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Huntingtin Lowering Strategies

for Disease Modification in Huntington's Disease

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Summary

Huntington's disease is caused by an abnormally expanded CAG repeat expansion in the *HTT* gene, which confers a predominant toxic gain of function in the mutant huntingtin (mHTT) protein. There are currently no disease-modifying therapies available, but approaches that target proximally in disease pathogenesis hold great promise. These include DNA-targeting techniques such as zinc-finger proteins, transcription activator-like effector nucleases, and CRISPR/Cas9; post-transcriptional huntingtin-lowering approaches such as RNAi, antisense oligonucleotides, and small-molecule splicing modulators; and novel methods to clear the mHTT protein, such as proteolysis-targeting chimeras. Improvements in the delivery and distribution of such agents as well as the development of objective biomarkers of disease and of HTT-lowering pharmacodynamic outcomes have brought these potential therapies to the forefront of Huntington's disease research, with clinical trials in patients already underway.

In this review, Tabrizi et al. describe new approaches to disease modification in Huntington's disease. These aim to reduce mutant huntingtin protein by targeting huntingtin DNA, RNA, and the mutant protein itself, in theory ameliorating all of its downstream pathogenic effects.

Huntington's disease, genome editing, zinc-finger proteins, transcription activator-like effector nucleases, CRISPR/Cas9, RNAi, antisense oligonucleotides, small-molecule splicing modulators, proteolysis-targeting chimeras, HD biomarkers, drug delivery

Main Text

Huntington's disease (HD) is an inherited autosomal-dominant neurodegenerative disorder characterized by a triad of motor, cognitive, and psychiatric features. HD typically displays onset in mid-life, with irreversible progression of symptoms over 10–15 years (Ross and Tabrizi, 2011). All cases of HD are caused by an abnormally expanded CAG repeat near the N terminus of the huntingtin gene (*HTT*), which leads to the production of mutant huntingtin protein (mHTT) on translation. It has now been 25 years since the identification of the genetic mutation in 1993, and intensive research efforts have described many cellular pathological mechanisms underlying disease development. Nearly all are driven by the presence of the mHTT protein, which is ubiquitously expressed and is thought to cause disease by a predominant toxic gain-of-function mechanism (Bates et al., 2015).

Currently, a major focus is development and testing of therapies that target proximally in HD pathogenesis; namely, by targeting HTT DNA, RNA, and protein. These approaches ultimately aim to reduce mHTT levels and, therefore, ameliorate all of its downstream pathogenic effects, which are multiple and varied. In animal models of HD, reduction of mHTT improves disease phenotypes and reverses neuropathology (Yamamoto et al., 2000; Wang et al., 2014), confirming the importance of mHTT lowering as a therapeutic aim.

This review will cover the methods we think are most important now in therapy development for HD; namely, HTT-lowering therapies that will mitigate all downstream pathogenic effects of mHTT protein (Figure 1). An overview of the treatments that have been or are being taken forward into clinical development is shown in Table 1.

Pathological Mechanisms in HD

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The CAG nucleotide repeat lies in exon 1 of the *HTT* gene; on translation, this creates an elongated polyglutamine (polyQ) stretch at the N terminus of the HTT protein. Expansion of the CAG repeat to 40 or more invariably leads to adult-onset HD, whereas patients with juvenile HD have an age of onset of less than 20 years and have generally inherited a mutation with more than 55 CAG repeats. The presence of HTT exon 1 mRNA has been confirmed in post-mortem brains and fibroblasts from patients with juvenile HD (Neueder et al., 2017). Wild-type HTT has a complex structure with multiple interaction sites (Guo et al., 2018), suggesting that it is a scaffolding protein that helps to co-ordinate other proteins and cellular functions (Zuccato et al., 2010). Its diverse roles include transcriptional regulation and enhancing brain-derived neurotrophic factor (BDNF) production, which is important for the survival of striatal and cortical neurons. Wild-type HTT also has a role in axonal transport, trafficking of endosomes and organelles, and vesicular recycling (Zuccato and Cataneo, 2014).

Mutant HTT is thought to cause disease by a predominant toxic gain-of-function mechanism (Bates et al., 2015; Ross and Tabrizi, 2011), and pathological mechanisms include early transcriptional dysregulation, synaptic dysfunction, altered axonal trafficking, impairment of the proteostasis network, aggregate pathology, impairment of the nuclear pore complex function, oxidative damage, mitochondrial dysfunction, and extra-synaptic excitotoxicity (Hughes and Jones, 2014; Grima et al., 2017). So far, attempts to target these downstream pathways for therapeutic benefit have not been successful, with initially promising candidates failing later in clinical trials (Travessa et al., 2017), suggesting that targeting just one of these pathogenic mechanisms is not sufficient to be of clinical benefit.

Deletion of the mouse huntingtin gene (*Hdh*) prior to neural development is embryonic lethal in mice (Nasir et al., 1995; Zeitlin et al., 1995). Conditional deletion of *Hdh* in the early post-natal period leads to a progressive neurological phenotype (Dragatsis et al., 2000); in 2-month-old mice, there is no neurological phenotype but death from acute pancreatitis, and deletion of *Hdh* at 4 or 8 months of age with normal HTT levels during development has no neurological or neuropathological phenotype in mice (Wang et al., 2016). The lack of phenotype in adult mice implies that reduction of wild-type HTT may not be detrimental in adult patients undergoing treatment with HTT-lowering agents. However, the role of wild-type HTT still needs to be considered when designing HTT-lowering therapies that reduce both mutant and wild-type protein in a non-selective manner to strike a balance between the benefits of lowering mutant HTT while maintaining levels of wild-type HTT sufficient to carry out its normal cellular functions, and this is discussed in detail below.

Putative Toxic Species in HD Pathogenesis

The expression of full-length mHTT in model systems has long been known to elicit neuropathology, including progressive motor deficits and striatal atrophy in the YAC128 mouse model (Slow et al., 2003) and impaired vesicular and mitochondrial trafficking in mammalian neurons (Trushina et al., 2004). The proteolytic cleavage of full-length mHTT by caspases, calpains, and other endoproteases is also known to yield N-terminal mHTT fragments that are thought to be responsible for generating neurotoxicity (Ratovitski et al., 2009; Landles et al., 2010). In addition, mHTT fragments have been isolated from human HD brains post-mortem (Lunkes et al., 2002). All pathologies resulting from the expression of full-length mHTT or its proteolytic cleavage products would be improved by therapies targeting HTT DNA or RNA.

However, a number of potential alternative toxic species have now been postulated to play a role in HD. The expression of exon 1 HTT protein causes HD-like pathology in mice in the absence of full-length mutant HTT (Mangiarini et al., 1996). Recently, an incomplete splicing mechanism for the generation of exon 1 HTT has been described. Mutant *HTT* transcripts allow read-through into intron 1, which has a stop codon at its start, and so, on translation, an exon 1 HTT fragment is produced (Sathasivam et al., 2013). *HTT* mRNA is also subject to repeat-associated non-ATG (RAN) translation, which leads to the expression of expansion proteins from all three reading frames without an AUG start codon. Four new homopolymeric expansion proteins (polyAla, polySer, polyLeu, and polyCys) have been shown to accumulate in HD human brains, particularly in the striatum and white matter. These proteins have been shown to be toxic to neural cells (Bañez-Coronel et al., 2015). Finally, the CAG-expanded *HTT* mRNA may itself contribute to HD toxicity (Rué et al., 2016). The relative contribution of these alternate mechanisms to overall HD pathogenesis is not yet clear but should be borne in mind because they might evade some of the current approaches used for HTT lowering (Figure 1). Targeting the most proximal cause of disease—that is, the mutated gene itself—would counteract all of these potentially disease-causing mechanisms.

Huntingtin-Lowering Therapies

DNA-Targeting Approaches

Huntingtin-lowering therapeutic agents currently in development that target *HTT* DNA can act by either modulating gene transcription or by direct modification of the *HTT* gene (genome editing). The standard

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approaches to DNA targeting use some form of specific DNA-binding element combined with effector elements such as nucleases, epigenetic modulators, or transcription factors. The DNA binding elements enable efficient and precise DNA targeting, and the effectors alter gene sequence or expression. Nuclease effectors act like genomic scissors and cleave the targeted DNA to produce a double-strand break that subsequently stimulates the cell's endogenous DNA repair mechanisms to repair the DNA break using either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is very error-prone and often results in the introduction of deletion or insertion mutations and functional disruption of the gene. HDR uses another DNA sequence as a template to correct the break. For genome editing applications, an exogenous DNA sequence with a desired sequence is included with the delivery of the nuclease and acts as a template for repair, directly introducing the new DNA sequence into the target region. There are three main classes of nucleases that can be engineered for DNA-targeting purposes: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and Cas9 or other RNA-guided bacterial nucleases. Each of these has a different mechanism of DNA binding and mode of action (Cox et al., 2015).

