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Invited Review

INVITED REVIEW

Gene suppression strategies for dominantly inherited neurodegenerative diseases: lessons from Huntington's disease and spinocerebellar ataxia

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Abstract

RNA-targeting approaches are emerging as viable therapeutics that offer an alternative method to modulate traditionally 'undrugable' targets. In the case of dominantly inherited neurodegenerative diseases, gene suppression strategies can target the underlying cause of these intractable disorders. Polyglutamine diseases are caused by CAG expansions in discrete genes, making them ideal candidates for gene suppression therapies. Here, we discuss the current state of gene suppression approaches for Huntington's disease and the spinocerebellar ataxias, including the use of antisense oligonucleotides, short-interfering RNAs, as well as viral vector-mediated delivery of short hairpin RNAs and artificial microRNAs. We focus on lessons learned from preclinical studies investigating gene suppression therapies for these disorders, particularly in rodent models of disease and in non-human primates. In animal models, recent advances in gene suppression technologies have not only prevented disease progression in a number of cases, but have also reversed existing disease, providing evidence that reducing the expression of disease-causing genes may be of benefit in symptomatic patients. Both allele- and non-allele-specific approaches to gene suppression have made great strides over the past decade, showing efficacy and safety in both small and large animal models. Advances in delivery techniques allow for broad and durable suppression of target genes, have been validated in non-human primates and in some cases, are currently being evaluated in human patients. Finally, we discuss the challenges of developing and delivering gene suppression constructs into the CNS and recent advances of potential therapeutics into the clinic.

Introduction

Gene suppression approaches for dominantly inherited neurodegenerative diseases have made great strides over the past decade, especially in the cases of the polyglutamine-repeat disorders. In particular, gene suppression approaches for Huntington's disease (HD) and the spinocerebellar ataxias (SCAs) have shown great promise and are quickly moving from testing in rodent models of disease into large animal models and, in the case of HD, into the first clinical trial in human patients. Polyglutamine-repeat disorders stem from abnormally long CAG expansions, which confer a toxic gain of function on the expressed protein, resulting in symptoms that are different for each disease. In this review, we will address the progress made in the fields of HD and the SCAs, with a specific focus on the use of RNA interference (RNAi) and antisense oligonucleotide (ASO) approaches. Additionally, we will discuss the hurdles to successful gene

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suppression approaches and clinical trial development for these disorders.

Therapeutic Approaches for Co-Opting Endogenous mRNA Degradation Pathways

RNA interference

RNA interference is an evolutionarily conserved process of post-transcriptional gene suppression that utilizes native non-coding double-stranded RNA sequences to reduce mRNA expression. MicroRNAs (miRNAs) are transcribed within the nucleus by pol II and pol III promoters to form stem-loop structures known as primary-miRNAs (pri-miRNAs). Pri-miRNAs are cleaved by the Drosha-DGCR8 microprocessor complex to form a precursor-miRNA (pre-miRNA) hairpin-like structure. Pre-miRNAs are then exported to the cytoplasm by Exportin-5 whereupon they are further processed by Dicer to create a mature miRNA duplex. The antisense, or 'guide' strand is loaded into the RNA-induced silencing complex (RISC), while the sense or 'passenger' strand is degraded. Upon entering RISC, the guide strand silences its target based on the degree of complementarity of the seed sequence to the target. Perfect complementarity, in the case of short-interfering RNAs (siRNAs), leads to Ago-2-mediated cleavage, whereas imperfect base pairing, in the case of miRNAs, leads to destabilization or translational repression (1).

Over the years, the endogenous cellular RNAi pathway has been co-opted to suppress specific genes of interest. RNAi effectors are generated in the laboratory and introduced into the cell as siRNAs, short hairpin RNAs (shRNAs) or artificial miRNAs (artificial sequences cloned into the context of an endogenous miRNA sequence, such as miR-30). siRNAs are 21 nucleotide-long duplexes that are processed by Dicer and silence target mRNAs through Ago-2-mediated cleavage. These can be delivered in a variety of flavors including chemically modified single-stranded siRNAs (2,3), liposome-formulated siRNAs (4) or nanoparticles (5). Early generation RNAi triggers were in the form of shRNAs embedded in a pri-miRNA scaffold, or miRNA backbone, and delivered by viral vectors (6–8). These shRNAs were extremely potent and achieved high levels of knockdown *in vivo*. However, the shRNAs were expressed at a high level and have been shown in certain cases to saturate endogenous RNAi machinery (9,10). Newer techniques use siRNAs embedded into artificial miRNA backbones to achieve stable and efficient mRNA suppression without the buildup of antisense precursor species in the cell.

The delivery and stable expression of RNAi suppression constructs remains a major challenge for gene therapy applications. siRNAs do not readily cross the blood–brain barrier (BBB) and when injected directly into the brain do not typically penetrate the plasma membrane without modification. To circumvent this issue, many studies have used shRNAs and artificial miRNAs expressed from viral vectors to achieve stable expression in particular brain regions of interest. Recombinant adeno-associated virus (AAV) and lentivirus (LV) are the most common gene therapy vectors for directed shRNA and miRNA delivery into the brain. They provide stable expression of RNAi triggers while being minimally immunogenic, non-pathogenic and replication incompetent (11). Recombinant LV can be pseudotyped with different glycoproteins to alter their cellular tropism (12), transduces both dividing and non-dividing cells and integrates into the host's genome unless the integrase is inactivated (13). However, LV-mediated transduction often results in low copy number, making it a weaker vehicle compared with AAV. AAVs provide strong and stable gene expression without integrating into the host genome, remaining as nuclear episomes. The numerous, variable capsid serotypes of AAV provide specific cell and tissue tropisms, making it a desirable choice for directed gene therapy (14,15). AAV serotypes bind to distinct cellular receptors, display patterns of retrograde and anterograde transport following injection and newer generation serotypes are being engineered with increased tropism to distinct tissues types and brain regions.

