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Recent Advances in RNA Interference Therapeutics for CNS Diseases

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Abstract Over the last decade, RNA interference technology has shown therapeutic promise in rodent models of dominantly inherited brain diseases, including those caused by polyglutamine repeat expansions in the coding region of the affected gene. For some of these diseases, proof-of concept studies in model organisms have transitioned to safety testing in larger animal models, such as the nonhuman primate. Here, we review recent progress on RNA interference-based therapies in various model systems. We also highlight outstanding questions or concerns that have emerged as a result of an improved (and ever advancing) understanding of the technologies employed.

Keywords RNAi · CNS · miRNA · shRNA · siRNA · polyQ

Introduction

The phenomenon of modulating gene expression by small RNAs is termed RNA interference (RNAi). Since the discovery of RNAi in plants and worms in the 1990s, scientists have made steady progress to understand the mechanisms of RNAi and use that knowledge to generate tools to study gene function. In addition, RNAi methodologies have been used to modulate the expression of disease genes with an intention of developing novel therapies. RNAi-based therapies are now in clinical trials for a variety of diseases, such as age-related macular degeneration, diabetic macular edema, solid tumors, and chronic myeloid leukemia (clinicaltrials.gov). For central nervous system (CNS) diseases in particular, early studies in model organisms and safety studies in nonhuman primates have shown that RNAi is an attractive therapy that warrants testing in patients. Initial pioneering studies using transgenic mice with inducible disease genes showed that it is possible to reverse disease phenotypes after onset [1–4], and set the stage for the therapeutic development of RNAi. In this review, we highlight recent discoveries in cell and animal models that have advanced the field of RNAi-based CNS therapeutics.

RNAi Mechanism and Function

RNAi is an innate gene regulatory mechanism that is essential to many cellular processes, such as proliferation, differentiation, cell death, and remodeling [5]. RNAi also plays an important role in host defense by protecting against viral infection and transposable elements [6]. In RNAi, the cell makes use of double-stranded (ds)RNA molecules to silence the expression of an messenger (m)RNA molecule by complementary base pairing. One form of naturally-occurring dsRNA molecules are microRNAs (miRNAs), which are transcribed in the nucleus as stem loop structured "primary miRNAs" (pri-miRNAs) from pol II or pol III promoters [7]. Pri-miRNAs are cleaved by the Drosha-DGCR8 microprocessor complex in the nucleus to form ~60–70 nucleotide hairpin-like structures called precursor-miRNAs (pre-miRNAs) [8, 9]. The pre-miRNAs are then exported to the cytoplasm by Exportin-5 and further processed by the Dicer (an RNase III

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endonuclease)-containing complex, which cleaves the loop structure of the pre-miRNA to release short, ~21-nucleotide, mature miRNA sequences [10, 11]. Dicer also processes exogenous long dsRNAs (such as viral RNAs) into smaller, 21-nucleotide small interfering RNAs (siRNAs) [12]. One strand of the miRNA or siRNA duplex, known as the antisense or guide strand, is selectively loaded into the Ago-containing RNA-induced silencing complex (RISC). This process is known as strand biasing; the non-loaded strand is referred to as the passenger strand [13]. This "activated" RISC carries out gene silencing, either by Ago2-mediated cleavage of the complementary target mRNA (in the case of siRNAs) or by target destabilization or translational repression (in the case of miRNAs) after imperfect base pairing to the 3' untranslated region (UTR) (Fig. 1) [14–16].

RNAi as a Tool for Directed Gene Silencing

RNAi has evolved rapidly as a tool for directed gene silencing. The RNAi machinery can be co-opted in many ways to achieve gene expression knockdown of a select target (Fig. 1). Synthetic siRNAs (~21 nucleotides) can be introduced into cells, which are loaded directly into RISC or, in the case of longer dsRNAs (25–27 nucleotides), first processed by Dicer and then loaded into the RISC to achieve gene silencing [17]. Gene-targeting siRNA duplexes can also be embedded in hairpin-based structures made to mimic the pri-miRNA (called artificial miRNAs) or the pre-miRNA (called short hairpin or shRNAs); when placed into expression vectors, they are transcribed in the nucleus and processed by the endogenous RNAi pathway to achieve gene silencing. shRNAs are typically expressed from strong Pol III promoters (such as U6 or H1), while artificial miRNAs can be expressed from pol II or pol III promoters. While shRNAs may have more potent silencing capability, they are often expressed at very high levels and can saturate the RNAi machinery, which disrupts endogenous miRNA processing and can induce toxicity [18, 19]. Artificial miRNAs, however, are generally safer and less toxic, and they do not appear to disrupt endogenous miRNA processing [18–23]. Although artificial miRNAs are less toxic, their safety profile is also dictated by the design of the RNAi sequence.

