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The prospects of CRISPR-based genome engineering in the treatment of neurodegenerative disorders

Jun Wan Shin and Jong-Min Lee

***Abstract*:** Over the past few decades, as gene discovery methods and sequencing technologies have evolved, many genetic variations that significantly increase the risk of or cause neurodegenerative diseases have been identified. However, knowledge of those pathogenic mutations and subsequent mechanism-focused studies has rarely yielded effective treatments, warranting alternative strategies for refining rational therapeutic targets. Nevertheless, with the evolution of gene targeting methods, it has been increasingly recognized that the disease-causing gene itself is the best therapeutic target even when we do not have a full understanding of its biological functions. Considering this, CRISPR/Cas gene editing technology offers the promise of permanently silencing or correcting the disease-causing mutations, potentially overcoming key limitations of RNA-targeting approaches. The versatile CRISPR/Cas-based strategies have the potential to become treatment options for challenging disorders such as neurodegenerative diseases. Here, we summarize recent reports of preclinical applications of CRISPR/Cas in models of neurodegenerative disorders to provide perspectives on therapeutic gene editing for diseases of the nervous system.

Keywords: allele-specific, CRISPR/Cas, gRNA, neurodegenerative disease, PAM, precision medicine

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Introduction

Neurodegenerative diseases collectively represent disease conditions involving loss of neurons in the nervous systems. Progressive neurodegeneration processes affect movement, balancing, breathing, cognition and behavior. Thus, neurodegenerative diseases gradually increase one's dependency over time, resulting in devastation for affected individuals and their family members. Despite numerous implicated underlying mechanisms and even known causal genetic mutations,[1–5] most neurodegenerative diseases have been defying the development of effective treatments, reflecting the difficulty in defining rational drug targets through mechanism-focused approaches. However, recent development of site-specific gene editing technologies such as CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated systems) offers new hope for the development of effective treatments for neurodegenerative diseases with known causative mutations.

In the literature, applications of CRISPR/Cas focused on the investigation of gene function, disease modeling and preclinical studies.[6–13] Of note, preclinical investigation or clinical use of CRISPR/Cas for disease treatment may offer several advantages over RNA-lowering approaches.[14] For example, CRISPR/Cas strategies can overcome key limitations of RNAi and/or antisense oligonucleotide (ASO) methods, such as a potentially high off-target activity[14] and requirement of repeated treatments,[15] which may increase the risk of complications in individuals compromised by chronic progressive neurodegenerative diseases. By contrast, most CRISPR/Cas approaches generate irreversible changes (e.g. inactivation or

Correspondence to:
Jong-Min Lee
Molecular Neurogenetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, 185 Cambridge Street, Boston, MA 02114, USA; Department of Neurology, Harvard Medical School, Boston, MA 02115, USA; Medical and Population Genetics Program, The Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
jlee51@mgh.harvard.edu

Jun Wan Shin
Molecular Neurogenetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA
Department of Neurology, Harvard Medical School, Boston, MA, USA

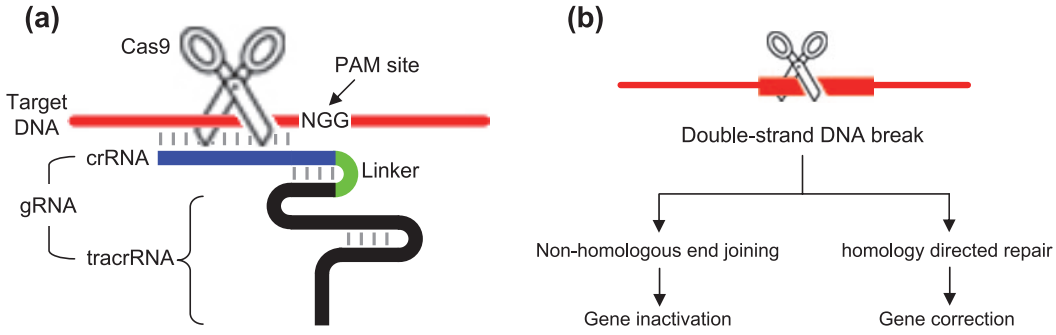

Figure 1. Components and mechanisms of CRISPR/Cas-mediated gene editing.
(a) CRISPR/Cas gene editing requires target DNA (red horizontal line), gRNA (crRNA and tracrRNA fusion), Cas endonuclease (scissors) and a PAM site. Cas9 and NGG PAM site of *S. pyogenes* are shown in this illustration.
(b) A double-strand break induced by CRISP/Cas is processed by two distinct pathways. Non-homologous end joining and homology-directed repair lead to gene inactivation and correction, respectively.