ZFNs consist of an active effector element, such as a transcriptional repressor protein or a specific nuclease, bound to a DNA-binding element that is composed of an array of multiple zinc-finger peptides. Each zinc finger can bind a sequence of three to five distinct nucleotides of the DNA strand. ZFN-targeting constructs usually contain a zinc-finger array that is several ZFN peptides long and highly specific to the DNA sequence of interest (Klug, 2010). Zinc-finger proteins (ZFPs) without nuclease activity can reduce the levels of gene expression by simply binding to DNA and preventing gene transcription. In the HD context, such ZFPs can be designed to bind selectively to expanded CAG repeats versus normal CAG repeat lengths, allowing relatively specific binding to the mutant *HTT* gene (Mittelman et al., 2009). One group has published data to show that ZFPs lower mHTT expression in cell lines without significantly altering expression from the non-expanded *HTT* allele or the expression of other non-expanded CAG repeat-containing genes (Garriga-Canut et al., 2012). Improved ZFPs containing an active repressor element introduced into the striatum of R6/2 mice using adeno-associated virus (AAV) vectors also reduced mHTT levels and improved some HD-like behavioral phenotypes.

These results support further development of allele-specific ZFP repressors and ZFNs as potential therapies for HD. Zinc-finger transcriptional repressor approaches might lower mHTT levels by targeting DNA without altering it, whereas ZFNs could add to the repressive effect of ZFPs by actually disrupting or correcting the mutant gene, similar to other direct genome editing strategies such as CRISPR/Cas9, and will have the potential to permanently correct the disease-causing CAG expansion in HD.

TALENs are very similar to ZFNs, using a nuclease effector domain linked to a DNA recognition domain. The TALEN DNA recognition domain use as series of specific amino acid repeats that bind to a specific nucleotide, and different combinations of these amino acid repeats can be generated to recognize specific DNA sequences. Although TALEN-based nucleases theoretically have higher efficiency and more specificity than ZFN-based approaches, TALENs require a specific nucleotide at the end of the DNA sequence that can limit potential targets (Malankhanova et al., 2017).

A TALEN construct designed to target a CAG trinucleotide repeat was both specific and highly efficient at shortening the CAG repeat tract in yeast (Richard et al., 2014). The only published HD work on TALENs used patient-derived fibroblasts (Fink et al., 2016). This group used two different approaches: a SNP-based (allele-specific) TALE without nuclease activity paired with a transcriptional repressor to decrease expression of the expanded *HTT* gene and CAG repeat-binding TALENs that required combined binding of two complementary TALEN monomers to re-constitute an active nuclease and cleave the target CAG repeat DNA. These TALEN constructs required at least 15 CAG repeats to bind both monomers, avoiding gene editing in normal-length CAG tracts. Using these two TALE-based approaches, a selective decrease in mHTT expression and aggregation was demonstrated in HD fibroblasts. Although the efficiency was extremely low, this work provides proof of principle that allele-specific *HTT* gene modification using TALEN-based methods is possible and supports further efforts in this area.

The CRISPR/Cas system forms the basis of a bacterial immune system that recognizes and destroys foreign (usually viral) DNA (Savić and Schwank, 2016). CRISPR stands for "clustered regularly interspaced short palindromic repeats," and the Cas9 protein is an RNA-guided nuclease that cleaves double-strand breaks in specific DNA sites. Therapeutic gene editing using the CRISPR/Cas9 system employs engineered versions of the Cas9 nuclease, but unlike ZFNs or TALENs, this enzyme does not use a protein-based DNA recognition domain. The Cas9 protein is guided by specific RNA constructs (guide RNAs) to target specific regions of DNA. For gene-editing applications, the DNA target sequence must be followed by a specific recognition site known as a protospacer-adjacent motif (PAM) sequence. Most PAMs contain 2 to 5 highly conserved nucleotides, and the PAM for the *Streptococcus pyogenes* Cas9, the initial protein used for gene editing, consists of NGG or NAG nucleotides (Jinek et al., 2012). Various forms of Cas9 nuclease can be combined with

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synthetic guide RNAs to produce ribonucleoprotein (RNP) constructs that can be targeted with high precision to selected DNA sites (Cox et al., 2015).

This is a rapidly evolving area of research and there are already a large number of different Cas9 variants (often derived from various organisms) that have differing PAM sequence specificity and differential (nickase) or inactive nuclease activity and that can be complexed to DNA effector molecules such as DNA methyltransferases or histone deacetylases (Malankhanova et al., 2017). There are a number of different potential mechanisms of action for *HTT* gene-directed editing using CRISPR/Cas9-type approaches, including direct excision of CAG repeats to correct the mutation and generate two wild-type *HTT* alleles; targeted inactivation of the mutant allele, leading to a hemizygous null state; or non-allele-specific targeting of the *HTT* gene that would lower total HTT levels. In addition, it is possible that CRISPR/Cas9-targeted epigenetic editing may also be an effective therapeutic approach for HD by altering *HTT* gene transcription without permanent genome modification.

Investigations of the use of CRISPR/Cas9 in HD are still in the early stages of preclinical development. Huntingtin lowering in principle was demonstrated using a non-catalytic CRISPR/Cas9 strategy that blocked *HTT* gene transcription in a human non-neuronal cell line (Heman-Ackah et al., 2016). Precise excision of the CAG repeats from the *HTT* gene has been accomplished using paired guide RNA (gRNA) constructs that target DNA flanking the CAG repeat and a modified “nickase” Cas9 approach and results in decreased mHTT expression in HD patient fibroblasts (Dabrowska et al., 2018). A similar approach using Cas9 was also successful in the Q140 transgenic mouse model of HD (Yang et al., 2017), producing selective mutant HTT reduction, attenuation of pathology, and improved motor function.

This system has also been used to selectively inactivate mutant *HTT* genes targeting unique PAM sites to SNPs associated with the CAG-expanded allele in patient-derived fibroblasts, resulting in a near-total reduction in both RNA and mutant HTT protein (Shin et al., 2016). CRISPR/Cas9 selectively inactivated mHTT expression in the brain of the BACHD mouse model using a similar approach, targeting SNPs associated with the CAG expansion (Monteys et al., 2017) and in human differentiated induced pluripotent stem (IPS) cells (Heman-Ackah et al., 2016; Xu et al., 2017). The use of CRISPR/Cas9-mediated strategies for targeted huntingtin lowering and *HTT* genome editing has significant potential for the treatment of HD. Preclinical findings to date support the feasibility of this approach for HD, but additional research is needed to bring these rapidly evolving technologies to human clinical trials.

Advantages, Disadvantages, and Safety Concerns

ZFN- and TALEN-based methods targeting the CAG expansion and using viral transduction will only need to develop a single agent for all HD gene mutation carriers and only require a single administration to provide long-term treatment that should effectively block all pathogenic pathways when treated early enough. Unfortunately, these treatments will be invasive, cannot be reversed, target only limited regions of the brain, may target other CAG repeat-containing genes, and, because of the long-term expression of exogenous proteins, have a long-term risk of inflammation from non-host repressor proteins (Agustín-Pavón et al., 2016). ZFNs and TALENs are based on protein-guided DNA binding and require special expertise and time-consuming design, assembly, selection, and validation. Designing these nucleases to be specific for one genetic sequence is a difficult and often expensive process because it requires linking together the right combination of zinc fingers in ZFNs or the right combination of amino acid repeats in TALENs. Moreover, as in RNA-targeted gene silencing, DNA target recognition by ZFNs or TALENs is imperfect and can result in significant off-target effects. ZFN and TALEN are also generally considered to be inferior to the CRISPR/Cas9 system in aspects such as design simplicity and ease of delivery (Fan et al., 2018).

There are currently very limited published data regarding CRISPR/Cas9 approaches in HD, and, apart from the aforementioned BACHD model experiments (Yang et al., 2017), most of this work is still being done in simple cellular model systems (Kolli et al., 2017). Potential limitations of CRISPR/Cas9 approaches include the need for a specific PAM adjacent to the target DNA sequence and off-target effects. Similar to concerns regarding ZFNs and TALENs, the expression of bacterial proteins in human brain might be immunogenic, there are still un-resolved issues related to delivery in the brain, and there is potential danger of prolonged nuclease expression because of the use of irreversible viral transduction systems (Fu et al., 2013). One group has directly addressed concerns regarding the risk of persistent Cas9 nuclease expression. Merienne et al. (2017) describe the KamiCas9 method, a self-inactivating Cas9 system for transient genome editing with the potential for improved safety. Although reducing long-term Cas9 expression, the lentivirus vector has the additional risk of insertional mutagenesis. This system was able to induce permanent disruption of *HTT* in an HD mouse model, demonstrating the feasibility and potential therapeutic utility of gene editing in the CNS (Merienne et al., 2017).