Designing RNAi Sequences

Designing a siRNA sequence to reduce target gene expression with efficacy and specificity is one of the key factors to achieve successful RNAi. Important steps in designing an efficient siRNA have been described by several groups [24–26]. Two important criteria include designing sequences for proper strand biasing and minimizing off-targeting.

To favor antisense strand incorporation into RISC, the antisense strand of the siRNA should have strong G–C base pairing at the 3' end and weak base pairing (A–U or G–U) at the 5' end, as RISC loads the strand with the lowest 5' thermodynamic stability [13]. There are many online tools and guidelines that help to design siRNA sequences to a gene target of interest [27–30]. Newer online tools also incorporate the very important aspect of siRNA off-targeting [31–33].

Off-targeting is a phenomenon by which a siRNA binds to and represses unintended targets owing to complementarity with the siRNA 'seed' sequence. In 2006, Fedorov et al. [34] reported toxic effects due to off-target effects of siRNAs. As miRNAs primarily target the 3'UTRs of genes, it was found that seed complementarity to hexamers in the 3'UTR's of genes is proportional to the number of off-target effects [35, 36]. Limiting off-targeting is particularly critical when designing siRNAs for therapeutic purposes. Our laboratory has designed a web-based program that designs highly-specific siRNA sequences to a target of interest by taking into consideration the off-targets of an siRNA seed sequence, and gives each siRNA a score depending on the number of potential off-targets [31]. Researchers can thus pick siRNA sequences with low potential off-targets and screen them *in vitro* to identify candidate sequences for delivery *in vivo*. Alternatively, the siRNA sequence can be incorporated into a miRNA or shRNA backbone for delivery *in vitro* and *in vivo*. The process of designing and screening of hairpin-based RNAi sequences (shRNAs and artificial miRNAs) has been described in detail previously [37].

Delivering RNAi Sequences to the CNS

RNAi delivery to the CNS faces unique challenges. For effective RNAi delivery via the blood, the presence of the blood–brain barrier (BBB) is an obstacle that must be overcome, while, for direct injection into the brain, steps must be taken to avoid toxic or inflammatory reactions. An ideal delivery system for RNAi to the CNS should be minimally immunogenic, nontoxic, target specific cells of the CNS, knockdown the specific target mRNA efficiently, and be easy to manufacture [38]. Two major types of delivery systems have emerged over the years, differing in production, safety, and efficacy. These are broadly classified as viral and non-viral delivery systems.

Non-viral Delivery

siRNAs can be delivered directly *in vitro* or *in vivo* to achieve knockdown of a gene of interest. However, exogenously delivered siRNAs are natural substrates for nucleases, which