correction) in the DNA, and therefore, if a cell is correctly targeted, the cell does not need repeated treatments. In addition, demonstration of the feasibility of genome editing in post-mitotic neurons and the mammalian brain[7,12] support the potential of CRISPR/Cas strategies as a therapeutic route for neurodegenerative disorders. Also, CRISPR/Cas is highly flexible and thus applicable to neurodegenerative diseases due to loss-of-function mutations, providing advantages over RNA-lowering methods, which is therapeutically meaningful to gain-of-function mutations. In contrast to other gene editing techniques, site-specificity for CRISPR/Cas is mediated by an interaction between guide RNA (gRNA) and target DNA. Thus, protein engineering is not required for CRISPR/Cas, making this approach highly feasible and affordable.[16,17]

The field has quickly applied various CRISPR/Cas strategies to models of neurodegenerative diseases, and demonstrated therapeutic potential in preclinical studies. CRISPR/Cas approaches for neurodegenerative diseases can be broadly grouped into (1) correction of disease-causing mutations; (2) inactivation of gain-of-function mutations; and (3) modulation of transcription. In this review article, we summarize recent applications of CRISPR/Cas9 to neurodegenerative diseases, focusing on describing intervention strategies based on CRISPR/Cas gene editing.

Background on the CRISPR/Cas gene editing system

The CRISPR/Cas system is a bacterial defense mechanism that inactivates foreign genetic material.[16–20] However, its recent modification, fusing CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) into a chimeric single gRNA (Figure 1(a))[21,22] has facilitated site-specific gene editing in mammalian cells, leading to widespread applications in biomedical research. Among three types of CRISPR, the type II system (i.e. CRISPR/Cas9 from *Streptococcus pyogenes*) is the most commonly utilized in the laboratories. In type II, the CRISPR locus is transcribed and processed, resulting in crRNA–tracrRNA dsRNA (double-stranded RNA) formation to direct Cas nuclease to the target sequence[16,17] and to generate double-strand breaks (DSBs).[21,23,24] DSBs are repaired by non-homologous end joining (NHEJ) repair mechanisms or homology-directed repair (HDR).[25] Although canonical NHEJ appears to result in conservative DSB repair,[26] NHEJ repair involved in CRISPR/Cas usually leads to frameshift mutations and thus is likely to produce downstream premature stop codons, resulting in inactivation of the target gene through a nonsense-mediated decay pathway[16,17] (Figure 1(b)). In the following sections, we describe how CRISPR/Cas strategies have been used to address various genetic mutations that cause neurodegenerative diseases.

Correction of disease-causing DNA repeats and single-nucleotide variants

Many forms of neurodegenerative diseases are due to genetic mutations,[1–5,27] and attempts have been made to correct disease-causing mutations using CRISPR/Cas strategies in various model systems.

Huntington's disease

Many neurodegenerative diseases [e.g. Huntington's disease (HD), spinocerebellar ataxias] are caused by expansions of unstable