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Significant progress has been made; however, many factors will still need to be considered before genome editing is a viable therapeutic option for HD. A major hurdle facing all HTT-targeted modalities has been delivery of the therapeutic agent to the CNS. Genome editing approaches targeting the CAG-expanded *HTT* DNA in HD have the potential for exceptional benefit, and correcting the underlying genetic defect should halt ongoing pathogenic mechanisms in HD. This would include all alternative toxic species of mHTT and other potential pathogenic mechanisms, such as those mediated by alternative splicing (Sathasivam et al., 2013), non-RAN translation (Bañez-Coronel et al., 2015), or RNA-mediated toxicity (Rué et al., 2016). In addition, if genome editing is used to correct the genetic mutation in the germ cells, then this would eliminate intergenerational transmission of the mutant allele and remove the risk of HD in future generations.

Genome editing for human neurologic diseases is a very rapidly evolving field that is still in its infancy. Although this approach is being pursued as a potential therapy for HD with promising results from many different groups, additional development and extensive pre-clinical studies will be required before this is ready for human clinical trials.

RNA-Targeting Approaches

At the post-transcriptional level, methods that serve to modulate translation efficiency include RNAi, antisense oligonucleotides (ASOs), and small-molecule modulators of RNA processing (Figure 1). Ultimately, all of these methods trigger cleavage, enhanced degradation, or translational suppression of mutant *HTT* mRNA, leading to a reduction in the amount of mutant HTT protein that is produced.

RNAi

RNAi technologies involve the manipulation of an endogenous and evolutionarily conserved process within cells to achieve the degradation of target mRNAs within the cytosol. Within the nucleus, native non-coding microRNAs (miRNAs) are transcribed by RNA polymerase to form stem-loop structures known as primary miRNAs (pri-miRNAs). These are cleaved by the Drosha enzyme to form a precursor miRNA (pre-miRNA) with a hairpin structure, which is exported to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNA is further processed by the Dicer enzyme to create a mature miRNA complex. The complex is loaded into the RNA-induced silencing complex (RISC), where its antisense or “guide strand” targets the RNA-induced silencing complex to its complementary target mRNA. Finally, the target mRNA is cleaved by Argonaut-2 (Ago-2), leading to translational repression (Ha and Kim, 2014). Small interfering RNAs (siRNAs) are similar to miRNAs but are derived from longer regions of double-stranded RNA and do not form short hairpins. Imperfect base-pairing complementarity in the case of miRNAs leads to translational suppression of multiple mRNA targets with similar sequences, whereas siRNAs typically exhibit perfect base pair matching and cause complete translational inhibition through cleavage of a single specific mRNA target (Keiser et al., 2016).

Synthetic siRNAs and artificial miRNAs have been generated to suppress the translation of particular proteins of interest, such as HTT. RNAi effectors have a more downstream site of action than ASOs and act on mature spliced mRNA species in the cytosol. siRNAs do not cross the blood-brain barrier (BBB) and do not readily cross the plasma membrane to enter CNS cells. Liposome formulations, nanoparticles, and chemical modifications to the siRNA itself have all been utilized to enhance cellular entry (de Fougerolles, 2008). More commonly, viral vectors are used to permanently transduce cells, achieving stable expression of siRNAs that suppress HTT translation.

RNAi was first shown to lower HTT levels and improve survival in cellular models of HD (Chen et al., 2005). Early *in vivo* studies carried out by Harper et al. (2005) in HD transgenic mice were encouraging; significant reductions in mutant *HTT* mRNA levels by 55%, on average, compared with untreated mice were seen in the N171-82Q model following bilateral striatal injections of anti-HTT short hairpin (shRNA) delivered by AAV vector, resulting in improvement in motor function, as measured by rotarod performance. Similar results have been reported using the R6/1 HD mouse model, with an increased presence of striatal markers (DARPP-32) and preproenkephalin (ppENK) following treatment (Rodriguez-Lebron et al., 2005). Intraventricular delivery of a liposome-siRNA complex reduced mutant *HTT* mRNA, HTT inclusions, and brain atrophy and also delayed onset of motor symptoms and improved survival in transgenic R6/2 mice (Wang et al., 2005). Cholesterol-conjugated anti-HTT siRNA duplexes delivered into the striatum of a viral transgenic mouse model of HD reduced neuronal pathology and delayed onset of the behavioral phenotype (DiFiglia et al., 2007).

These studies highlight the potential importance of the timing of HTT-lowering therapies in the treatment of HD. All of the RNAi treatments described above were administered in pre-symptomatic mice; treatment in symptomatic transgenic HD mice (HD190QG) has also been shown to reduce HTT protein levels, reduce inclusion formation, and improve histopathology (Machida et al., 2006), but the effect (if any) on actual reversal of the disease phenotype remains to be seen. Ideally, HTT-lowering therapies will be introduced as early as possible in the disease course to prevent or delay the onset of symptoms because it is unclear from pre-clinical

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animal studies whether a complete reversal of all symptoms, when they manifest, will be possible. The availability of predictive testing for HD allows for trials in pre-manifest populations, but initial studies will still have to be performed in early symptomatic individuals.

In vivo studies in transgenic mice have all served to lower the amount of the transgenic protein, but endogenous HTT levels (both wild-type and mutant) were not affected. Targeting endogenous full-length mutant HTT while leaving wild-type HTT mRNA unaffected is known as "allele selectivity," and although desirable, has presented considerable challenges (see Allele Selectivity of Huntingtin-Lowering Approaches). An alternative strategy is to non-specifically lower both mutant and wild-type HTT levels to a degree that improves HD pathology but is also safe and well tolerated in terms of wild-type HTT loss. Experiments in fragment (Boudreau et al., 2009b) and full-length (McBride et al., 2008) mouse models of HD have shown that 40%–60% lowering of both endogenous and mutant HTT extends the lifespan and prevents motor symptoms without resulting in increased toxicity. This was also the case in rodents that were already symptomatic (Drouet et al., 2009; Stanek et al., 2014).

Partial lowering of wild-type HTT by RNAi has also been shown to be safe and tolerated in non-human primates. Bilateral putaminal injection of AAV vectors expressing artificial miRNAs were delivered to adult rhesus monkeys (McBride et al., 2011), and a 45% reduction in HTT levels did not result in any observed neuropathology or behavioral symptoms. Sustained knockdown was still evident 6 months after treatment with no ill effects (Grondin et al., 2012).

Work is underway to plan for clinical trials of miRNA therapy in HD (Table 1). A phase 2 trial of stereotactically guided intracerebral injections of AAV2-encapsulated nerve growth factor RNA in patients with Alzheimer's disease showed virally delivered gene therapy to be safe and well tolerated in principle (Rafii et al., 2018). A clinical trial, sponsored by Spark Therapeutics, of AAV1-delivered anti-HTT miRNA that has shown promise in non-human primates (NHPs) (McBride et al., 2011) is being planned to initiate in the next few years. Additionally, new research highlights that AAV miRNAs targeting HTT can be safely delivered to the non-human primate brain using a Food and Drug Administration (FDA)-approved intra-MRI delivery platform that is similar to the neurosurgical platform that can be used in human patients, increasing the predictive validity of these studies (McBride, 2018, CHDI Foundation, conference). Intracranial administration of AAV5-delivered anti-HTT miRNA into a transgenic HD minipig model reduced mHTT levels in widespread brain regions (Evers et al., 2018), and UniQure NV plans to file an investigational new drug (IND) application for this later in 2018. Voyager Therapeutics has a similar program under development for an AAV-delivered anti-HTT RNAi therapy (VY-HTT01) that has recently been shown to lower HTT mRNA in brains of non-human primates following intra-cranial delivery and was well tolerated (http://ir.voyagertherapeutics.com/phoenix.zhtml?c=254026&p=irol-newsArticle&ID=2371804).

Off-target effects are unintended consequences following administration of protein-lowering therapies. RNAi effectors have the potential to bind mRNA sequences that share sequence homology with the desired target, causing unintended downregulation of unrelated proteins (Jackson and Linsley, 2010). However, although endogenous miRNAs bind their targets with imperfect base-pairing complementarity, most artificial miRNAs are designed to have perfect homology with their target, reducing the likelihood of this occurrence.