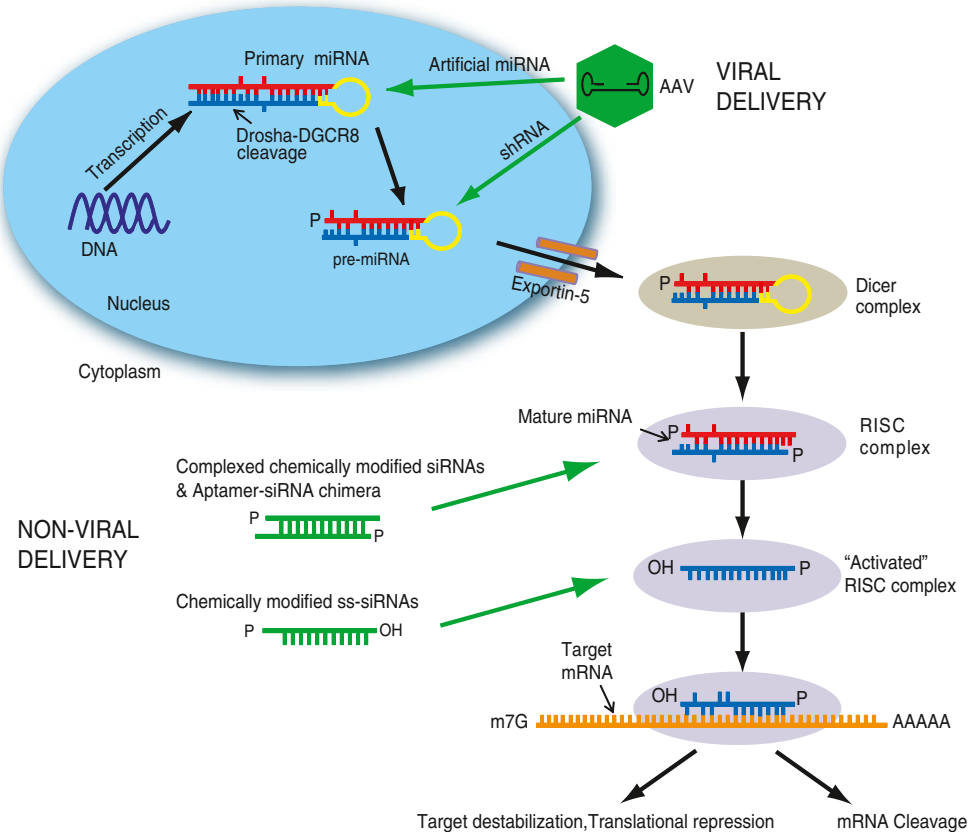

Fig. 1 Co-opting the microRNA (miRNA) pathway for delivery of RNA interference triggers to the central nervous system (CNS). Primary miRNAs are transcribed in the nucleus and are processed by the Drosha-DGCR complex to give rise to precursor (Pre)-miRNAs. Pre-miRNAs are exported out of the nucleus by Exportin-5 and undergo further processing by Dicer in the cytoplasm to give rise to mature miRNAs. The mature miRNA is then loaded into RNA-induced silencing complex (RISC) to carry out silencing by binding to complementary messenger RNA (mRNA) sequences. Artificial miRNAs or short hairpin RNAs (shRNAs) can be delivered via an adeno-associated virus (AAV) and enter the miRNA pathway at different stages of the miRNA pathway. Small interfering RNAs (siRNAs) that are complexed or delivered directly into cells, enter the pathway at the Dicer-to-RISC stage or can be incorporated directly into RISC to carry out gene silencing

makes them inherently unstable. This can be overcome by chemically modifying siRNAs to resist nucleases. In addition, chemical modifications can increase cell uptake, strand biasing, and efficiency of gene knockdown, while reducing immunogenicity and off-target effects [39]. Different types of chemical modifications have been introduced into the backbone of the siRNA, the most popular of which are 2'-Fluoro, 2'-O-methyl and locked nucleic acids [39]. A recent study demonstrated allele-specific silencing of mutant Htt in Huntington disease (HD) mice using chemically-modified single-stranded siRNA (ss-siRNA) molecules [40, 41]. ss-siRNAs are advantageous as they avoid off-target effects associated with passenger strand loading, although some other issues remain [42]. In addition to chemical modifications, siRNAs can be complexed into liposomes or nanoparticles, or can be incorporated into aptamer structures for delivery *in vivo*. Owing to their ability to cross the BBB and their small size, nanoparticles delivered intravenously or via carotid artery can gain entry to the CNS [43, 44]. Recently, aptamers functionalized with nanoparticles targeted glioblastomas for potential therapy [45]. Nanoparticles can target specific cell types in tissues, but have low transfection efficiency [46].

While non-viral methods have been used *in vivo*, they are generally less efficacious and are, by nature, transient, requiring repeated delivery. This transient nature can be advantageous for therapies that do not require long-term treatment, such as in antitumor and antiviral therapies. In addition, this provides an important safety measure; in the case of adverse side effects, treatment can simply be terminated.

While methods to improve the efficacy of non-viral molecules are currently under development, viral vectors have been used successfully from mouse models to human studies in gene replacement strategies.

Viral Delivery

There are a number of viral vectors that can be used for gene delivery to the CNS, as discussed in a prior review [38]. The two main viral vector systems that are used to

transduce the CNS are lentiviruses (LV) and adeno-associated viruses (AAV). Both viruses are minimally immunogenic and can transduce a number of CNS cell types. Recombinant lentiviruses are pseudotyped with various glycoproteins that can impart different tropisms after directed delivery into brain [47, 48], and they have been used successfully in gain-of-function [49] and loss of function studies [50–53]. One difference between lentivirus and AAV or adenovirus-based systems is the level of expression. This is due, in part, because LV-mediated transduction often results in low copy numbers of transgene/cell. Also, the placement of the expression cassette in the LV genome can affect expression levels [54]. Most LV vectors integrate unless the integrase activity has been inactivated. As integrase-deficient vectors often have low titers compared with their integrase competent counterparts, their production for use for therapeutic applications is impractical. Integration competency for CNS applications may be less of an issue than in the setting of stem cell transduction (most cells in the CNS are not dividing), where integration and activation of an oncogenic gene provides a growth advantage for the transformed cell [55, 56].

AAV belongs to the genus *Dependovirus* and in its wild type state requires a helper virus, such as adenovirus, to replicate. A number of factors make AAV suitable for gene delivery *in vivo*. AAV can be manufactured easily and it is scalable for human use [57, 58], particularly for the relatively low volumes needed for brain-expressed targets. Additionally, AAV rarely integrates into the genome. In general, AAVs are non-pathogenic and have low immunogenic properties, which make them ideal for gene delivery *in vivo* [38]. AAVs confer robust expression, efficiently transduce neurons and other cell types, and, in the absence of an immune response to what is being expressed, can afford long-term expression [59–61].