It should be noted that viral vector-mediated delivery can induce a neutralizing antibody response against the viral capsid, diminishing transgene expression in target tissues (16,17). Several animals used in preclinical research, as well as humans, have varying levels of pre-existing antibodies to many of the AAV serotypes, with highest levels against AAVs 1 and 2 compared with AAVs 5, 6, 8 and 9 (18,19). Research animals and human patients can be pre-screened for the presence of anti-AAV neutralizing antibodies and current research is investigating novel engineered AAV capsids that may be able to evade neutralizing antibody responses. In the case of AAVs, sustained expression has been seen over 6 years in canine and non-human primates (20), making the need for re-delivery unlikely. An outstanding example of AAV tolerability and re-delivery, should it be necessary, is the work by Bennett *et al.* (21) which demonstrated that re-administration of AAV to the eye of patients was extremely well tolerated several years after the original delivery.

Antisense oligonucleotides

ASOs are single-stranded nucleotides, typically 12–22 bases in length, that bind complementary target RNA through Watson and Crick hybridization resulting in modulation of the target RNA. ASOs are primarily used to modulate RNA either through the recruitment of RNase H, an endogenous enzyme that recognizes RNA/DNA heteroduplexes and degrades the target RNA (22), or by obstructing interactors of the target RNA to inhibit translation or modulate splicing. The antisense mechanism invoked depends on both the ASO's binding location on the target RNA and the chemical modifications of the ASO (23).

More than 30 years of optimization of oligonucleotide modifications have generated molecules with characteristics amenable to the treatment of neurodegenerative diseases. Most, if not all, ASOs in development or in the clinic today contain a substitution of sulfur for non-bridging oxygen atom in the phosphate backbone, transforming the phosphodiester linkage to a phosphorothioate (24). This improves pharmacokinetics by increasing resistance to nucleases and improves distribution and pharmacodynamics by increasing protein binding (25). Another common modification is a 2′ alteration to the sugar moiety. This includes 2′-*O*-methoxyethyl (MOE) modifications, as well as introduction of bicyclic nucleosides such as locked nucleic acids (LNAs) and constrained 2′-*O*-ethyl nucleic acids (cEts). Sugar modifications increase binding affinity to the target RNA, increase resistance to nucleases and can decrease non-specific toxicities, each to a different degree (26,27). For the higher affinity modifications, like LNAs, increased potency can come at the cost of tolerability (28). Importantly, most sugar modifications render the modified nucleotide resistant to RNase H activity.

Each modification, with its unique properties, can be used as a building block to assemble an ASO with a desired set of characteristics. If RNA degradation is required, a gapmer design can be used. This is done by incorporating sugar modifications into the 5′ and 3′ residues to improve binding affinity and resistance to nucleases (the wing) while leaving a stretch of uninterrupted, unmodified deoxynucleic acids in the center (the gap) to engage RNase H (27). Alternatively, ASOs with fully modified sugars are

used to bind RNA with high affinity without degrading the target RNA. This is typically done to obstruct binding of other interactors, such as hnRNPs to alter splicing, or inhibit translation by blocking ribosomes (29–31). Higher affinity modifications, like cEts, can be employed to target tissues or sites on a gene that are normally less amenable to ASO activity (32,33). Thus, each ASO has a common set of class properties that can be partially predicted based on the modifications used as well as the unique set of properties determined by the sequence of the ASO.

Like siRNAs, ASOs do not cross an intact BBB. However, ASOs are soluble in artificial cerebrospinal fluid (CSF) and can be delivered directly into the CSF surrounding the brain and spinal cord. Once introduced into the CSF, modified ASOs have sufficient stability and uptake to distribute to the brain parenchyma and enter cells, both in preclinical models and in human patients (34–37). MOE-modified gapmers have long half-lives (35,37,38); therefore, it is likely that only intermittent and infrequent delivery of ASOs to the CSF will be required. Finally, ASOs behave in a dose-dependent manner, making it possible to moderate the degree of target modulation to a level that is safe yet still effective (35).

Gene Suppression for Dominantly Inherited Diseases

Huntington's Disease

HD is an autosomal dominantly inherited neurodegenerative disorder that results from an expanded CAG repeat in the HTT gene (*mHTT*) on chromosome 4 (39). The encoded protein, mutant huntingtin (mHTT), contains an expanded polyglutamine (PolyQ) tract at the N-terminus, which leads to aberrant folding and the inability to be processed appropriately by the cell. mHTT takes on a toxic gain of function leading to cellular dysfunction, inclusion body formation, gliosis and brain atrophy (40). In general, non-affected individuals have fewer than 36 glutamine repeats in their huntingtin gene (*HTT*), and 36–39 repeats results in a reduced-penetrance of the disease, with a later onset and slower progression of symptoms. Repeats of 40 or higher result in full expression of disease phenotypes. The number of CAG repeats is directly related to the age of onset of symptoms, and patients with exceptionally high repeat lengths have the juvenile form of HD (41). While numerous brain regions are affected, the striatum and overlaying cortex are the most-heavily affected regions. HD symptoms have a devastating impact on the patient's quality of life. Motor symptoms include involuntary hyperkinetic movements, an abnormal gait and difficulty with speech and swallowing (42,43). Personality changes are defined by wide mood swings, aggression, anxiety and clinical depression (44–46). Cognitive decline is characterized by both long- and short-term memory dysfunction and loss of executive function (46–49). Eventually, patients require help with all activities of daily living and end up wheelchair-bound and bedridden. HD is invariably fatal and patients typically succumb to the disease 10–15 years following diagnosis. Currently, all treatments for HD are palliative and thus, finding a therapeutic that ameliorates neuropathology and related behavioral dysfunction would be of tremendous benefit to patients. Because the genetic mutation that causes HD is known, gene suppression strategies have garnered a great deal of interest as a potential interventional therapeutic.