Mammalian cells can mount an immune response against double-stranded RNA species, such as siRNAs, that are mistaken for viral byproducts. siRNAs have been shown to upregulate the expression of interferon (IFN)-stimulated genes (Sledz et al., 2003) and to activate certain Toll-like receptors (TLRs), leading to the expression of pro-inflammatory cytokines (Sioud, 2005; Hornung et al., 2005; Karikó et al., 2004). These effects can sometimes be mitigated by careful engineering to generate high complementarity, enhanced stability, and reduced immunogenicity (Godinho et al., 2015).

The endogenous RNAi pathway within cells may be overwhelmed by exogenous shRNAs that overload exportin-5 (Barik, 2006; Martin et al., 2011), and this, in turn, can lead to other to detrimental effects, such as liver toxicity (Grimm et al., 2006; Borel et al., 2011). Co-expression of recombinant exportin-5 or selection of weaker promoters for shRNAs can help with this (Grimm et al., 2006; Yi et al., 2005). Artificial miRNAs are better tolerated, and synthetic siRNAs are able to bypass the nuclear processing step altogether (Boudreau et al., 2009a; McBride et al., 2008).

ASOs

ASOs have a more upstream site of action than RNAi effectors. They are short, synthetic, single-stranded oligonucleotide analogs consisting of 16–22 bases that bind to complementary pre-mRNA targets in the nucleus through Watson-Crick base-pairing and lead to the modulation of gene expression through a number of pathways (Figure 2). One such pathway is through RNase H1 recruitment. Upon ASO binding, an RNA-DNA

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hybrid is formed that becomes a substrate for RNase H1, which degrades the target mRNA through hydrolysis. Cleavage products are then cleared through normal cellular mechanisms in the nucleus and cytoplasm.

The HTT-targeting ASO IONIS-HTT_{Rx} (RG6042) has recently been shown to lower the levels of mHTT protein in the cerebrospinal fluid (CSF) of patients with early-stage HD in a phase 1/2a clinical trial sponsored by Ionis Pharmaceuticals (Tabrizi et al., 2018). This has led to considerable hope that ASOs may lead to a viable disease-modifying therapy for HD in the near future.

The HTT-Rx (RG6042) ASO is thought to act through the RNase H1 mechanism and targets both wild-type and mutant HTT levels, leading to non-specific HTT lowering. Preclinical studies of HTT-targeting ASOs administered to symptomatic YAC128 and BACHD mouse models of HD have shown amelioration of key striatal gene expression changes and reversal or improvement of motor phenotypes (Kordasiewicz et al., 2012; Stanek et al., 2013). Phenotypic improvements were greatest when treatment was initiated earlier in the disease course and were sustained for many months following termination of treatment (Lu and Yang, 2012). Suppression of the target mRNA itself also lasted for 12 weeks after a single administration of ASO in these mice (Kordasiewicz et al., 2012). This has implications for the timing of intervention and dosing frequency that would be required for HD patients. Lumbar intrathecal infusion of a similar ASO into non-human primates has also been shown to effectively lower HTT in many brain regions targeted by HD (Kordasiewicz et al., 2012). Intrathecal delivery of the IONIS-HTT_{Rx} (RG6042) ASO via lumbar puncture into the CSF has been shown to be safe and well tolerated in HD patients, and its effects on disease modification will soon be assessed as part of a larger phase 3 trial sponsored by Roche. In addition, as shown in Table 1, allele-selective ASOs targeting single nucleotide polymorphisms in linkage to the CAG expansion have been developed (Skotte et al., 2014), and a clinical trial by Wave Life Sciences is already underway (Hersch et al., 2017). Biomarin also has an allele-selective ASO in pre-clinical development that directly targets the expanded CAG repeat (Datson et al., 2017).

Other mechanisms of ASO action depend on the location of ASO binding to target mRNA (Figure 2; Rinaldi and Wood, 2018). ASOs binding to the AUG translation start site can cause steric hindrance of the ribosomal machinery and, therefore, lead to complete translational inhibition; this would not be desirable in the case of HD because the long-term effect of complete HTT elimination in humans is unknown. ASO binding to intron-exon junctions may modulate splicing through the disruption of splice sites or recruiting or inhibiting splicing factors. This type of ASO may prove to be useful in targeting the production of the neurotoxic mutant exon 1 HTT protein generated by alternative splicing (Neueder et al., 2017). ASOs can also be chemically modified to directly cleave the target mRNA after hybridization through incorporation of intrinsic catalytic nucleic acids such as ribozymes and DNAzymes.

Since their inception, ASOs have undergone a number of chemical modifications to improve their suitability as therapeutic agents. The original phosphoribose backbone was susceptible to rapid breakdown by endonucleases. Substitution of sulfur for non-bridging oxygen atoms to generate a phosphorothioate backbone resulted in nuclease resistance and improved protein binding as well as an increased half-life. In addition, multiple alterations at the 2′ position of the ribose sugar moiety have led to improved safety and efficacy of ASOs with increased binding affinity to the target mRNA, further resistance to nucleases, and decreased immunogenicity (Rinaldi and Wood, 2018). The IONIS-HTT_{Rx} ASO incorporates both phosphorothioate and 2′-O-methoxyethyl (sugar) modifications.

Trials of ASOs in humans have shown positive outcomes in other neurodegenerative diseases, albeit in infants and with a different mechanism of action as that of the current HTT ASOs in clinical testing for HD. Dramatic improvement was observed in infants with spinal muscular atrophy (SMA) type 1 treated with lumbar intrathecal bolus injections of nusinersen (Finkel et al., 2017). This methoxyethyl (MOE)-modified ASO alters mRNA splicing of the *SMN2* gene, leading to production of functional SMN protein that is lacking in patients because of mutations of the *SMN1* gene. This trial was terminated early following interim analysis, which showed such convincing results that the drug (marketed as Spinraza) received expedited approval from the FDA for treatment of all forms of SMA (US Food and Drug Administration, 2016). Strikingly positive results were also seen in patients with later-onset SMA (Mercuri et al., 2018). It is worth noting that SMA is an aggressive disease with rapid progression; therefore, clinical improvement could be more quickly discerned in the clinical trial setting. An ASO targeting SOD1, for the treatment of SOD1-positive amyotrophic lateral sclerosis (ALS), has also been shown to be safe and well tolerated following lumbar intrathecal infusion (Miller et al., 2013); a more potent ASO (ISIS-$SOD1_{Rx}$) is therefore being taken forward into a phase 1/2a clinical trial by Ionis Pharmaceuticals in conjunction with Biogen (https://clinicaltrials.gov/ct2/show/NCT02623699).

The unintended binding of mRNA sequences with shared homology to the target is less of an issue with ASOs than for RNAi because it is possible to carefully select unique target sequences that do not appear anywhere else in the genome. ASOs can be designed to target intronic regions as well as exonic regions because they bind to

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pre-mRNA rather than mature transcripts. Thus, there is more RNA "real estate" from which to select the ideal drug candidate. ASOs also have the advantage that they do not saturate any endogenous pathways within the cell and have clear dose-dependent and reversible effects. However, repeated administration of ASOs will likely be required to maintain therapeutic benefits.

Small-Molecule Approaches for HTT Lowering

Intrathecal delivery is a relatively invasive procedure, particularly when administered repeatedly, and a small molecule that is orally bioavailable and distributed to the brain is a more attractive treatment option for HD. Phenotypic screening in HD patient-derived embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has been used to identify small molecules that modulate HTT (or mutant HTT) protein levels (Doherty, 2017, CHDI Foundation, conference). Optimizing compounds that alter pre-mRNA splicing and lower HTT levels is an approach being taken forward by PTC Therapeutics.

There is an increased risk of off-target effects because small molecules do not have the specificity afforded by Watson-Crick base-pairing that is utilized by RNAi effectors and ASOs. Small molecules that alter *SMN2* pre-mRNA splicing were identified through screening and, when applied to mouse models of severe SMA, led to improved motor function and extended lifespan (Naryshkin et al., 2014). However, when the lead compound RG7800 was tested in a phase 1b/2a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02240355), ocular complications emerged in on-going monkey studies, and the trial was terminated. Nevertheless, a phase 1 study of a second compound, RG7916, was recently completed (https://clinicaltrials.gov/ct2/show/NCT02633709), and three phase 2 studies of this drug are now underway in adults and children with different forms of SMA. Preliminary results of RG7916 in patients with SMA type 2 and 3 have shown a dose-dependent increase in SMN protein, suggesting that small-molecule splicing modifiers can, in theory, achieve target engagement similar to ASOs (PTC Therapeutics, 2017).

A potentially promising novel approach to blocking transcription of mHTT mRNA has been developed by Nuredis. Transcription through DNA regions with long expanded CAG repeats requires binding of specific transcription elongation cofactors (Liu et al., 2012). Genetic interference in this process selectively decreased mHTT but not wild-type HTT levels in HD patient lymphoblastoid cells and resulted in improved rotarod performance and extended lifespan in the R6/2 mouse model of HD (Liu et al., 2012). Small-molecule blockers of this pathway are currently in pre-clinical development.