Tissue tropism of AAV is dictated by the capsid serotype. AAV capsids with different cell/tissue tropisms have been identified and, depending on the capsid serotype, AAV can transduce neurons, astrocytes, glia, and ependymal cells with a high transduction efficiency [62–66]. The AAV capsid can be modified to alter its tropism in several ways, including directed evolution, capsid shuffling, and incorporation of targeting peptides. Directed evolution involves mutagenesis of the capsid, which may alter tropism [67, 68]. Capsid shuffling involves the assembly of variant capsid sequences to give rise to recombinant capsids with tropisms to different cell types [69–71]. AAV tropism can also be altered by the incorporation of targeting ligand into the capsid, to mediate ligand specific receptor binding [72–74].

Gene transfer after direct delivery of AAV vectors by intraparenchymal, intraventricular, or intrathecal injections to target cells of the brain is used for RNAi delivery. Direct delivery by intraparenchymal injections has proven effective for targeting neurons in various neurodegenerative diseases and it limits transduction to those tissues most relevant to disease. Widespread exposure of a transgene product occurs after intraventricular or intrathecal delivery of AAVs, when the transgene product is a secreted molecule [75]. Recently, intrathecal injection of AAV9 or AAV2.5 showed robust transduction of the brain and spinal cord in nonhuman primates [76].

Peripheral delivery of AAVs for brain targeting has also been used [72–74, 77–79]. Concerns about this approach for clinical application are the high doses needed, the transduction of peripheral organs (which may not be desirable), and the induction of a robust anti-AAV and likely anti-transgene response. Nonetheless, using vectors that can cross the BBB may be beneficial for some applications. Intravenous delivery of AAV9, a recently identified serotype, can cross the BBB after, and transduce neurons in neonatal mice, and astrocytes and scattered neurons in adult mice and rhesus macaques [77, 78]. Also, variants of AAV9 transduce motor neurons and astrocytes after systemic delivery by intravenous injection to adult mice [78, 79].

Although AAVs have a small packaging capacity (~4.7 kb), they are ideally suited to deliver the small RNAi expression cassette. RNAi sequences delivered to the brain after AAV injection have shown therapeutic promise in mouse models of dominantly inherited polyglutamine (polyQ) diseases and other neurological disorders, as discussed in the following.

Emerging Therapies

RNAi therapy is well suited for diseases where the disease-causing gene acquires a toxic ‘gain of function’ effect. The identification of such disease-causing genes (modifiers or mutant genes) has allowed researchers to design RNAi molecules to target the disease-causing allele and demonstrate therapeutic potential (Table 1).

Huntington’s Disease

Huntington’s Disease (HD) is caused by a polyQ expansion in the coding region of the gene Huntingtin (*HTT*). HD is a gain-of-function autosomal dominant disease with neuronal dysfunction occurring prior to cell death in medium spiny neurons within the striatum, as well as other brain regions. Patients exhibit involuntary hyperkinetic movements, coordination difficulties, and cognitive disturbances [50, 80]. Both nonallele-specific (targeting the mutant and wild type alleles) and allele-specific (targeting only the mutant allele)

Table 1 Progress in RNA inteference (RNAi) therapeutics demonstrating therapeutic potential in cell and animal models