RNAi studies for HD in rodents and non-human primates

Over the past decade, numerous potential therapeutic approaches have emerged to reduce the expression of *mHTT* in the brain, including the use of siRNAs, shRNAs, artificial miRNAs, zinc finger transcriptional repressors and ASOs (Table 1). It has been more difficult than first anticipated to specifically target the mutant allele, while preserving expression of the unaffected allele. Several early proof-of-concept studies targeted only the human *mHTT* transgene in both transgenic and AAV-mediated rodent models of HD. These studies demonstrated that solely reducing expression of the mutant transgene, while leaving the endogenous rodent huntingtin (Hdh) alleles unaffected, was efficacious in reducing neuropathology and behavioral readouts germane to each disease model, including striatal inclusion formation, neuronal atrophy and motor dysfunction (7,50,52,53). These initial studies were incredibly important in highlighting the overall efficacy of an RNAi approach in preventing HD disease symptomology. In an attempt to specifically target the *mHTT* allele, other groups have identified particular single-nucleotide polymorphism (SNP) variants that reside only on the mutant allele using DNA collected from HD patient lymphocytes and fibroblasts (75,76). In 2009, Pfister *et al.* (77) identified a set of five allele-specific siRNAs corresponding to three SNP sites that could potentially be used to treat a large portion of HD patients in the US and Europe. More recently, Monteys *et al.* (59) demonstrated partial allele specificity *in vivo* using an artificial miRNA designed to target an SNP in a double transgenic engineered HD mouse model. Aside from targeting SNPs in the *HTT* gene itself, Becanovic *et al.* (78) have shown that targeting an SNP in the *HTT* promoter effectively reduces NF-kB binding and reduces huntingtin protein (HTT) expression in HD patient cells and in transgenic mouse striatum.

An alternative gene suppression strategy for HD that has made substantial progress over the last decade is the partial reduction in both mutant and native *HTT* alleles (known as non-allele-specific RNAi). While it is clear that normal HTT protein plays an important functional role in the neuron and is necessary during development, the goal of non-allele specific RNAi is to establish the lowest level of knockdown that leads to amelioration of neuropathology and behavioral deficits while maintaining a positive safety and tolerability profile. Numerous reports have shown that a partial reduction in endogenous huntingtin in the adult brain of multiple species is well tolerated and this approach is now being pursued by many teams as a viable clinical approach to treat HD. Reducing both mutant and endogenous huntingtin in both transgenic HD mouse and rat striatum by 40–60% prevents motor deficits, extends lifespan and does not lead to toxicity in both fragment and full-length mouse HD models in both short- and long-term studies (8,54,55). Encouragingly, reducing both *HTT* alleles in post-symptomatic transgenic HD mice was well tolerated and resulted in reduced inclusion formation and rotorod dysfunction, suggesting that RNAi may be beneficial in HD patients that already present with disease phenotypes (55,58).

Following success in rodent models of HD, the safety of partially reducing endogenous *HTT* has been evaluated in non-human primate studies following the delivery of AAV vectors expressing artificial miRNAs and shRNAs. McBride *et al.* (60) demonstrated that a 45% reduction in rhesus *HTT* did not induce neuronal loss, gliosis, behavioral dysfunction or weight loss and these results were further extended when Grondin *et al.* (61) showed that sustained *HTT* suppression out to 6 months post-infusion was well tolerated. Two follow-up studies found that siRNA infusion into the rhesus putamen reduced *HTT* to similar degrees and that partial *HTT* suppression lasted for up to 39 days after the infusion of siRNA was terminated (62,63). Similar tolerability has recently been shown following a 50% reduction in *HTT* in the sheep putamen by Neil Aronin's laboratory (poster presentation, CHDI conference, 2015). Together, these