Approaches to Target Alternative Toxic Species in HD

Exon 1 HTT protein generated through incomplete splicing of mutant HTT mRNA (Sathasivam et al., 2013) may not be lowered by current RNAi or ASO drugs being tested in the clinic, which act on sites downstream from the activated cryptic polyA signals. The effect of this on overall therapeutic benefit remains to be seen and has so far not been proven to be a significant issue in animal studies. In contrast, RNAi or ASOs that are complementary to *HTT* exon 1 mRNA may still be effective. Currently, work is ongoing to pursue biologic and small molecule approaches to target this HTT exon 1 mRNA specifically (Gillian Bates, personal communication).

Previously described toxic RAN proteins (Bañez-Coronel et al., 2015) may also not be fully targeted by RNAi, particularly RAN proteins that arise from the antisense strand. ASOs acting at the pre-mRNA level, however, would likely lead to a reduction in all RAN proteins and would also reduce the amount of potentially toxic CAG-expanded mRNA in the cytosol (Rué et al., 2016). The formation and targeting of alternative toxic species are summarized in Figure 1, and it can be seen that interventions at the level of HTT DNA would inhibit the formation of all these downstream products.

Protein Clearance Approaches to Lowering Mutant Huntingtin

In addition to therapeutic approaches that reduce mHTT levels by targeting either *HTT* DNA or RNA, there are potential small-molecule huntingtin-lowering technologies based on increasing the cellular clearance of the mHTT protein (Lin and Qin, 2013). Misfolded and defective proteins such as mHTT are cleared from neurons using two main pathways: the ubiquitin-proteasome system (UPS), which removes soluble and short-lived proteins by tagging them with ubiquitin and targeting them to the proteasome, leading to breakdown into individual amino acids, and autophagy, a process in which larger cytoplasmic structures, such as aggregated proteins and damaged organelles, are degraded within double-walled vesicle structures called autophagosomes and shuttled to the lysosome. Impairment of neuronal proteostasis, the cellular protein quality control system, has been well described in HD, and there is abundant evidence that ineffective or disrupted degradation of mHTT and other misfolded proteins by the UPS and/or autophagic pathways may contribute to HD pathogenesis and result in the accumulation of toxic forms of mHTT and intracellular aggregates of mHTT in neurons (Zhao et al., 2016).

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These therapeutic approaches aim to use the cell's own protein quality control mechanisms to increase the degradation and clearance of mHTT through modulation of the UPS and/or autophagic pathways. Many small-molecule therapeutic agents that can alter mHTT proteostasis have been assessed in both *in vitro* and *in vivo* HD models, with some modest success (Harding and Tong, 2018), but none of these agents have been brought to human clinical trials. Small-molecule huntingtin-lowering drugs that are orally bioavailable are of considerable interest because they would have simplified administration compared with other therapies we have discussed, good systemic distribution, and likely a much lower cost, making them accessible to all HD patients, including those in developing countries.

Of particular interest is a recently developed technique that allows researchers to specifically harness the UPS to target particular proteins in the cell. This approach uses proteolysis-targeting chimera proteins (PROTACs) to selectively tag specific proteins for UPS degradation, leading to decreased cellular levels of the targeted protein. PROTAC-based approaches selectively target proteins for ubiquitylation using chimeric molecules that link a targeting element that selectively binds to the protein of interest with ligands that bind cellular ubiquitin ligases. PROTAC-based approaches may allow additional specificity when the chimeric molecule uses ligands that only recruit specific ligases expressed in a certain tissue or cell type. This approach has now been applied to HD, and several small molecules have been identified that ubiquitinylate mHTT and target the toxic protein for breakdown by the proteasome. Decreased levels of mHTT in HD patient-derived fibroblasts are caused by PROTAC-induced degradation (Tomoshige et al., 2017). Additional modifications of the system increased mHTT lowering efficacy, but many issues remain to be resolved before this approach is ready for human clinical trials (Tomoshige et al., 2018). These studies provide initial pre-clinical proof of principle in HD cells for mHTT lowering using PROTAC-based approaches, but this effect has not yet been demonstrated *in vivo*, and delivery to the CNS will be an important issue to overcome. Despite these caveats, this remains a promising avenue for future therapeutic agent development.

Allele Selectivity of Huntingtin-Lowering Approaches

Approaches aimed at lowering the levels of toxic mHTT protein have substantial therapeutic potential for HD. Reversal of mHTT expression in a conditional transgenic HD mouse model, even after symptom onset, rescued neuropathological and motor phenotypes (Yamamoto et al., 2000). It is clear, based on the known pathogenesis of HD, that a neurotoxic gain-of-function mHTT is principally responsible for the pathological and clinical features of HD (Hughes and Jones, 2014), that mHTT levels modulate the HD phenotype (Graham et al., 2006), and that the degree of mHTT lowering is the key variable that will influence the clinical efficacy of HTT-lowering therapy. Total HTT-lowering approaches to HD therapy result in a reduction of both mHTT and wild-type HTT levels, and current mHTT allele-specific approaches target either the CAG triplet repeat expansion directly or SNPs linked to the causative mutation (Wild and Tabrizi, 2017).

Huntingtin is a highly conserved protein that plays an essential role in embryonic neurodevelopment, transcriptional regulation, mitochondrial function, axonal vesicular trafficking, endocytosis, and neuronal survival (Saudou and Humbert, 2016). The many identified cellular functions of wild-type HTT in development raise important theoretical risks of total HTT lowering, even in the adult brain, and the effect of partial lowering levels of total HTT on these important cellular roles in the adult human brain has yet to be fully elucidated (Liu and Zeitlin, 2017). Rare individuals who are genetically null for one *HTT* allele do not develop HD (Ambrose et al., 1994), and carriers of homozygous CAG repeat expansions develop normally, with a similar age of HD onset as heterozygotes CAG repeat expansion carriers, suggesting that HD is a fully dominant disorder and is not primarily caused by a loss of HTT function (Lee et al., 2012).

A non-coding SNP in a nuclear factor κB (NF-κB) transcription factor binding site of the *HTT* promoter reduces *HTT* transcriptional activity and has a bidirectional effect on HD age of onset. When present on the expanded allele, the transcription-lowering SNP was associated with a highly significant 9.3-year mean delay in HD age of onset. The presence of the transcription-lowering SNP on the wild-type *HTT* allele was associated with a modest 3.9-year mean earlier age of onset that was significant in one of two cohorts (Bečanović et al., 2015). These results suggest that total HTT lowering should have a net beneficial effect in HD. It is currently unclear which of the available total HTT or allele-selective mHTT-lowering ASO drugs in clinical testing is optimal because both approaches have different strengths and limitations. Ultimately, appropriately performed clinical efficacy trials will be required to answer this question.

Issues and Challenges

A significant advantage of total HTT silencing approaches compared with SNP-based allele-specific targeting is the potential to develop a single therapeutic agent for the entire HD population. In addition, these drugs can be developed to target any region in HTT, not limited to SNP associated sequences, dramatically increasing the

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likelihood of developing a highly potent and specific HTT-targeting drug. Concerns about the effects of lowering wild-type HTT in the setting of mHTT have arisen from pre-clinical studies that found worsening of HD phenotypes in the YAC128 transgenic mouse model of HD when wild-type mouse *Hdh* was genetically deleted throughout development (Van Raamsdonk et al., 2005). In these studies, mouse huntingtin was deleted throughout life, including critical developmental periods. Heterozygous inactivation of *HTT* is not associated with any detectable abnormal phenotype in humans (Ambrose et al., 1994). A 46-year-old woman with one intact *HTT* allele and one disrupted *HTT* allele and one of her children, who also carried the disruption, were phenotypically normal. These individuals showed no signs of HD, suggesting that 50% levels of total HTT throughout life were not associated with pathology. Current total HTT-lowering ASO treatments in clinical trials do not cause complete ablation of all HTT expression, targeting about 50% of normal HTT levels (Tabrizi et al., 2018), and are initiated in adulthood, avoiding the potential developmental effects of HTT lowering. In a fully humanized transgenic mouse model of HD in which both *Hdh* alleles were deleted and functionally replaced by human *HTT* transgenes expressing both mutant and wild-type HTT, the benefits of ASO-mediated lowering of total HTT levels (both mHTT and wild-type HTT [WTHTT]) by 75% were similar to that of decreasing mHTT alone, suggesting that the degree of mHTT lowering is the most critical parameter for preclinical efficacy (Southwell et al., 2018). It would appear from these studies that a reduction of total HTT of 50%–75% in the adult brain is likely to be safe and well tolerated. Decreases of mutant HTT of more than 50% are consistently associated with benefits in preclinical models, providing potential guidance for the degree of HTT lowering likely to be safe and effective in human trials of HTT-lowering therapies.