Disease	Target gene	Approach used	Delivery vehicle	Study demonstrating therapeutic potential	References
Huntington's Disease (HD)	*HTT*	Allele-specific (AS) and nonallele-specific (NAS)	AAV1, AAV2, chemically modified ss-siRNAs	Silencing of endogenous HTT by shRNAs and artificial miRNAs in rhesus is tolerated up to 6 months without toxicity. Potent allele-specific silencing of mutant HTT is demonstrated in HD mice using chemically modified ss-siRNAs targeting expanded CAG repeats.	[40, 41, 80–82, 97]
SCA1	*ATXN1*	NAS	AAV1	Silencing of ATXN1 using shRNAs and, more recently, artificial miRNAs in SCA1 transgenic and knock-in mouse models show improvement of motor coordination without toxicity.	[94]
SCA2	*ATXN2*		AAV1	Partial suppression of $InsP_3R$ in the cerebellum improved motor coordination, reduced Purkinje cell degeneration in SCA2 transgenic mice.	[97]
SCA3	*ATXN3*	NAS, AS	Lentivirus	Nonallele-specific silencing and allele-specific silencing of mutant ATXN3 was well tolerated and reduced neuropathology in a rat model of SCA3.	[104, 105]
SCA6	*CACNA1A*	AS		Splice-isoform specific RNAi using artificial miRNAs demonstrated allele-specific silencing of mutant CACNA1A *in vitro*	[113]
SCA7	*ATXN7*	AS		Allele-specific silencing of mutant ATXN7 is demonstrated *in vitro* using shRNAs.	[117]
Parkinson's disease	*SNCA*		Lentivirus, AAV2	Allele-specific silencing of α-syn using shRNAs was observed in the rat brain and ameliorated behavioral deficits, but was also toxic in dopamine neurons.	[22, 132]
	LRRK2	AS		Allele-specific silencing of mutant α-syn and LRRK2 was achieved *in vitro* using artificial mirtron mimics.	[134]
Alzheimer's disease	*BACE1*		Lentivirus	shRNAs silence BACE1 to reduce amyloid production and behavioral deficits in a transgenic mouse model.	[122]
	Tau	AS		Allele-specific silencing of mutant Tau demonstrated using shRNAs *in vitro*.	[127]
	APP	AS	AAV5	Allele-specfic silencing of APP in a transgenic AD mouse model mitigated phenotypic progression.	[124]
	PS1	AS		Allele-specific siRNAs silence mutant PS1 *in vitro* and reduced amyloid β42 production.	[124, 125]
	CDK5		Lentivirus	shRNAs targeting CDK5 reduced neurofibrillary tangles in a transgenic mouse model.	[126]
	PLK1		Lentivirus	RNAi silencing of Plk1 *in vitro* reduced amyloid β-induced cell death.	[129]
	MSUT2			Silencing of MSUT2 using siRNAs decreased tau aggregation *in vitro*.	[128]
Dystonia	*TOR1A*	AS	Lentivirus	Allele specific silencing of TorsinA(ΔE) by shRNAs worked well *in vitro*, but when moved into a mouse model, the shRNAs proved to be toxic.	[21, 53]
SBMA	*CELF2*		AAV9	Overexpression of naturally occurring miR-196a indirectly enhances decay of androgen receptor through silencing of CELF2 *in vivo*.	[141]
ALS	*SOD1*	AS	Lentivirus	Silencing SOD1 slows progression and extends survival in rodent models of ALS	[51, 52, 147, 148]

ss-siRNA = single-stranded small interfering RNA; shRNA = short hairpin RNA; miRNA = microRNA; SCA1 (2, 3, 6, 7) = spinocerebellar ataxia type 1 (2, 3, 6, 7); $InsP_3$ = inositol 1,4,5 phosphate receptor; APP = amyloid precursor protein; siRNA = small interfering RNA; PS1 = presenilin-1; CDK5 = cyclin-dependent kinase 5; SBMA = spinobulbar muscular atrophy; ALS = amyotrophic lateral sclerosis

approaches for HD therapy are under development. It has been shown recently that nonallele-specific silencing, using AAV-mediated delivery of RNAi, provides benefits in a mouse model of HD and 2 studies assessing the effect of knockdown of endogenous HTT in rhesus found no adverse effects up to 6 months post-injection [81, 82]. However, HTT is necessary for embryonic development and is involved in cellular pathways in differentiated neurons [83–88]. Thus, long-term therapies may require targeting only the mutant allele. This has led to the development of strategies for targeting only mutant

HTT [83–88]. An example is the work done by Hu et al. [89] targeting the CAG repeat sequence expansion. Their target strategy capitalized on the concept that siRNAs bind to their targets with full complementarity, while miRNAs exhibit one or more mismatches to their targets. Mimicking the miRNA mechanism, one or two mismatched bases were introduced into therapeutic RNAi sequences. These miRNA-like sequences showed greater inhibition of the mutant HTT in patient-derived cells whereas the siRNAs showed little selectivity between wild type and mutant alleles. This study also looked at the CAG containing mRNAs (*TBP* and *FOXP2*) and found no off-target silencing [89]. Other allele-specific techniques have targeted single nucleotide polymorphisms (SNPs) occurring in some, but not all, mutant *HTT* alleles [90–92].

Spinocerebellar Ataxia Type 1

Spinocerebellar ataxia type 1 (SCA1) is a late onset, autosomal dominant neurodegenerative disease caused by a polyQ expansion in Ataxin1 (*ATXN1*), which encodes the ATXN1 protein. The average age of onset is within the fourth decade of life, although juvenile cases have been documented [93]. Symptoms include loss of coordination, dysarthria, and cognitive impairment. Purkinje cell (PC) death and brain stem neuronal death is characteristic of SCA1 [93]. In 2004, RNAi was established as a potential therapy for SCA1 after successful rescue of the disease phenotype in SCA1 transgenic mice expressing the human ATXN1 protein containing 82 pathogenic polyQ repeats. AAVs expressing shRNAs against human 82Q ATXN1 were injected to SCA1 mice cerebellar cortices, improving molecular and behavioral phenotypes [94]. Further testing of RNAi triggers using artificial miRNAs is being pursued.