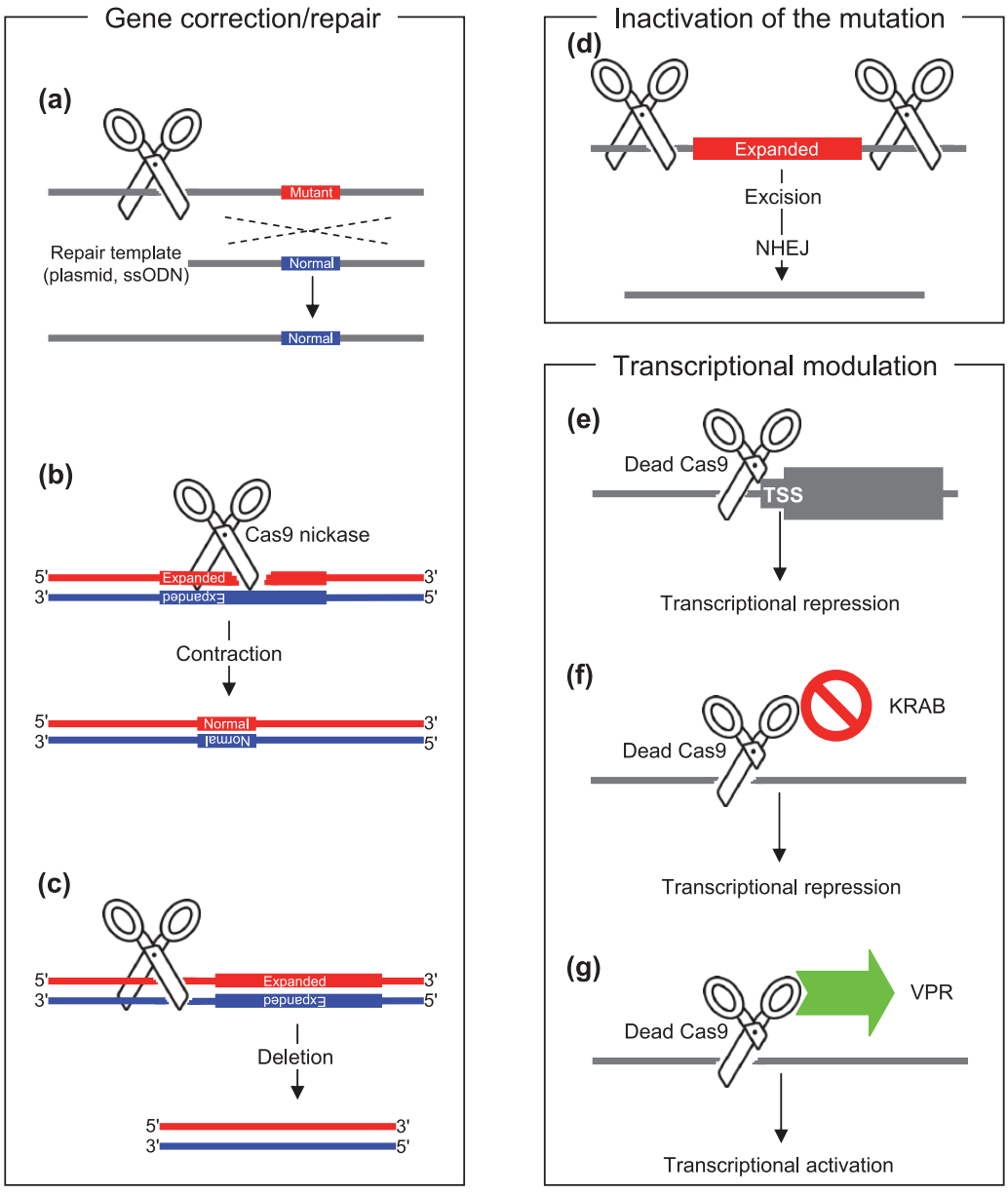

Figure 2. CRISPR/Cas strategies for neurodegenerative diseases.
Broadly, three CRISPR/Cas strategies were applied to model systems of neurodegenerative diseases: gene correction; inactivation of mutation; and transcriptional modulation. Depending on the objective of the gene editing, Cas9 endonuclease ([a], [c], [d]), Cas9 nickase (b), or dead Cas9 ([e], [f], [g]) were used to correct the genetic defects ([a], [b], [c]), inactivate the gain-of-function mutation ([d]), or modulate transcription of disease-related genes ([e], [f], [g]).