Table 1. *In vivo* gene reduction strategies for HD and SCAs

Disease	Target gene	Therapeutic construct	Injection site	Animal model	Synopsis of study results	Ref.
HD	Human *mHTT*	AAV2/1-shRNA	Striatum	N171–82Q Tg mouse	Reduction in human *mHTT* prevented inclusions, gait deficits and rotorod dysfunction	(50)
HD	Human *mHTT*	AAV2/5-shRNA	Striatum	R6/1 Tg mouse	Reduction in human *mHTT* reduced inclusion burden, normalized striatal transcripts and prevented clasping phenotype	(7)
HD	Human *mHTT*	siRNA	ICV	R6/2 Tg mouse	Reduction in human *mHTT* reduced inclusion burden, prolonged longevity, improved motor dysfunction and slowed weight loss	(51)
HD	Human *mHTT*	siRNA	Striatum	AAV-HTT-injected mouse	Reduction in human *mHTT* prolonged striatal neuron survival, reduced aggregates and prevented motor dysfunction	(52)
HD	Human *mHTT*	AAV1/2-shRNA	Striatum	AAV-HTT-injected rat	Reduction in human *mHTT* prevented neuronal loss and forelimb impairment	(53)
HD	Human *mHTT*/ Mouse *Hdh*	AAV2/1-shRNA/ AAV2/1-miRNA	Striatum	CAG140 KI mouse	Placing an siRNA construct in the construct of an artificial miRNA compared with an shRNA alleviated toxicity due to high expression of antisense RNAs	(8)
HD	Human *mHTT*/ Mouse *Hdh*	AAV2/1-miRNA	Striatum	N171–82Q Tg mouse	Reducing expression of both human *mHTT* and endogenous mouse *Hdh* reduced inclusion formation, prevented rotorod deficits and extended lifespan	(54)
HD	Human *mHTT*/Rat *Hdh*	LV-shRNA	Striatum	LV-HTT-injected rat	Reducing expression of both human *mHTT* and endogenous rat *Hdh* lowered inclusion formation, prevented rotorod deficits and was well tolerated out to 9 months post-injection	(55)
HD	Human *mHTT*	AAV2/5-Cre	Hypothalamus	BACHD Tg mouse	Reducing human *mHTT* expression prevented weight gain and impaired glucose metabolism	(56)
HD	Human *mHTT*	AAV2/9-miRNA	Jugular vein	BACHD, N171–82Q Tg mouse	Vascular delivery of an miRNA reduced *mHTT* in several brain regions, reduced aggregates, prevented regional cortical and striatal atrophy and prevented weight loss	(57)
HD	Human *mHTT*/ Mouse *Hdh*	AAV2/5-miRNA	Striatum	YAC128 Tg mouse	Reducing expression of both human *mHTT* and endogenous mouse *Hdh* after symptom development lowered inclusion burden, normalized striatal transcripts and prevented motor phenotypes	(58)
HD	Human *mHTT*	AAV2/1-miRNA	Striatum	HTT double Tg mice	Partial allele selectivity was achieved by targeting an SNP that resides on the *mHTT* transgene	(59)
HD	Human *mHTT*	AAV2/1-miRNA	Hypothalamus	N171–82Q Tg mouse	Reducing human *mHTT* expression prevented inclusion formation, neuropepetide dysregulation, glucose dysregulation and weight loss	[a]
HD	Rhesus *HTT*	AAV2/1-miRNA	Putamen	NHP	Reduction in rhesus *HTT* did not induce neuronal loss, gliosis, an immune response, motor dysfunction or weight loss	(60)
HD	Rhesus *HTT*	AAV2/2-miRNA	Putamen	NHP	Reduction in rhesus *HTT* did not induce neuronal loss, gliosis, an immune response, motor dysfunction or weight loss	(61)
HD	Sheep *HTT*	AAV2/9-miRNA	Striatum	Sheep	Reduction in sheep *HTT* did not induce neuronal loss, gliosis, an immune response, motor dysfunction or weight loss	[b]
HD	Rhesus *HTT*	siRNA	Putamen	NHP	siRNA treatment for 28 days reduces rhesus *HTT* expression throughout the putamen and was well tolerated	(62)
HD	Rhesus *HTT*	siRNA	Putamen	NHP	siRNA treatment for 4 days resulted in sustained *HTT* suppression out to an estimated 27–39 days post-infusion	(63)
HD	Human *mHTT*	ASO	ICV	BACHD, YAC128 R6/2 Tg mouse	Reduction in human *mHTT* in young and aged mice reversed rotarod deficits in YAC128 and BACHD mice, ameliorated hypoactivity and anxiety in aged BACHD mice and prevented loss of brain mass in R6/2	(35)
HD	Human *mHTT*/ Mouse *Hdh*	ASO	ICV	BACHD Tg mouse, non-Tg mouse	Simultaneous suppression of human *mHTT* and normal mouse *Hdh* reversed rotarod and open-field deficits for 8 months post-treatment. No difference from mutant selective suppression. No behavioral changes in ASO treated non-transgenic littermates	(35)
HD	Monkey *HTT*	ASO	IT	NHP	Monkey *HTT* was reduced in the cord and cortex for >8 weeks post-treatment termination	(35)

