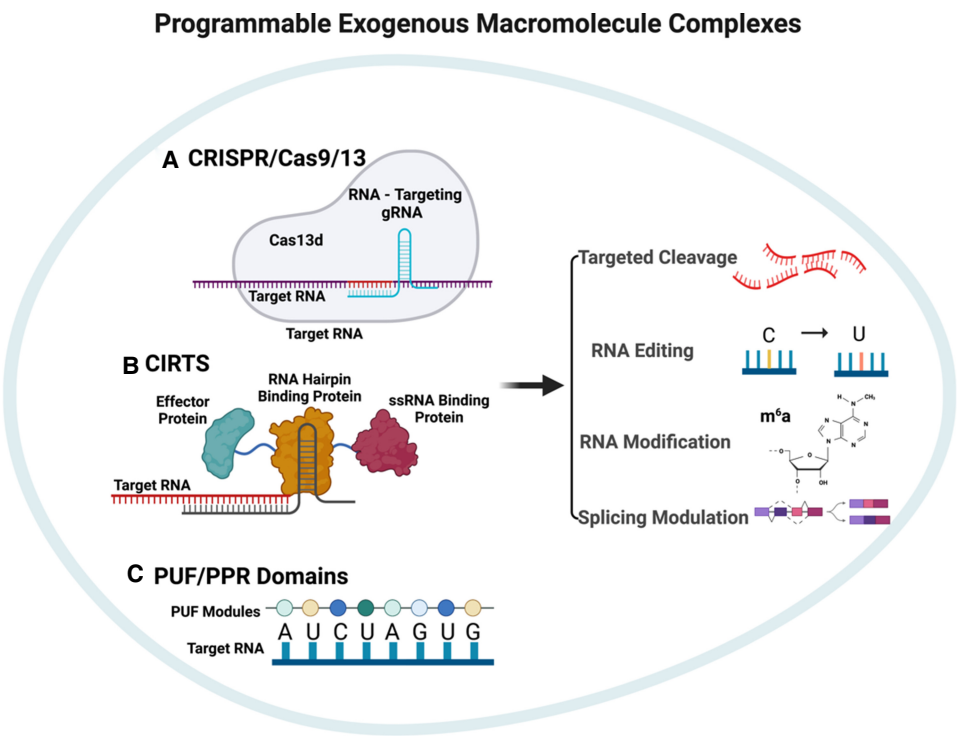

FIGURE 1. Overview of different mechanisms of action of RNA therapeutics developed from exogenous macromolecule machinery. They include RNA-targeting (*A*) CRISPR–Cas9/13 systems, (*B*) CIRTS, and those developed from (*C*) PUF domains. Each system can be engineered to degrade, edit, modify, or alternatively splice RNA molecules. This schematic was created with BioRender.

U snRNAs and their partner proteins that mediate the splicing and 3′-UTR processing of pre-mRNAs, offer one such opportunity (Will and Luhrmann 2011). In principle, U snRNAs can be engineered by replacing their native guide sequences with target guide sequences to execute exon skipping or inclusion of alternative exons, exon skipping of constitutive exons to induce or correct premature stop codons, exon inclusion of mutated constitutive exons whose splice-site mutations preclude recognition by the native U snRNA, or displacement of pre-mRNA binding factors (Fig. 2).

Modified U7 snRNP (U7smOPT), the simplest of such engineered systems that recruits only the Sm core proteins, shows great potential as an RNA-targeting therapeutic construct that can safely modify splicing variations for several reasons: its relatively small genetic payload (~500 bp from gene promoter to terminator) enables single AAV delivery; it does not elicit immunological reactions due to lack of protein components; it possesses no inherent catalytic activity; and, unlike shRNAs, it localizes to the nucleus and thus can target nuclear pre-mRNA (Gadgil and Raczynska 2021).

One notable disease application for U7smOPT is Duchenne muscular dystrophy (DMD), a severe neuromuscular disorder caused by mutations in the *dystrophin* gene (*DMD*) that disrupt its open-reading frame, yielding a nonfunctional truncated protein. As a result, antisense-mediated exon skipping is one of the most promising approaches for the treatment of DMD. Unlike ASOs, which also act through antisense RNA targeting, U7smOPT vectors can be genetically encoded in AAV vectors engineered to transduce muscle tissue more efficiently (El Andari et al. 2022). Bifunctional U7 snRNAs harboring silencer motifs induce complete skipping of DMD exon 51, and thus restore dystrophin protein expression to near wild-type levels, in both DMD patient cells and in vivo mouse models (Goyenvalle et al. 2009). Other exciting potential applications for U7smOPT therapies include SOD1-linked ALS and spinal muscular atrophy (Dal Mas et al. 2015). In both cases, unlike gene therapy approaches, U7smOPT vectors can directly target deficient endogenous genes for repair.

Since U7smOPT technology operates solely through binding and steric hindrance, its main limitation is an inability to induce exon inclusion in a generalizable manner. Engineered U1 snRNAs, which possess all the benefits of U7smOPT, overcome this barrier and have been exploited for robust exon-specific inclusion (Rogalska et al. 2016; Hatch et al. 2022). In addition, the high endogenous expression level of U1 snRNPs (estimated at approximately 1 million copies per cell) suggests that engineered U1 snRNA overexpression may not significantly perturb cellular processes.