nucleotide repeats in certain genes.[28] Reducing the sizes of disease-causing expanded repeats using CRISPR/Cas is therefore hypothesized to ameliorate associated disease pathogenesis. HD is a dominantly inherited neurodegenerative disease, caused by an expansion of CAG trinucleotide repeat in the first exon of huntingtin gene (*HTT*).[3,29] *HTT* CAG repeat is highly polymorphic in the normal population[30]; once their lengths become greater than 35, various characteristic neurological symptoms occur.[29] The first attempt to reduce the size of the disease-generating CAG repeat in induced pluripotent stem cells (iPSCs) derived from an individual with HD (carrying 72 and 19 CAGs) was based on homologous recombination using a repair template of bacterial artificial chromosome (BAC) containing the entire *HTT* with a normal CAG repeat (21 CAGs).[31] Expression profiling analysis and apoptosis assays showed that genetically corrected iPSC clonal lines

showed normalization of various cellular pathogenic signaling pathways (e.g. cadherin, TGF-β, BDNF) and disease phenotypes (e.g. susceptibility to cell death),[31] supporting its therapeutic benefits. Subsequently, CRISPR/Cas9 was used to improve the efficiency of homologous recombination (Figure 2(a)) when making isogenic allelic series of HD cell models.[32] More recently, an expanded *HTT* CAG repeat in HD patient-derived iPSC line was corrected using CRISPR/Cas9 in combination with a *piggyBac* transposon-based selection system through HDR[33]; neural rosette formation deficit and increased cell death following growth factor withdrawal were reversed in corrected HD iPSC lines.[34] Contracting expanded CAG/CTG repeats using CRISPR/Cas9 D10A nickase without using a repair template was also reported. DSBs generated by CRISPR/Cas9 directly targeting the repeat sequence induced both expansion and contraction of the repeat. However, when single-strand breaks were produced by Cas9 D10A nickase, the repeat in the reporter vector tended to contract due to the activation of distinct DNA repair mechanisms (Figure 2(b)).[35] CRISPR/Cas approaches directly targeting disease-causing repeats have to be thoroughly evaluated for their target gene specificities to avoid permanent modification of other repeat-containing genes.

Amyotrophic lateral sclerosis/frontotemporal dementia

An expansion of GGGGCC hexanucleotide (G4C2) repeats in the *C9orf72* gene is the most common cause of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[27,36] An expanded G4C2 repeat in an iPSC line from a subject with *C9orf72* mutation was corrected via homologous recombination using CRISPR/Cas9 and a plasmid DNA donor template (Figure 2(a)); motor neurons differentiated from gene-corrected iPSC lines showed amelioration of disease-associated cellular phenotypes such as abnormal protein aggregation and stress granule formation.[37] Applications of CRISPR/Cas to correct pathogenic single-nucleotide variants causing neurodegenerative diseases (Figure 2(a)) are also described in the literature. Mutations in several genes including *SOD1* and *FUS* are found in familial cases of ALS,[4,38–40] and targeted gene correction methods using CRISPR/Cas9 were able to convert pathogenic alleles such as *SOD* A272C and *FUS* G1566A into non-pathogenic normal alleles in iPSCs. The repair template was provided by either (1) a donor plasmid containing wild-type allele and homology arm or (2) a linear single-stranded oligodeoxynucleotide (ssODN).[41] Corrected iPSC clones maintained pluripotency markers and differentiation capability.

Frontotemporal dementia and Alzheimer disease

Similar CRISPR/Cas-mediated correction approaches (Figure 2(a)) were applied to cell models of FTD and Alzheimer's disease in an effort to generate an isogenic cell panel of disease models. Briefly, R406W and P301L mutations in microtubule associated protein tau (*MAPT)* were corrected using ssODN-mediated CRISPR/Cas9 in iPSCs.[42–44] Similarly, A79V and L150P mutations in presenilin 1 (*PSEN1*) were corrected using CRISPR/Cas and ssODN repair templates.[45,46]

Fragile X-associated tremor ataxia syndrome

A neurodegenerative disease, Fragile X-associated tremor ataxia syndrome (FXTAS) is caused by an expansion of CGG repeat in the 5′-untranslated region of the fragile X mental retardation gene (*FMR1*). The size of expanded repeats determines the type of disease; a full mutation (>200 repeats) and pre-mutation (55–200 repeats) result in Fragile X syndrome (FXS) and FXTAS, respectively.[47–50] Application of a CRISPR/Cas9 strategy aiming at targeting the end of the upstream sequence of the CGG repeat of *FMR1* could cause large deletions of abnormally expanded repeats (Figure 2(c)), and reverse *FMR1* methylation in patient-derived iPSC and pluripotent stem cells.[51]

Inactivation of gain-of-function mutation

Allele-specific CRISPR/Cas approaches to (selectively) inactivate the disease allele (Figure 2(d)) have been tested in model systems of a dominantly inherited neurodegenerative disorder.