HD	Human *mHTT*	ASO	ICV	YAC128 Tg mouse	Reduced *mHTT* improved motor deficits and this correlated with improvements in several striatal dysregulated transcripts	(64)
HD	Human *mMTT*/ Mouse *Hdh*	ASO	ICV	BACHD, YAC128 Tg mouse	Single bolus injection of non-allele-selective huntingtin ASO decreased mHTT and mouse huntingtin protein levels >80% and did not induce gliosis or astrocytosis	(65)
HD	Human *HTT* SNP targeting	ASO	ICV	BACHD Tg mouse	Single bolus injection of SNP targeting ASO decreased mHTT protein levels 50% and did not induce gliosis or astrocytosis	(65)
HD	Human *HTT* SNP targeting	ASO	ICV	Hu97/18 double Tg mouse	Single bolus achieved 75% reduction in human mHTT protein 4 weeks post-dose, with no change in normal human HTT protein	(66)
HD	Human *HTT* SNP targeting	ASO	IT	Rat	No gliosis, astrocytosis, change in body weight, grip strength or open field	(58)
HD	Human *HTT* SNP targeting	ASO	ICV	Hu97/18 double Tg	Selective human mHTT suppression >90%. mHTT protein suppression maintained at >50% for 36 weeks after single ICV bolus injection	(38)
HD	Human *HTT* SNP targeting	ASO	ICV/IT	Mouse, rat	Lead SNP ASOs did not induce gliosis, astrocytosis, weight loss or alteration in neurological exams in mice or rats	(38)
HD	CAG	ss-siRNA	ICV	Q150 KI mouse	Selectively decreased CAG expanded huntingtin protein 50–75% in various regions throughout the mouse brain	(2)
SCA1	Human *mATXN1*	AAV2/1-shRNA	CBL CTX	SCA1 Tg mouse (B05)	Suppression of cerebellar *mATXN1* prevented cerebellar degeneration and motor phenotypes	(6)
SCA1	Human *mATXN1*/ mouse *Atxn1*	AAV2/1-miRNA	DCN	SCA1 Tg mouse (B05)	Suppression of cerebellar human *mATXN1* and mouse *Atxn1* levels improved behavioral and molecular phenotypes	(67)
SCA1	Mouse *Atxn1*	AAV2/5-miRNA	DCN	SCA1 KI mouse	Suppression of cerebellar *Atxn1* levels improved behavioral and neuropathological phenotypes for out to 1 year post-injection	(68)
SCA3	Human *mATXN3* SNP targeting	LV-shRNA	Striatum	SCA3 rat	Allele-specific targeting of a SNP on human *mATXN3* prevented inclusion formation and neuron loss	(69)
SCA3	Human *mATXN3*/ Rat *Atxn3*	LV-shRNA	Striatum	SCA3 rat	Non-allele-specific targeting of human *mATXN3* and rat *Atxn3* reduced neuropathology	(70)
SCA3	Human *mATXN3*	AAV2/1-miRNA	DCN	SCA3/MJD84.2 mouse	Allele-specific suppression of *ATXN3* resolved molecular phenotypes	(71)
SCA3	Human *mATXN3*	AAV2/1-miRNA	DCN	SCA3 rat	Lifelong suppression of *mATXN3* was well tolerated but did not prevent motor impairment or prolong lifespan	(72)
SCA7	Human *mATXN7*/ Mouse *Atxn7*	AAV2/1-miRNA	Retina	SCA7 Tg	Suppression of *mATXN7* and mouse *Atxn7* was well tolerated, did not induce neuropathology and normal retinal function was preserved	(73)
SCA7	Human *mATXN7*/ Mouse *Atxn7*	AAV2/1-miRNA	DCN	SCA7 Tg	Suppression of human *mATXN7* and mouse *Atxn7* improved behavioral and molecular phenotypes	(74)

Tg, transgenic; inj., injected; LV, lentivirus.

[a]McBride, oral presentation, Gordon Research Conference on CAG Repeat Disorders, 2015.

[b]Aronin, poster presentation, CHDI conference, 2015.

results show that a partial reduction in both mutant and wild-type *HTT* alleles may be a viable therapeutic strategy to treat HD.

Historically, RNAi constructs evaluated in animal models of HD have been administered via a direct injection into the striatum, the primary site of degeneration in HD, or into the adjacent lateral ventricle (Fig. 1). However, many extra-striatal brain regions are also affected in HD and have become recent targets for gene suppression therapy. Hult *et al.* (56) lowered *mHTT* expression in the BACHD transgenic HD mouse hypothalamus using an AAV-Cre-based approach and effectively prevented the characteristic metabolic phenotype. More recently, McBride *et al.* found that a partial reduction in both human *mHTT* and mouse *Hdh* using an AAV-miRNA approach in the N171–82Q transgenic mouse hypothalamus partially prevented neuropeptide dysregulation, glucose homeostasis and altered energy metabolism (McBride, oral presentation, Gordon Research Conference on CAG Repeat Disorders, 2015). Because neurodegeneration is seen throughout many regions of the brain, a global reduction in *mHTT* expression throughout the brain would be expected to provide a greater benefit compared with targeting individual affected regions. In 2014, Dufour *et al.* (57) showed that AAV serotype 9 expressing an artificial miRNA targeting *mHTT* crossed the BBB following injection into the jugular vein of transgenic HD mice and significantly reduced *mHTT* expression in the cortex, striatum, hypothalamus, hippocampus and thalamus (Fig. 2). This widespread *mHTT* knockdown was associated with prevention of cortical and striatal atrophy as well as reduced inclusion body formation. This study was the first to show a reduction in *mHTT* expression in brain following vascular administration of a gene suppression construct and highlights the possibility of sustained *mHTT* knockdown from a single, systemic injection.

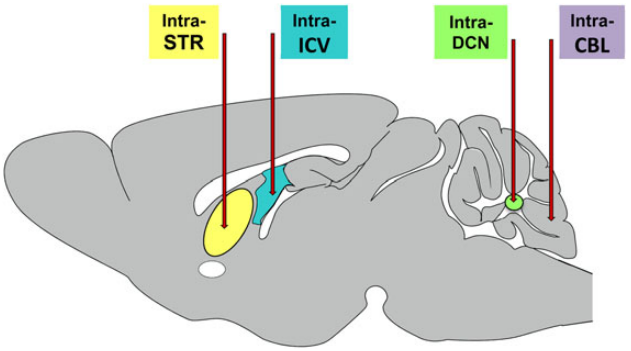

Figure 1. Cartoon of common delivery sites for gene suppression constructs evaluated in mouse models of HD and the SCAs. Primary delivery sites include the striatum, lateral ventricle, DCN and the cerebellar cortex. Delivery into the striatum (STR) (7,8,50,53,54,58,59,79), DCN (67,68,71,74,80) and cerebellar cortex (CBL) (6) result largely in focal expression of the injected construct, with evidence if anterograde and retrograde transport when AAVs are used as the delivery vehicle. In contrast, injection into the CSF via injections into the lateral ventricle (ICV) (2,35,38,51,64,66) or the cisterna magna (CM) result in more widespread delivery throughout the brain.