Development of SNP-based allele-specific targeting can suffer from decreased potency of the agents because of a limited number of potential target sequences around SNPs. Also, selectivity for the mutant *HTT* allele in CAG-targeting approaches is relative and often demonstrated in very large expansions but with reduced specificity for expanded CAG repeat lengths in the common HD-causing range (40–50 CAG repeats). Appropriate HD animal models should always be developed to assess allele-specific agents *in vivo* prior to going to clinical trials (Southwell et al., 2014). In addition, genotyping to phase the SNP must be highly accurate, and each SNP-based allele-selective HTT-lowering agent will only target a single SNP. It is estimated that 5 agents targeting 3 different SNPs will be required to treat a maximum of 75% of the HD population, with many individuals left untreated by this approach because of absence or homozygosity of the targeted SNPs (Pfister et al., 2009). Each agent will require independent clinical efficacy and safety trials at significant cost, with each successive trial providing only incremental increases in the numbers of treatable patients. At the current time, no CSF or plasma biomarker exists for non-expanded HTT, preventing the assessment of mHTT allele-specific approaches in human clinical samples (see Biomarkers of Target Engagement).

Both total HTT and mutant allele-selective HTT-silencing ASO-based therapies are currently in active clinical trials, and although both approaches have different strengths and weaknesses, the most advanced clinical program appeared to be safe in a short phase 1 trial, was well tolerated, and holds great promise as a potential HTT-lowering therapy for HD (Tabrizi et al., 2018). This ASO-based therapy provides partial, transient, dose-dependent, and reversible total HTT lowering and appears to be safe and well tolerated in rodents, non-human primates, and now humans (Leavitt et al., 2016; Tabrizi et al., 2018). Ultimately, the maximum degree of total HTT lowering and the safety of long-term total HTT lowering that will be safely tolerated in humans will require further investigation, but alternative total HTT-lowering approaches that are non-reversible and not truly dose-dependent or titratable (for example, those requiring viral mediated transduction) may not have a similar safety profile.

Drug Delivery and Administration

Delivery of all HTT-lowering therapies has proven to be a considerable challenge. HD is primarily a disease of the CNS (although a peripheral phenotype is well recognized), and the most striking neuronal loss is seen in the striatum. This is a deep-seated area of the brain and, therefore, is difficult for therapeutic agents to access. Another issue is that the human brain is unique in terms of its anatomy and complexity; therefore, promising results from animal studies do not necessarily translate into successful treatments for patients, as has been the case for virtually all HD therapies tested to date. The human brain is 3,000 times larger than the mouse brain, and any CNS-delivered therapies would have a completely different pattern of distribution throughout the parenchyma. Studies in large animal transgenic HD models, such as sheep and pigs, are helpful (Pouladi et al., 2013) because their brains are closer in size to those of humans. Studies in non-human primates have been most useful to study the distribution of HTT-lowering therapies following delivery (Kordasiewicz et al., 2012; Grondin et al., 2012; McBride et al., 2011) and are likely to be more reflective of the CNS distribution that could be achieved in humans. Another advantage of non-human primates is that safety studies can monitor small changes in phenotype, such as fine motor skills or chorea and dystonia, which may not be possible in sheep or pigs. Most research has been carried out in the delivery of RNAi and ASOs, as described below.

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Delivery of RNAi Therapies

Naked siRNAs do not readily cross the BBB or the plasma membrane of cells; therefore, viral vectors are most commonly used to transport siRNAs into neurons. Recombinant AAVs (rAAVs) and lentiviruses (LVs) are often used; both are non-pathogenic, cannot replicate in the host, and trigger a minimal immune response. AAVs do not integrate into the host genome but remain as nuclear episomes; however, they still result in stable gene expression at higher levels than LVs. Thus, siRNAs may not require repeated administration; a single treatment could, in theory, achieve a permanent effect. Although convenient, this is at present irreversible, and there are no antidotes.

Multiple capsid serotypes of AAVs exist, with differing cellular tropisms, allowing specific cell-directed therapy (Murlidharan et al., 2014; Kantor et al., 2014). Many patients have pre-existing antibodies to certain AAV serotypes that would neutralize the viral vector (Louis Jeune et al., 2013). It may be possible to screen patients for these antibodies or to engineer AAV capsids that evade the host's immune system. In any case, direct intra-parenchymal infusions of virally delivered RNAi are not as affected by circulating antibodies (McBride et al., 2011).

Expression of RNAi effectors in desired brain regions is generally achieved in animal models by bilateral direct injection or infusion into the striatum (Keiser et al., 2016). However, to accomplish this in humans would require stereotactic neurosurgery, which is highly invasive and carries risks such as infection, bleeding, and death. Notwithstanding any operative risk, there is limited tissue distribution following injection, necessitating multiple potential injection sites or choosing one injection site over another. The striatum would be the most obvious choice because it is the most severely affected area in HD; this is likely to confer significant benefit, but HD is a whole-brain condition and may require treatment of additional brain regions. Recent data from non-human primates have shown both striatal and cortical (although to a lesser extent) reduction of HTT mRNA following putaminal and thalamic injection of AAV miRNA (VY-HTT01), suggesting a degree of AAV transport from injected brain regions (http://ir.voyagertherapeutics.com/phoenix.zhtml?c=254026&p=irol-newsArticle&ID=2371804).

RNAi that can be delivered via peripheral administration is therefore a very appealing potential alternative. AAV serotype 9 (AAV9) crosses the BBB and transduces neurons and glia following peripheral intravenous injection (Foust et al., 2009). Transgenic HD mice treated with jugular vein injection of AAV9 expressing artificial miRNA showed reduced mHTT expression throughout the brain (including the cortex, striatum, hypothalamus, and hippocampus), with subsequent decreased inclusion formation and cortical and striatal atrophy (Dufour et al., 2014). Most recently, Deverman et al. (2016) have generated AAV variants with very high CNS tropism using a capsid selection method termed Cre recombination-based AAV-targeted evolution (CREATE). In mice, intravenous injection of the variant AAV-PHP.B transduced the majority of neurons and astrocytes across the CNS with 40-fold greater efficacy than AAV9 (Deverman et al., 2016). However, vascular infusion of AAV-PHP.B did not perform any better than AAV9 in transducing the marmoset brain (Matsuzaki et al., 2018). Overall, these newer viral vectors offer hope of non-invasive gene therapy for HD in the future, but trials using traditional viral vectors (Table 1) are imminent and will, if successful, provide proof of principle for RNAi gene therapy in HD.

Finally, patisiran, a double-stranded siRNA targeting transthyretin encapsulated in a lipid nanoparticle (LNP) for the treatment of hereditary transthyretin amyloidosis, represents the first successful approved human therapy using LNP-based delivery (Adams et al., 2018). LNPs are highly efficient in delivering nucleic acid constructs to the CNS (Cullis and Hope, 2017), and this is a very promising potential avenue for the delivery of siRNA, CRISPR/Cas9, and other gene-editing agents in HD.

Delivery of ASOs

MOE ASOs also do not cross the BBB but are soluble in artificial CSF and can therefore be delivered into the CSF through intrathecal (as is the case for the IONIS-HTT_{Rx} ASO and other clinical trials of ASOs) or intraventricular injection. Following injection, ASOs are distributed to the brain parenchyma and taken up by neuronal and glial cells. Unlike siRNAs, they do not require a viral vector and do not transduce the cell; therefore, repeated administration dependent on the specific half-life of each drug is required to maintain therapeutic levels. ASOs therefore have predictable, dose-dependent pharmacokinetics, allowing controlled titration of HTT protein lowering, although multiple repeat administrations over time are required, with the associated risks of injection into the CSF. Approaches to deliver ASOs via other routes (for example, implantable Omaya reservoirs) will need to be investigated for long-term, life-long administration.

Kordasiewicz et al. (2012) showed that intraventricular delivery of anti-HTT ASOs in rodents led to a more than 75% reduction of target *HTT* mRNA throughout the brain and spinal cord. In non-human primate brains, the

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presence of anti-HTT ASOs was observed in the spinal cord, cortex, and, to a lesser extent, the deeper brain structures following lumbar intrathecal injection (Kordasiewicz et al., 2012). The beneficial effects of HTT lowering were seen to persist for many months beyond the period of HTT lowering itself, a phenomenon dubbed the “huntingtin holiday” (Lu and Yang, 2012). The distribution of ASOs within the CNS is known to be affected by the anatomy of the intrathecal space, CSF dynamics, CSF clearance routes, and the location and volume of the injected bolus (Wolf et al., 2016). Notably, the highest tissue levels are in areas adjacent to the CSF, suggesting that passive diffusion plays an important role in drug distribution. Cardiac and respiration-associated oscillations of CSF that propel the CSF along paravascular routes may account for distribution to deeper structures. Active transport of ASOs is also likely to play a part because, even in areas with overall low ASO levels, neuronal populations containing higher levels of ASO can be found (Keiser et al., 2016).