Spinocerebellar Ataxia Type 2

Spinocerebellar ataxia type 2 (SCA2) is a polyQ disease caused by an expansion of >31 CAG repeats in the coding region of Ataxin2 (*ATXN2*), while normal individuals have ~20 CAG repeats (CAG_8-CAA-CAG_4-CAA-CAG_8) [95]. SCA2 is characterized by initial hyper-reflexia followed by hyporeflexia with disease progression, ophthalmoplegia, dysphagia, ataxia, and, occasionally, symptoms associated with Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple system atrophy [95]. RNAi against the mutant allele may be beneficial as in other polyQ diseases (HD, SCA1, SCA3, SCA6). Nonallele-specific silencing will likely be tolerable as SCA2 knockout mice are viable and fertile [96]. In addition to targeting the mutant allele, modifier genes can also be targeted for treatment. Studies in the SCA2-58Q transgenic mouse showed an increase in inositol 1,4,5 phosphate ($InsP_3$)-mediated calcium release in the PCs resulting in dysregulated PC function [97]. When presymptomatic SCA2 mice were treated with a calcium stabilizer drug (Dantrolene), or by partial suppression of the $InsP_3$ receptor, PC degeneration was significantly reduced and significant motor improvement was seen [97, 98]. These studies are promising and suggest that suppressing $InsP_3$-mediated calcium release by targeting the $InsP_3$ receptor in PCs by RNAi may be therapeutic, particularly if regulated RNAi systems are developed. Dantrolene was also found to be neuroprotective in a HD mouse model (YAC128) and in an SCA3 mouse model [99, 100]. Dysregulated calcium signaling has emerged as a theme in many ataxias and other neurodegenerative diseases, and could provide a novel therapeutic target for RNAi therapies [101, 102].

Spinocerebellar Ataxia Type 3

Spinocerebellar ataxia type 2 (SCA3), also known as Machado–Joseph disease, is a dominant polyQ disease caused by a CAG expansion in the coding region of Ataxin3 (*ATXN3*). SCA3 is characterized by impairment of gait, vision, and speech [103]. The therapeutic utility of RNAi for SCA3 was first tested in the rat by targeting a SNP in the mutant allele. Reducing levels of the mutant allele rescued diseased phenotypes [104]. Later, work from the same group showed that shRNAs designed to be nonallele-specific, silenced both wild type and mutant ataxin-3, and rescued SCA3 phenotypes. Thus, nonallele-specific silencing for SCA3 may be a safe and effective treatment [105]. Alternate methods of gene silencing used peptide nucleic acid conjugates and achieved mutant ATXN3 silencing *in vitro* [106].

Spinocerebellar Ataxia Type 6

Spinocerebellar ataxia type 6 (SCA6) is caused by a polyQ expansion of the *CACNA1A* gene, which encodes the α1A ($Ca_v2.1$) subunit [107]. SCA6 requires a relatively small expansion of glutamine (19–33 CAGs) to manifest disease. SCA6 is characterized by progressive ataxia, dysarthria, and nystagmus. Onset generally occurs in the fifth decade of life and lifespan is not shortened [108, 109]. Neurodegeneration occurs selectively in the PCs of the cerebellum with no neuropathy in other neurons, making it a pure cerebellar ataxia [110]. Unlike other polyQ diseases, only certain isoforms of $Ca_v2.1$ contain the expanded glutamine tract [111, 112]. A recent experiment took advantage of this isoform-specific mutation. Tsou et al. [113] used a novel splice isoform-specific RNAi strategy to target the polyQ calcium channel splice-variant. The splice isoform-specific miRNA mimics achieved allele selective silencing *in vitro* [113]. These results suggest a potential therapy for SCA6 that could be tested *in vivo*.