Huntington's disease

HD results from a CAG expansion mutation in *HTT* through gain-of-function mechanisms. Complete lack of *Htt* leads to embryo lethality in mice,[52–54] and compound heterozygous nullifying variants in *HTT* are associated with a neurodevelopmental disorder in humans.[55] Whether or not huntingtin is dispensable in the adult brain is

controversial.[56,57] However, loss of one copy of the gene due to balanced translocation does not cause HD,[58] supporting therapeutic CRISPR/Cas gene silencing. These reinforce that therapeutic CRISPR/Cas silencing strategy for HD needs to be mutant allele-specific to avoid adverse outcomes due to collateral silencing of the normal allele. Recently, mutant allele-specific CRISPR/Cas strategies using SNPs that create or eliminate PAM sites were developed.[59] By mapping all CRISPR PAM sites on *HTT* gene haplotypes and comparing haplotypes in a pair-wise manner, the mutant *HTT* gene haplotype-specific CRISPR PAM sites for a given diplotype were identified. In a proof-of-principle experiment, two mutant allele-specific gRNAs whose targets encompass (1) important regions for gene transcription including promoter region and transcription start site and (2) the expanded CAG repeat in the first exon, were used to selectively inactivate the mutant allele. The simultaneous use of two mutant allele-specific gRNAs eliminated an approximately 44 Kb DNA segment including the promoter region, transcription start site and expanded CAG repeats from the mutant allele without affecting the normal *HTT* allele in patient-derived fibroblast, iPS and NPC cells. This excision ultimately prevented the generation of mutant *HTT* mRNA and protein, indicating complete and permanent inactivation of the HD chromosome.

Subsequently, a similar gene silencing approach to inactivate the mutant allele by excising the first exon of *HTT* in an allele-specific manner was reported.[60] Using one allele-specific and one non-allele-specific gRNA simultaneously, the first exon of *Htt* including the CAG expansion mutation was excised, leading to reduced expression levels of the mutant *Htt* in a population of cells. Although moderate, the normal *HTT* expression levels were also reduced by a dual gRNA CRISPR approach possibly due to the interference of transcription of normal allele by non-allele-specific gRNA. Nevertheless, the delivery of dual gRNAs and Cas9 using a rAAV shuttle vector system into BACHD transgenic mice significantly lowered the mutant *HTT* mRNA levels, demonstrating the effectiveness of CRISPR/Cas *in vivo* for the first time.

Novel concepts such as (1) allele specificity using PAM-altering SNPs, (2) targeting the haplotype carrying the mutation, rather than the mutation itself and (3) preventing transcription of the mutant allele to inactivate the gene were integrated to permanently silence the mutant gene in a completely allele-specific manner in HD. Although strategies of transcription prevention by CRISPR/Cas may be less efficient compared to single gRNA CRISPR/Cas approaches because of (1) excision of a rather larger region and (2) requirement of two gRNAs, haplotype-targeting CRISPR/Cas silencing strategies are broadly applicable to other dominant diseases.

Transcriptional modulation of neurodegenerative disease-related genes

Targeting specific regions of a gene using (modified) CRISPR/Cas systems permits modulation of expression levels of genes that cause or are associated with neurodegenerative diseases.

Huntington's disease

It has been recently demonstrated that the expression levels of *HTT* could be lowered by CRISPR/Cas approaches using a single gRNA, targeting non-coding regions of the gene.[61] In mesenchymal stem cells extracted from the bone marrow of the YAC128 HD mouse model,[62] single gRNA-mediated CRISPR/Cas9 DNA editing at the 5′ untranslated region (UTR) or exon1–intron1 junction of *HTT* resulted in reduced *HTT* mRNA and protein expression levels.[61] A significant reduction of *HTT* mRNA expression levels was also achieved by targeting the transcription start site of *HTT* using a single gRNA and dead Cas9 (dCas9) (Figure 2(e)).[63] If genetic variations that permit allele-specific CRISPR/Cas9 targeting are available in the region important for transcriptional and/or translational regulation of *HTT*, this approach may provide therapeutic benefits without producing significant side effects in HD.