ASO strategies for HD

Since the identification of the single causative mutation in HD, multiple ASO approaches using different antisense mechanisms to modulate mHTT have been tested. The most advanced of these approaches, total HTT suppression using traditional MOE gapmer ASO designs, is currently being tested in the clinic. Here, an MOE gapmer ASO complementary to the human huntingtin RNA is introduced directly into the CSF. This is predicted to result in partial suppression of both the mutant and normal *HTT* alleles. As ASOs behave in a dose-dependent manner, *HTT* suppression can be targeted from 0 to 75% (35). In preclinical models, huntingtin targeting MOE gapmer ASOs have both prevented and reversed disease (35,64). Suppression of human *mHTT* in the YAC128 and BACHD models of HD reversed existing behavioral phenotypes and prevented progressive loss of brain mass in R6/2 mice (35). Similarly, ASO-mediated human *mHTT* suppression in YAC128 mice ameliorated key striatal gene expression changes (64). Interestingly, the earlier in disease the treatment was initiated, the more robust the disease reversal. Perhaps the finding of greatest significance from these rodent studies was that transient ASO suppression of *mHTT* (~4 months) was sufficient to obtain a long-term benefit (>9 months). One can imagine a treatment where intermittent suppression of *HTT* ('a huntingtin holiday') is all that is required to provide continuous benefit (81).

ASOs can be introduced into the CNS by direct injection in the CSF either by intrathecal (IT) injection in non-human primates and human patients or (due to surgical limitations) intracerebral ventricular (ICV) injection in rodents (Fig. 1). Human huntingtin ASOs delivered ICV in rodents suppress human *HTT* mRNA >75% throughout the brain and spinal cord (35). In larger non-human primate brains, huntingtin ASOs complementary to monkey *HTT* RNA delivered IT are present in the spinal cord, cortex and to a lesser degree deeper brain structures (35). Tissue adjacent to the CSF has the highest total tissue levels of ASO,

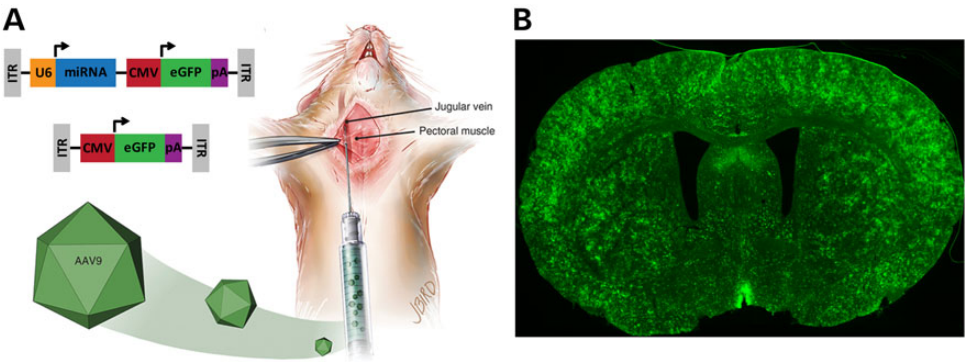

Figure 2. Global suppression of *HTT* in the transgenic HD mouse brain. (**A**) Cartoon depicting the delivery of an AAV9 vector expressing an *mHTT*-specific miRNA and a GFP control gene (top construct, AAV9-mi2.1-GFP) or AAV9 vector expressing GFP alone (bottom construct, AAV9-GFP) into the mouse jugular vein. (**B**) Vascular delivery of AAV9-mi2.1-GFP resulted in widespread expression of both the therapeutic miRNA and GFP throughout the N171–82Q transgenic mouse brain and a concomitant reduction in *mHTT* in several brain regions including the cortex, striatum, hypothalamus, hippocampus and thalamus (57).

suggesting most ASO reaches the tissue by passive diffusion. ASOs are also found in and diffusing radially from the Virchow–Robins space, a fluid-filled area that surrounds veins and arteries in the brain parenchyma and is likely another route of ASO distribution. In areas of low bulk ASO levels, populations of neuronal cell bodies that contain high levels of ASO are often found, making it unlikely that ASOs arrived to these neurons by passive diffusion. One possibility is that ASOs are actively transported to these cell bodies by anterograde or retrograde transport. However, the exact mechanisms of ASO uptake and transport in the CNS have not been fully elucidated and warrant further study. Regardless of mechanism, what is clear is that delivery of huntingtin ASOs to the CSF results in distribution of active ASO to much of the CNS.

Modified ASOs have long half-lives in the CNS. In BACHD mice expressing the human *mHTT* gene, human *mHTT* mRNA is suppressed up to 12 weeks following termination of treatment (35). Similar longevity of suppression is achieved in non-human primates treated with a huntingtin ASO. This is further supported by clinical data, as ASO was detectable in the brain and spinal cord of an amyotrophic lateral sclerosis (ALS) patient 3 months after an IT delivery of ASO (34). The long half-lives of ASOs in the CNS are particularly beneficial for slowly progressing diseases, where patients will likely require treatment for years, if not decades. In cases where targeting inherited disease causing mutations is possible, as in HD, infrequent dosing becomes even more valuable, as ideally patients could be identified and treatment initiated before disease manifests, but would likely require nearly a lifetime of treatment.