Spinocerebellar Ataxia Type 7

Spinocerebellar ataxia type 7 (SCA7) is unique among the SCAs, as patients experience vision loss in addition to ataxia. A polyQ expansion of >32 CAG repeats in the Ataxin7 (*ATXN7*) gene causes the slowly progressing ataxia, while > 52 CAG repeats in *ATXN7* results in retinal degeneration in addition to cerebellar ataxia. ATXN7 is present in a transcriptional coactivator complex, STAGA or TFTC, which modulates Gcn5 histone acetyl transferase activity and deubiquitinase activity [114]. Mutant polyQ ATXN7 alters STAGA recruitment to target genes, altering its activity [115, 116]. The exact function of ATXN7 in the STAGA complex is unknown. As there is no knockout model for SCA7, the consequences of complete or partial ATXN7 knockdown by RNAi remain to be tested. Recently, shRNA sequences were designed to target a SNP found linked to mutant *ATXN7* in an affected South African population, resulting in allele-specific silencing *in vitro* [117]. This SNP (G to A) is found in the 3' region of the mutant gene in 50% of South African SCA7 patients. However, neither allele-specific nor nonallele-specific approaches for SCA7 have been investigated *in vivo*.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative dementia. AD is characterized by the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles that cause brain atrophy [118]. Amyloid plaques are created by the misfolding of amyloid-β (Aβ) peptides, while neurofibrillary tangles are comprised of hyperphosporylated-tau. AD pathogenesis is not completely understood; Aβ aggregation or hyperphosphorylation of tau may be the primary cause of AD pathogenesis [119, 120]. The majority of work using RNAi as a therapy for AD has focused on proteins involved in amyloid plaque formation. Aβ is formed by proteolytic processing of amyloid precursor protein (APP) by β-secretase (BACE1) and γ-secretase, a large enzyme complex containing presenilin-1 (PS1) [121]. APP, BACE1, and PS1 have all been targeted in studies to prevent Aβ formation. In 2005, siRNAs designed to suppress BACE1 reduced APP cleavage and lowered Aβ formation, improving neuropathy in APP transgenic mice [122]. Allele-specific shRNAs designed to silence APP directly also improved phenotypes and decreased levels of soluble Aβ in transgenic mice [123]. Other studies targeting the amyloid cascade have focused on PS1. Both nonallele-specific and allele-specific silencing of PS1 decreased toxic Aβ formation in cell culture providing a possible *in vivo* strategy [124, 125]. Piedrahita et al. [126] targeted cyclin-dependent kinase 5, an enzyme required for the phosphorylation of tau using RNAi. Also, studies have targeted tau specifically [127]. cyclin-dependent kinase 5 suppression reduced phosphorylated tau levels and blocked neurofibrillary tangle formation in transgenic AD mice. Additional novel studies have targeted *PLK1* and *MSUT2*, two genes whose suppression by RNAi reduces toxic aggregation *in vitro* [128, 129]. Thus, both the Aβ and the tau hyperphosphorylation pathways provide many potential targets for RNAi therapy for AD.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by resting tremor, bradykinesia, and rigidity. Predominant cell death is seen in dopaminergic neurons of the substantia nigra pars compacta. Two of the known genes associated with PD are the aggregate-forming alpha-synuclein (*α-syn*) seen in Lewy Bodies, and leucine-rich repeat kinase-2 (*LRRK2*) [130, 131]. Most RNAi studies in PD have focused on targeting SNPs in α-syn or *LRRK2*. In 2006, Sapru et al. [22, 132] designed shRNAs targeting a SNP in mutant α-syn. Allele-specific silencing was achieved *in vitro* and in the rat brain. Sibley et al. [133] have also achieved efficient allele-specific silencing of *LRRK2* using shRNAs or using an RNAi trigger mimicking miR-1224 *in vitro* [134].

Dystonia

Primary early-onset dystonia (DYT1) is the most common form of inherited dystonia. The cause of DYT1 in most patients is the result of a glutamic acid deletion in the torsinA protein [135]. TorsinA is an ATPase associated with diverse cellular activities [136], whose mutant form (TorsinA(ΔE)) is thought to have a dominant negative effect [137, 138]. Allele-specific TorsinA(ΔE) suppression was first achieved by shRNA delivery *in vitro* [53]. However, when AAV expressing the shRNAs were delivered into the striatum of transgenic DYT1 mice, the shRNAs induced lethal neurotoxicity [21]. This study is intriguing in that it raises the question as to whether the DYT1 brain is intolerant to the exogenous expression of RNAi triggers or if the particular sequences tested were toxic. Mutant TorsinA forms perinuclear aggregates that may contribute to this lack of tolerability. Importantly, this work was done prior to general utility of artificial miRNAs for directed gene silencing in brain. However, RNAi sequences to target the common TorsinA(ΔE) mutant allele are confined to those surrounding the mutation, and may, by nature, induce deleterious off-target effects, regardless of the platform used to elaborate the final siRNA product.

Spinobulbar Muscular atrophy

Spinobulbar muscular atrophy (SBMA) is caused by a polyQ expansion of the X-linked androgen receptor (*AR*) gene [139].

SBMA is characterized by proximal muscular weakness and atrophy, and facial muscle fasciculations, difficulty in speech, and swallowing [140]. Recently, Miyazaki et al. [141] demonstrated a potential SBMA therapy using a novel approach exploiting the miRNA pathway. They identified miR-196a expression as significantly upregulated in the spinal cord of transgenic mice at advanced disease stages. miR-196a does not target the *AR* gene directly, but rather silences CUGBP, Elav-like family member 2 (CELF2). CELF2 is responsible for the stability of *AR* transcripts by binding the CUG repeat sequence upstream of the polyQ expansion in *AR* mRNA. AAV delivery to hindlimb skeletal muscle for miR-196a overexpression decreased CELF2 expression in SBMA transgenic mice. This, in turn, enhanced decay of *AR* mRNA and ameliorated disease phenotypes. With a clinical setting in mind, experiments were done in human patient fibroblasts. miR-196a overexpression in an SBMA mouse model significantly downregulated both *CELF2* and *AR* mRNA levels, improving SBMA phenotypes, suggesting miR-196a-mediated treatment as a potential clinical therapy for SBMA patients [141].