Yucatan pigs treated with lumbar intrathecal injection of IONIS-HTT_{Rx} showed substantial brain distribution, and these animals are known to have a spinal column of similar length as humans. Non-human primates administered lumbar intrathecal bolus doses of IONIS-HTT_{Rx} showed a 50% reduction in cortical HTT, which was associated with a 15%–20% reduction in the striatum (Leavitt et al., 2016). The lowering of striatal HTT in humans is also expected to be much lower than in the cortex because of the dynamics of CSF flow; however, small reductions in striatal HTT may still lead to significant clinical benefits, and ongoing clinical trials of ASOs will inform on this. HTT lowering in the cortex is still likely to be helpful, particularly because the cortex is known to lend trophic support to the striatum (Zuccato and Cattaneo, 2007). Important preclinical work in this area was undertaken by Wang et al. (2014) and Estrada-Sánchez et al. (2015). Both of these studies in BACHD mice showed that cortical lowering of HTT alone was more beneficial that striatal lowering alone but that lowering in both compartments produced the greatest improvement. They also showed that, when full-length mhtt was genetically reduced in cortical output neurons, including those that project to the striatum, there was concomitant improvement in striatal function, suggesting that cortical HTT lowering may directly improve cortico-striatal connectivity (Estrada-Sánchez et al., 2015). These preclinical studies lend support to the current ASO therapy approaches that primarily lower HTT in the cortex and, potentially to a lesser extent, in the caudate nucleus and putamen (Leavitt et al., 2016). Post-mortem brain tissue from patients treated with nusinersen revealed the presence of the ASO in the cortex and brain stem in both neurons and glial cells. This is the first confirmation that lumbar intrathecal delivery of ASOs is distributed to the brain in humans (Finkel et al., 2016).

Medical devices—for example, implantable pumps—may one day replace the need for repeated lumbar punctures to achieve regular intrathecal dosing of ASOs. Although surgical placement of such devices would likely be required, ultimately the burden of administration for both patients and medical professionals would be reduced. Peripheral administration of ASOs through intravenous injection may also be a possibility in the future; development of a peptide-conjugated ASO has been shown to have broad peripheral and CNS distribution in a mouse model of severe SMA following systemic administration (Hammond et al., 2016).

Biomarkers of Target Engagement

The establishment of effective biomarkers to evaluate target engagement in the CNS will be critical for the efficient clinical translation of HTT-lowering therapies. Numerous potential HD pharmacodynamic biomarker candidates have been proposed for development in HD, including biofluid biomarkers; structural, functional, or biochemical imaging; electrophysiologic measures; quantitative clinical measures; and digital biomarkers (Weir et al., 2011). At the current time, only CSF biomarkers have been developed to a stage where they are useful for target engagement in HTT-lowering clinical trials (Byrne et al., 2018). The ideal biomarker of target engagement for HTT-lowering trials in HD would be a direct measure of brain mHTT levels, but this is not feasible at present without resorting to brain biopsies. The analysis of biomarkers in HD patient CSF represents the most accessible opportunity to biochemically sample the brain. Brain imaging techniques, especially mHTT-specific positron emission tomography (PET) ligands (which are under development by the CHDI Foundation) could potentially provide useful surrogates for direct measurement of brain mHTT levels, but despite considerable interest and effort in developing imaging brain biomarkers of mHTT levels, there are no well-established ligands available. Measurement of disease-relevant biomarkers is an important element of modern therapeutic trial design. Biofluid biomarkers that accurately measure mHTT levels or biomarkers that reflect neuronal damage or other disease-related processes, such as neuroinflammation, will be useful for target engagement, determining therapeutic efficacy, or following disease progression.

The CSF is a clinically accessible biofluid that is enriched for brain-derived proteins that are altered in HD (Fang et al., 2009), but soluble mHTT is present in extremely low concentrations in CSF, making detection very difficult. Meaningful quantification of CSF mHTT levels required development of a novel single-molecule counting immunoassay (Wild et al., 2015). This assay demonstrated that CSF mHTT levels from HD patients are associated with clinical severity, proximity to disease onset, diminished cognitive function, and motor

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symptoms. A second technique confirmed these clinical results and provided evidence that CSF mHTT levels reflect brain mHTT levels (Southwell et al., 2015). Reduction of brain mHTT levels in BACHD mice, either by genetic manipulation or following ASO-mediated mHTT lowering, correlated with decreased CSF mHTT levels. Additionally, this study provided evidence that mHTT is released by dying neurons, suggesting that CSF mHTT levels may reflect ongoing neurodegeneration (Southwell et al., 2015). The single-molecule counting immunoassay was recently validated according to clinical regulatory standards (Fodale et al., 2017). Together, these studies provide a basis for the use of CSF mHTT levels to assess CNS target engagement in HTT lowering studies and were critical in generating the first evidence of successful mHTT lowering in the Ionis ASO trial (Leavitt et al., 2016).

There are good data to suggest that other specific components of the neuronal cytoskeleton are released into the CSF as a result of neuronal damage and, ultimately, cleared in the blood (Zetterberg et al., 2013). Neurofilament light protein (NfL) and tau are important neuron-specific components of the neuronal cytoskeleton. Similar to mHTT, NfL and tau levels in CSF have been shown to correlate with HD stage and may reflect useful markers of neurodegeneration (Constantinescu et al., 2009; Rodrigues et al., 2016). It is of great interest in HD and other neurodegenerative diseases that plasma NfL levels appear to directly correlate with CSF levels, making this measure a potential peripheral biofluid biomarker of neuronal injury and disease progression in HD (Byrne et al., 2018). Plasma NfL also correlated with rates of brain atrophy and cognitive decline in HD patients and may predict disease onset in premanifest HD mutation carriers (Byrne et al., 2017). A recent report also demonstrated that plasma NfL predicts regional atrophy in the HD brain, suggesting that, with further development and regulatory validation, this may be a useful peripheral biomarker for future HD clinical trials (Johnson et al., 2018).

To the Future

A genome-wide association study has recently shown that genetic variants in DNA handling and repair proteins affect age of onset (AAO) in HD (Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium, 2015). This includes *FAN1*, a nuclease involved in DNA interstrand cross-link repair, in which two different SNPs have a bidirectional effect on AAO. FAN1 has since been shown to have a stabilizing effect on somatic expansion of the CAG repeat in a range of HD cell models (Goold et al., 2018). The rate of HD progression has also been shown to correlate with variants in DNA repair proteins, including in MSH3, which is a neuronally expressed DNA mismatch repair protein (Moss et al., 2017). MSH3 forms a heteromeric complex with MSH2 to form MutSβ, which recognizes insertion-deletion loops of up to 13 nucleotides and promotes somatic expansion; deletion of MSH3 is known to stabilize trinucleotide repeat tracts (Williams and Surtees, 2015). Somatic expansion has been shown to occur in the human striatum (Kennedy et al., 2003) and is known to be associated with an earlier age of disease onset (Swami et al., 2009). Therefore, MSH3 and other DNA repair proteins are promising targets for disease modification in HD (Figure 3), and lowering MSH3 (whether by ASOs, RNAi, or other means) may slow somatic expansion of the CAG repeat and delay or even prevent the onset of symptoms.

With all HTT-lowering therapies, the timing of intervention is critical; ideally, treatment must be started early, stopping the disease in its tracks before the appearance of clinical symptoms. Predictive genetic testing is available for patients at risk of HD because of a positive family history and can identify pre-manifest individuals who are otherwise fit and well. The challenge is to start treatment in this group of people just prior to disease onset, and objective biomarkers are needed to assess this. Large prospective longitudinal natural history and deep phenotyping studies have been undertaken for HD (Ross et al., 2014), and data from the TRACK-HD study suggest that pathology may be detectable on volumetric neuroimaging 10.8 years prior to symptom onset (Tabrizi et al., 2013). Our HD Young Adult Study (HD-YAS) is currently ongoing and aims to identify the earliest time point when any HD-related changes can be found in young adult gene carriers, using a combination of MRI, assessment of cognitive and emotional function, and measurement of biomarkers in the blood and CSF.