Parkinson's disease and Alzheimer's disease

In addition, a mutant form of Cas9 and various fusions were used to modulate the expression levels of neurodegenerative disease-related genes. The use of dCas9 and a gRNA targeting the transcription start sites significantly reduced the expression levels of disease-associated genes such as *SNCA, MAPT* and *APP*.[63] Also, mRNA and protein expression levels of *SNCA* could be precisely up- and downregulated in the human iPSC-derived neurons by CRISPR/Cas9 systems using dCas9-KRAB (Figure 2(f)) and dCas9-VPR effector domain fusion (Figure 2(g)).[63]

Challenges and opportunities

Various CRISPR/Cas strategies tested in the model systems of neurodegenerative disease demonstrated good target engagement and amelioration of disease-associated cellular phenotypes, supporting their therapeutic potential in clinical trials. Still, most reported CRISPR/Cas strategies in the scientific literature are not immediately applicable for treating humans with neurodegenerative diseases. Various technical hurdles and biological questions need to be addressed before therapeutic use of CRISPR/Cas in humans.

Off-targeting

Most CRISPR/Cas approaches aim at generating permanent changes in DNA, and therefore off-targeting is one of the major concerns of clinical applications of CRISPR/Cas. Cas9 requires extensive homology between sequences of target DNA and gRNA for cleavage.[17,64] Still, Cas9 can bind with off-targets with mismatches,[64] and such transient binding may result in variable off-target activities.[65] Thus, levels of off-targeting may vary widely depending on the CRISPR/Cas strategies, and it may be very difficult to develop ones without any off-target activity. However, levels of off-target activities of CRISPR/Cas can be significantly reduced by using (1) optimized gRNA design methods,[65] (2) truncated gRNA,[66] (3) Cas9 with increased fidelity,[67,68] (4) Cas9 nickase mutant,[69,70] (5) low levels of Cas9[23] and (6) controllable Cas9.[71,72] Combinatorial approaches may further lower off-target activity of CRISPR/Cas to the extent that therapeutic benefits exceed off-targeting-mediated side effects.

Delivery

The most promising CRISPR/Cas delivery vector for neurodegenerative disease is adeno-associated viruses (AAVs), as they are relatively safe and efficient in generating a gene of interest, without genome integration.[73,74] Due to their limited transgene capacities, smaller endonucleases such as saCas9[75] or Cpf1[76–78] may be better suited for AAV vectors. Nevertheless, dual AAV vector systems for spCas9 and gRNA efficiently generated DNA modification in mouse brains.[12,60] However, prolonged expression of high levels of Cas9 may lead to increased levels of off-target activity.[23] Alternatively, Cas9 protein may be delivered for efficient DNA targeting with minimal off-targeting in the context of protein therapy.[79,80]

Homology-directed repair versus non-homologous end joining

CRISPR/Cas strategies for genetic correction through HDR are believed to be less efficient, and may instead induce NHEJ, potentially resulting in knocking-out the target gene. In the worst-case scenario, CRISPR/Cas strategies aimed at correcting the mutant allele may inactivate the normal allele. In preclinical studies, screening assays were performed to select genetically corrected cell clones for subsequent molecular characterization. Since selection is not desirable in therapeutic applications of CRISPR/Cas, development of genome editing strategies that induce high-fidelity HDR with low NHEJ is critical.[23,81] The use of mutant allele-specific CRISPR/Cas strategies designed for HDR of the mutant allele will also contribute to minimizing the levels of NHEJ of the normal allele.