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a dominant neurodegenerative disease characterized by the progressive loss of motor neurons. Although many genes contribute to ALS pathogenesis, as reviewed by Strong [142], RNAi for ALS therapy has focused on gain-of-function mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene [143, 144]. Because wild type SOD1 is important for normal cell function allele-specific silencing is important [145, 146]. Allele-specific shRNAs targeting mutant SOD1 improved disease phenotypes in transgenic mice [51, 52, 147]. More recently, asymmetric siRNAs with mismatched base pairs achieved allele-specific silencing *in vitro*, but these have not been tested in animal models [148]. While SOD1 therapies show promise for ALS, other target genes have been documented. SNPs within *OPTN* [149], *TARDBP* [150–156], *FUS* [157–160], and *ANG/VEGF* [161–165] provide additional opportunities for potential allele-specific ALS therapies.

Taking RNAi to the Clinic

As RNAi therapies for CNS diseases approach the clinic, there are obvious considerations to moving each potential drug forward.

1) Allele-specific versus non-allele-specific silencing. While targeting the mutant allele is always desirable, it may not be necessary for some diseases, as RNAi reduces, but does not fully remove, the targeted gene product. Moreover, wild type levels of the gene being targeted may not be required for maintenance of cell viability. For example, SCA1, SCA2, and SCA3 knockout mice are viable and fertile, indicating that knockdown of the wild type allele function may be tolerable [96, 166, 167]. Nonallele-specific silencing of HTT in HD mice resulted in a significant rescue of the HD phenotype, and 2 studies have shown that reducing levels of wild type HTT in the adult rhesus macaque striatum is safe and well tolerated for at least 6 months [80–82]. However, as the HD null mice are embryonic lethal, and the levels of HTT required for cell viability of adult neurons is unknown, researchers are also investigating allele-specific silencing options [83]. For every disease being tested by nonallele-specific silencing, it is important to consider whether partial loss of function of the wild type allele is sufficient to retain function long-term.
2) Dose, delivery, and distribution of RNAi in the human brain. Ultimately, the goal of developing RNAi therapies and testing them in animal models is for treatment in humans. Thus, it is important to understand what kind of dose may be appropriate and how long the RNAi efficacy is retained in the human brain. A number of studies have focused on determining an appropriate dose of RNAi that is efficacious and safe in the primate brain using viral and nonviral methods [81, 82, 168, 169]. Nonviral delivery systems used cannulas implanted in the brain or convection enhanced delivery systems that use flow pressure to increase the volume of distribution. For long-term effects, repeated dosing would be required. For AAVs, directed delivery can be done, or, if the targeted structure is larger, convection enhanced delivery is effective [168]. Various methods are used to inform investigators about the distribution of the drug after delivery. To detect siRNAs after delivery, Stiles et al. [168] used radiolalebeled siRNAs, which allowed comparison of the volume of brain for which there was target suppression, as a function of dose and spread. For viral vectors, a common strategy to determine the distribution of transduced cells takes advantage of reporter genes, such as eGFP [80, 82]. In the future, targeting the brain after systemic delivery may be possible. Alternatively, researchers may be able to take advantage of the impaired BBB in some neurological diseases, allowing for diffusion of viruses, drugs, and other small molecules into the brain that are delivered systemically [67, 170].
3) Duration of silencing. For non-viral methods of delivery, targeting the brain with RNAi molecules will require repeated dosing as their effect will eventually wane. Thus, indwelling ports for brain access will be required. However, expression of transgenes after AAV delivery has been observed to last many years (>8

years) in the primate brain [60]. Whether these same platforms can provide for lasting expression of RNAi triggers sufficient to last the life of the patient, or at the least, many years, is not yet known. The longevity of expression from viral vectors may vary depending on the vector type, the promoter, the cell types transduced, and the pathology of the particular disease. While longevity of the transgene expression by viral vectors is important, it is also important to consider regulating its expression in the case of adverse effects from off-targeting. In this regard, regulating expression of the transgene by exogenous factors such as the erythromycin based on–off system may be an important parameter to consider. This system has the benefit of using the clinically approved drug erythromycin and uses erythromycin-responsive *Escherichia coli* operator or repressor elements to either turn on or off a transgene [171].

In summary, cumulative and ongoing studies with RNAi delivery for CNS therapies are encouraging. RNAi activity in neurons continues to show efficacy in animal models for treatment of "gain-of-function" neurodegenerative diseases, and with careful choice of siRNA to avoid off-target effects, holds much promise for translation to the clinic.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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