U1 and U7smOPT represent a small fraction of potential engineered snRNAs, and undoubtedly future studies will

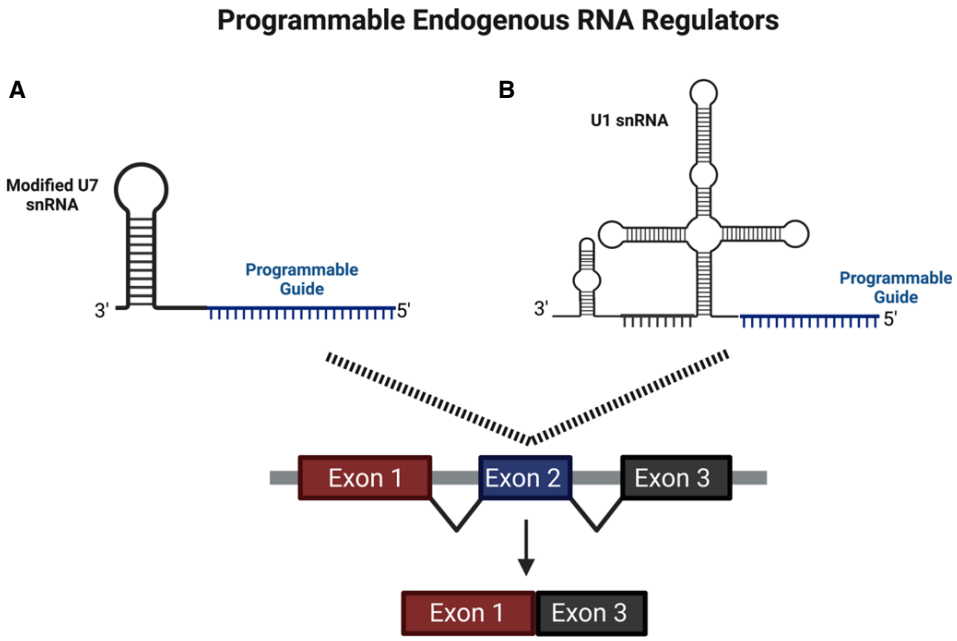

FIGURE 2. Schematic overview of U7 (*A*) and U1 (*B*) snRNA-based therapeutic designs. Placement proximal to and downstream from a 5′ splice site can lead to enhanced exon inclusion. This schematic was created with BioRender.

explore alternatives. Given that U1 and U7smOPT partner proteins are either upstream of other spliceosomal components or ubiquitously involved in splicing however, other instantiations such as engineered U2 or U5 snRNAs may lead to more undesirable off-target splicing effects. Nevertheless, such engineering opportunities are worth considering.

FUTURE RNA-TARGETING THERAPEUTIC APPLICATIONS

Thus far, programmable RNA-targeting therapeutic applications have included knockdown, A-to-I and C-to-U editing, m^6A modification, and exon inclusion and exclusion. Unexplored areas include other RNA modifications, robust RNA transcript stabilization, relocalization, and precise edits beyond A-to-I and C-to-U. Conceivably, both exogenous macromolecules and endogenous RNA regulators will play roles in realizing these applications. With a growing and evolving menu of RNA-targeting therapeutic modalities available for a given neurological disease target, balancing efficacy and safety will be critical in the selection process.

In addition to properly modifying its target RNA, each new RNA-targeting modality must be adequately delivered to and continuously expressed in the CNS to be effective. RNA degradation by endogenous RNases and limited uptake of biotherapeutics through the BBB are two major challenges when it comes to developing RNA-targeting therapeutics to treat neurological disorders. AAV delivery vehicles have circumvented these obstacles, with their ability to cross the BBB and longstanding expression (years). However, a large fraction of the population has pre-existing immunity to current FDA-approved AAVs, excluding these individuals from potential life-saving treatment (Weber 2021). AAV administration also cannot be halted if negative side effects are observed at any point during a patient's lifetime or readministered if therapeutic effects diminish overtime.

Alternative delivery strategies for RNA-targeting therapies have also been developed, including lipid-based and polymer-based nanoparticles. Yet neither is capable of crossing the BBB, limiting delivery options to invasive methods (Paunovska et al. 2022). Thus, new delivery methods must also be developed for RNA-targeting therapies to be safe, effective, and durable in potential patients. Despite all their challenges, RNA-targeting therapeutics are advancing at a rapid pace to meet the prospective treatment demands of a panel of neurological diseases.

COMPETING INTEREST STATEMENT

G.W.Y. is a visiting professor at the National University of Singapore. G.W.Y. is a cofounder, member of the board of directors, equity holder, and paid consultant for Locanabio and Eclipse BioInnovations, and a paid scientific advisor to Jumpcode Genomics. The terms of these arrangements have been reviewed and approved by the University of California San Diego in accordance with its conflict-of-interest policies. The authors declare no other competing interests.

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