As with siRNAs, one primary question for any gene suppression approach is the necessity for allele selectivity. Also, like siRNA and miRNA approaches, in preclinical models, ASO-mediated huntingtin suppression in normal mice and non-human primates does not induce a phenotype (35). In disease models, simultaneous reduction in endogenous mouse *Hdh* along with human *mHTT* yielded the same phenotypic reversal as reduction in *mHTT* alone (35). However, despite lack of evidence that partial suppression of normal huntingtin will be detrimental, an allele-selective approach is appealing and remains tractable.

An alternative approach is to use RNase H ASOs to target SNPs linked to the CAG expanded *HTT* allele and not present in the normal *HTT* allele. More than 90% of *mHTT* linked SNPs are present in intronic regions, making targeting of these sites with siRNAs unfeasible. ASOs, however, are active against pre-mRNAs as well as mature mRNAs and can target intronic sequences. Using population genetics, researchers have identified heterozygous SNPs that are linked to the CAG allele in nearly half of the HD population (65). Strategic incorporation of high affinity modifications (cEts) into MOE gapmers and targeting SNP sites, yielded >50-fold selectivity of the *mHTT* allele over the normal allele containing a one base mismatch at the SNP site (38,65,66). Although promising, one limitation is that 3–5 ASOs at unique sites would be required to target 80% of the HD population allele selectively (82).

To allele selectively target all HD patients, fully 2′ sugar modified ASOs complementary to the CAG repeat can preferentially obstruct the translation of the expanded CAG containing transcript, resulting in 6-fold selectivity over unexpanded HTT (83–86). Selectivity is likely achieved through steric hindrance, where longer transcripts allow for binding of multiple CAG targeting ASOs. A similar approach targeting the CAG repeat, but employing the miRNA pathway utilizes a single-stranded siRNA (ss-siRNA) that contains a mismatch to the CAG results in the recruitment of Ago2 to the expanded transcript, and suppression of translation without altering mRNA levels (2). The CAG targeting ss-siRNAs were validated both *in vitro* in patient fibroblasts and *in vivo* in a knock-in (KI) HD mouse model (2). One consideration when developing these approaches is to determine the potential and safety of unwanted suppression of other CAG-containing transcripts. Another consideration is that the current CAG targeting ss-siRNAs are not as potent as the other siRNA and ASO approaches. Despite the challenges, CAG targeting is particularly attractive as a single drug has the potential to treat all polyglutamine diseases.

RNAi studies for the SCAs in rodents and non-human primates

Like HD, many of the SCAs are part of a subset of disorders known collectively as polyglutamine-repeat diseases. In particular, SCA 1, 2, 3, 6, 7 and 17 are categorized as autosomal-dominant polyglutamine diseases.

Spinocerebellar ataxia type 1 (SCA1) is a late onset neurodegenerative disease caused by a CAG expansion in the coding region of ataxin-1 (*ATXN1*) which affects 1–2 in 100 000 people worldwide (87,88). Symptoms include loss of coordination, slurred speech, difficulty swallowing and mild cognitive impairment. Neurodegeneration occurs predominantly in cerebellar Purkinje cells, deep cerebellar nuclei (DCN), brainstem nuclei and spinocerebellar tracts (Fig. 1) (89–92). Reliable mouse models have been integral in understanding disease pathology (93–95). RNAi as a potential therapeutic was first demonstrated using AAV expressing shRNAs targeting *ATXN1* in the transgenic mouse model of SCA1. Directed injections to the medial cerebellar lobules led to significant knockdown of ataxin-1 and an improvement of behavioral and neuropathological phenotypes (6). It has since been shown that delivery to the DCN, versus the cerebellar cortex itself, leads to a wider biodistribution of therapeutic load by trafficking retrograde up the axons to the Purkinje cell soma (67,68,72). More recently, artificial miRNAs targeting human *ATXN1* (in a transgenic model) or mouse *Atxn1* (in a knock-in model) were delivered using AAV serotypes 2/1 and 2/5, respectively (Fig. 3A) (67,68). Directed stereotaxic injections of RNAi constructs to the DCN of pre-symptomatic SCA1 mice prevented motor deficits and improved cellular and molecular phenotypes. Significant knockdown of ataxin-1 was achieved 35 weeks (67) and 60 weeks (68) post-delivery with no overt glial response in either case. Following success in mouse models, the feasibility and safety was evaluated in non-human primates. AAV2/1 expressing the same artificial miRNA used in transgenic SCA1 mice was stereotaxically injected to the DCN of adult, naive rhesus macaques using magnetic resonance imaging-guided techniques (Fig. 3B). The DCN, cerebellar cortex, brainstem nuclei and projections to the ventral lateral thalamus were safely transduced with significant reduction in endogenous *ATXN1* mRNA levels ≥30% in transduced areas (96).

Spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease, is the most common of the SCAs. SCA3 is a late onset, progressive neurodegenerative disease caused by a CAG expansion in the ataxin-3 (*ATXN3*) gene. Symptoms are characterized by ataxia as well as dystonia and peripheral neuropathy (97). Previously, LV expressing both allele-specific and non-allele-specific shRNAs targeting *ATXN3* in rats and mice were both beneficial well tolerated (69,70,98). More recently, two complementary papers studied the effects of artificial siRNAs targeting *ATXN3* in transgenic mice. Both studies used stereotaxic injections of AAV2/1 to target the DCN of SCA3 mice. Combined, the studies demonstrated that directed delivery of siRNAs achieved prolonged knockdown of *ATXN3* and cleared nuclear