Despite the loss of brain volume seen with neuroimaging in premanifest HD patients, normal cognitive and motor task performance is maintained. This indicates the presence of active compensatory mechanisms, and an operational model for longitudinal compensation has recently been described (Gregory et al., 2018). Real-time fMRI neurofeedback training has also been shown to induce plasticity and enhance performance in HD patients and may be a useful adjunctive therapy for the treatment of HD (Papoutsi et al., 2018).

Finally, there has been enormous progress in PSC technology (including iPSC and human ESC [hESC] generation and differentiation) in recent years. In addition to providing opportunities for disease modeling and experimentation, such cells could provide a source of material for neuronal transplantation in HD patients. Previous trials of fetal striatal transplantation in HD have displayed mixed results. However, human embryonic stem cell-derived neural stem cells (hNSCs) transplanted into the striatum of HD mice (both R6/2 and Q140

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knockin) resulted in symptomatic improvement (Reidling et al., 2018). Protocols for the development of clinical-grade striatal neurons have now been published (Wu et al., 2018). There is the potential to carry out *ex vivo* genetic modification of autologous iPSC-derived cells to produce neurotrophic factors and subsequently transplant them back into the CNS of patients (Gowing et al., 2017), an approach that is being taken forward for the treatment of ALS. These cell-based therapies offer hope for patients who are already displaying symptoms of HD.

Conclusions

Worldwide collaborative efforts have led to the progression of HTT-lowering approaches from pre-clinical development to clinical application. A phase 3 clinical trial of an HTT-lowering ASO is about to begin, with trials of RNAi and ZFNs due to commence soon. Challenges of drug delivery and distribution have been extensively researched and largely overcome; intrathecal delivery of ASOs has been proven to be a viable method, and intracranial delivery of viral vectors for RNAi has demonstrated feasibility in animal models and patients.

Lessons learned from work in HD could be used to tackle other neurodegenerative diseases in which the causative mutation or toxic species is known. Recent work has opened up the potential for tau lowering as a therapy for Alzheimer's disease and other tauopathies (DeVos et al., 2017), and clinical trials of ASOs targeting *MAPT* mRNA are underway.

Objective biomarkers have been discovered that accurately track HD progression. Combined with the facility for predictive genetic testing in HD, this provides an opportunity to initiate treatment just prior to symptom onset and prevent neurodegeneration. The next few years will see further expansion in HTT-lowering therapies, and there is the tantalizing possibility of a disease-modifying therapy for HD in the near future.

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Declaration of Interests

In the past 2 years, S.J.T. has undertaken consultancy services, including advisory boards, with F. Hoffmann-La Roche Ltd., Ixitech Technologies, Shire Human Genetic Therapies, Takeda Pharmaceuticals International, and Teva Pharmaceuticals. All honoraria for these consultancies were paid to UCL, S.J.T.'s employer. Through the offices of UCL Consultants Ltd., a wholly owned subsidiary of University College London, S.J.T. has undertaken consultancy services for Alnylam Pharmaceuticals Inc., F. Hoffmann-La Roche Ltd., GSK, Heptares Therapeutics, Takeda Pharmaceuticals Ltd., Teva Pharmaceuticals, UCB Pharma S.A., University College Irvine, and Vertex Pharmaceuticals Inc. B.R.L. is on the scientific advisory board of sRNAlytics and reports scientific consultancy fees from Teva, Mitoconix, Roche, Nuredis, Lifemax, Ionis, and PTC. B.R.L.'s laboratory has obtained previous research grants from CHDI, Teva, and uniQure.

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Figure 1. Production of Potential Toxic Species in HD and Mechanisms for HTT Lowering

Yellow sections of DNA, RNA, and protein represent the pathogenic expanded CAG tract and its polyglutamine product. Pink boxes, therapeutic approaches; yellow boxes, widely accepted toxic species; gray boxes and dotted arrows, proposed mechanisms for the production of alternative toxic species; ZFP, zinc-finger protein; TALEN, transcription activator-like effector nuclease; ASO, antisense oligonucleotide; RISC, RNA-induced silencing complex; RAN, repeat-associated non-ATG; PROTACS, proteolysis-targeting chimera. Adapted from Wild and Tabrizi (2017) with permission from Elsevier.

Figure 2. Targeting Huntingtin Translation for HD Treatment

(A) ASOs bind to form an RNA-DNA hybrid that triggers RNase H1-induced degradation of the target HTT pre-mRNA.

(B) ASOs binding to the AUG translation start site of HTT mRNA may cause steric hindrance of the ribosomal machinery and translational arrest.

(C) ASOs binding to intron-exon junctions modulate splicing and could potentially inhibit the formation of alternatively spliced *HTT* exon 1 mRNA.

(D) Cleavage of the HTT mRNA is carried out by siRNAs as part of the RNA-induced silencing complex. In addition, ASOs incorporating ribozymes or DNAzymes could also directly cleave the target *HTT* mRNA after hybridization.

(E) ASOs binding to upstream open reading frames (uORFs) increase the amount of protein translated from the downstream ORF; in theory, this could increase the amount of neuroprotective proteins such as brain-derived neurotrophic factor (BDNF).

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Figure 3. Identification of Novel Therapeutic Targets in HD

Genetic modifiers of HD were identified through genome-wide association studies, suggesting a key role for proteins of the DNA repair pathway, including MSH3. Defects in DNA repair lead to somatic instability of the CAG repeat, which is known to occur in HD. Targeting MSH3 and other DNA repair proteins may open up new therapeutic avenues in HD. DNA mismatch repair proteins: MSH3, MutS homolog 3; MSH2, MutS homolog 2; MutSβ, complex formed by MSH3 and MSH2; MLH1, MutL homolog 1; PMS2, Mismatch repair endonuclease.

Table 1. Huntingtin-Lowering Programs Targeting DNA and RNA

Sponsor	Stage	Delivery	Allele Selectivity	Advantages	Disadvantages	**References**
DNA-Targeting Approaches						
Zinc-Finger Transcription Factor						
Shire and Sangamo	preclinical	intracranial (AAV)	CAG repeat	single drug for all carriers of the HD mutation; single administration to provide long-term treatment; targeting transcription should ameliorate all pathogenic pathways	invasive; cannot be deactivated; small treatment volumes; risk of inflammation from non-host repressor proteins	Zeitler et al., 2014
Imperial College London	preclinical	intracranial (AAV)	CAG repeat	as above, plus use of host species proteins reduces inflammatory effects	use of human proteins in clinical candidate compound might limit utility of animal work	Garriga-Canut et al., 2012; Agustín-Pavón et al., 2016
CRISPR/Cas9						
Harvard University University of Pennsylvania	preclinical	direct to fibroblasts	SNP-targeted	permanent removal of genetic cause; highly specific and targeted	very early work in model systems only; irreversible, ethical concerns for germline alteration, delivery problems as with other virally delivered approaches, immunogenicity of bacterial proteins	Shin et al., 2016; Monteys et al., 2017
Emory University	preclinical	intracranial	nonselective HTT depletion by polyglutamine domain deletion	as above	as above	Yang et al., 2017
RNA-Targeting Approaches						
ASOs						
Ionis Pharmaceuticals	phase 1/2a completed	intrathecal	none	single drug for all HD mutation carriers	potential risk from reducing wild-type HTT; repeated administration	Bennett and Swayze, 2010; Kordasiewicz et al., 2012; Tabrizi et al., 2018
Wave Life Sciences	phase 1b/2a	intrathecal	SNP-targeted	selective silencing of mutant allele	several drugs required to treat majority of patients SNP-targeting limits choice of RNA binding sequences, increasing risk of side effects; repeated administration	Butler et al., 2015; Hersch et al., 2017

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Biomarin	pre-clinical	intrathecal	CAG repeat	selective silencing of mutant allele with a single drug for all mutation carriers	will reduce expression of other important CAG-containing genes, risking off-target effects; repeated administration	Datson et al., 2017
RNAi						
Spark	pre-clinical	intracranial (AAV1)	none	single treatment provides sustained HTT reduction	invasive delivery limited treatment volume cannot reverse if adverse events occur	Harper et al., 2005; Franich et al., 2008; McBride et al., 2011
Voyager	pre-clinical (plan to launch phase 1 in 2019)	intracranial (AAV)	none	as above	as above	Stanek, 2015, CHDI Foundation, conference
UniQure NV	pre-clinical (plan to launch phase 1 in 2019)	intracranial (AAV5)	none	as above	as above	Miniarikova et al., 2016, Evers et al., 2018
Small-Molecule Splicing Modulators						
PTC Therapeutics	preclinical	oral	unknown	highly accessible route of delivery potentially readily reversible	more difficult to achieve selectivity for HTT over other genes with non-nucleotide therapeutic	Doherty, 2017, CHDI Foundation, conference

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