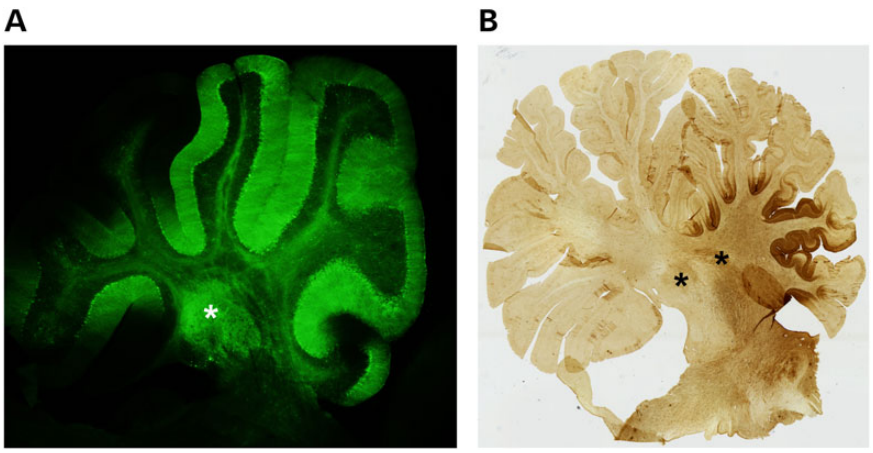

Figure 3. Transgene expression in the mouse and NHP cerebellum following AAV-mediated expression in the DCN. (**A**) Sagittal section of mouse cerebellum following direct delivery of AAV2/5 expressing an miRNA targeting *Atxn1* and eGFP to the DCN. Green staining demonstrates biodistribution to multiple lobules from single injection site. (**B**) Sagittal section of NHP cerebellum following direct delivery of AAV2/1 expressing and an miRNA targeting *ATXN1* and eGFP to the DCN. Brown staining shows biodistribution throughout multiple lobules. *DCN.

aggregates characteristic in the mouse model (71). However, despite significant knockdown of cerebellar *ATXN3* and apparent tolerability of the constructs, the mice still developed motor phenotypes (72).

Spinocerebellar ataxia type 7 (SCA7) is caused by a CAG expansion in ataxin-7. In addition to the characteristic cerebellar neurodegeneration and ataxic symptoms, patients also lose their vision (99). Recently, non-allele-specific artificial miRNAs targeting ataxin-7 (*ATXN7*) were injected into either the eye or the DCN of SCA7 Tg mice. AAV2/1 delivery to the DCN resulted in improvement of motor phenotypes as well as improvement of cerebellar Purkinje cell dendrites, known to be affected in the model. In addition, long-term knockdown of *ATXN7* was achieved in the cerebellum and brainstem without toxicity (74). Artificial miRNA expressed from AAV2/1 injected to the retina of SCA7 mice achieved ≥50% suppression of *ATXN7* in the retina with no adverse effects in retinal functioning (73).

Ongoing and future clinical trials

The success of the last decade of work has led to the first in man gene suppression therapy in HD patients. ASOs have previously been delivered IT to both ALS (34) and spinal muscular atrophy patients (100). Recently, a phase I clinical trial delivering a huntingtin ASO has been initiated in early-stage HD patients. This is the first potentially disease-modifying therapy to enter trials for HD, and certainly the first to target *HTT*, the underlying cause of the disease. Additional Phase I clinical studies, utilizing AAV-delivered miRNAs that have made substantial progress in rodent and NHP studies, are likely to follow in the next few years. The first HD gene suppression trial is focused on safety and tolerability of an IT-delivered RNase H MOE-modified ASO targeting human *HTT*. In addition to safety endpoints, neuroimaging assessments, as well as exploratory endpoints quantifying cognitive, motor, and neuropsychiatric aspects of disease will be performed.

One key challenge in the clinic for centrally delivered gene suppression approaches is identification of a target engagement biomarker. Blood, urine and other easily accessible matrices are not likely to reflect changes limited to the CNS, as is the case for most CNS gene suppression approaches. Numerous groups have been working to determine if HTT is detectible in the CSF. This year, two groups have successfully detected mHTT in the CSF (101,102); mutant HTT increases with disease burden and tracks with tau and neurofilament light chain load in the CSF (102). Southwell *et al.* (101) has demonstrated, in a humanized mouse model, that mHTT levels in the CSF decrease after ASO-mediated suppression of HTT in the CNS, suggesting that CSF HTT can be used to track changes in HTT levels in tissues. Analysis of HTT levels in CSF will allow for confirmation of target engagement in the CNS, and aid in dose selection. Similar attempts to detect and quantify other polyQ disease proteins in the CSF are also underway.

As discussed, there are multiple methods for delivery of RNA therapeutics to the CNS. In diseases where pathology is restricted to a distinct brain region or tissue, or if global suppression is unfavorable for a given target, then directed intracranial injections can be employed (7,8,50,53,54,58,59,67,68,71,74,79,80). If broader suppression is needed, injections directly into the CSF (2,35,38,51,64,66) or the vasculature (57,103) can provide a more global distribution to the brain and spinal cord. These advances are encouraging and future studies are warranted to identify alternative methods to deliver RNA therapeutics across the BBB.

This is an exciting time for gene suppression therapies in neurodegenerative diseases. It is clear from the preclinical data that suppression of the underlying cause of disease, particularly in dominantly inherited diseases like polyQ diseases, can have a dramatic effect on disease course in animal models. We are entering a time where technologies have advanced and delivery techniques have been refined to a point where treatment of human patients is tractable. With human clinical trials ongoing, and more imminent, it is becoming increasingly likely that gene suppression approaches will have a real chance at altering the course of these devastating diseases.

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