Requirement of personalization for allele-specific CRISPR/Cas

Allele-specific CRISPR/Cas strategies targeting genetic variations that are linked to the mutations require customized designs because each patient may have different combinations of mutant and normal allele haplotypes. Considering the cost associated with clinical trials, personalized allele-specific CRISPR/Cas strategies may sound unrealistic. However, certain disease subjects share the founder mutation,[82] and therefore alleles on the ancestral haplotype with low allele frequency in the normal population may allow a single mutant allele-specific CRISPR/Cas gene editing to be applicable to most patients who share the founder mutation. In addition, when distributions of haplotypes of mutant allele and normal allele are quite distinct (e.g. HD),[83] a set of a small number of mutant allele-specific CRISPR/Cas strategies may be developed for the majority of patients. Identification of broadly applicable allele-specific target sites will facilitate the efficacy testing of CRISPR/Cas strategies in humans.

Limitations of transcriptional modulation

The use of dead Cas9 would generate temporary effects due to the lack of DNA modification, and therefore it remains to be investigated whether CRISPR-mediated transcription modulation strategies using dCas9 (CRISPRi) provide advantages over conventional RNA-lowering approaches such as ASO. Also, the likelihood of allele-specific

application would be variable, depending on the availability of human genetic variations near transcription start sites of target genes. Still, transcriptional activation using dCas9–VPR fusion indicates versatility of CRISPR approaches and supports its utility in treating human diseases due to haploinsufficiency.

Treatment window

Since the CRISPR field is actively developing highly efficient and gene-selective CRISPR genome editing methodologies and delivery mechanisms,[12,67,68,72,84,85] many of the technical problems including off-targeting are expected to be solved in the future. With a promising forecast for technical aspects of CRISPR/Cas, integration of allele-specific strategies with efficient delivery methods is likely to open routes for intervening in neurodegenerative diseases in humans. However, important biological questions of neurodegenerative disorders still remain to be addressed. Considering the nature of the disease, primary objectives of treatments for neurodegenerative disease are to prevent the loss of neurons and their functions, and therefore maximum therapeutic benefits may be achieved when the treatments are applied reasonably early. Symptomatic interventions were able to attenuate related pathological markers and behavioral phenotypes in animal models of neurodegenerative diseases, but neuronal loss was not reverted.[86–89] In support of stage-dependent therapeutic efficacy, disease was completely and partially reversed when the production of mutant ataxin-1 was halted at an early stage and at a later stage, respectively, in a model system of spinocerebellar ataxia type 1 (SCA1).[90] When it comes to CRISPR/Cas therapeutics for chronic and progressive diseases of the nervous system, it is particularly important to choose an optimal treatment window to obtain maximum therapeutic benefits without generating unwanted side effects. For example, if CRISPR/Cas treatments are delivered too late, damaged neurons may not be able to restore their cellular integrity and functions; if gene silencing CRISPR/Cas treatments are applied too early, unwanted adverse consequences may arise due to unexpected compromised gene functions. Despite its importance, the optimal treatment window for each neurodegenerative disease is unclear due to the lack of preclinical CRISPR/Cas experiments on relevant animal models. Thus, generation of genetically faithful animal models with relevant human genetic variations that allow tests for allele-specific CRISPR/Cas strategies, and subsequent time-series analysis, will provide valuable insights into temporal aspects of CRISPR/Cas therapeutics, facilitating the clinical application of powerful CRISPR/Cas genome engineering technology in treating neurodegenerative diseases.

Conclusions

The field has witnessed promises and limitations of CRISPR/Cas as a therapeutic means for challenging health issues such as neurodegenerative disorders. Technical barriers and safety issues certainly must be solved before applying CRISPR/Cas strategies to treat human diseases. Nevertheless, conceptual foundations and key components necessary for the application of CRISPR/Cas to neurodegenerative diseases have been addressed in preclinical studies, and the potential of integrative approaches was also demonstrated. Now, personal genomics becomes more affordable than ever, facilitating the discovery of disease-causing genes and providing knowledge of targets for CRISPR/Cas. Conversely, development of broadly applicable and efficient therapeutic CRISPR/Cas genome engineering tools will permit targeting the root cause of the disease, facilitating genetic tests and the identification of disease-producing mutations through personal genomics. The synergistic interaction between two disciplines will eventually support widespread applications of CRISPR/Cas in precision medicine for neurodegenerative diseases and more, significantly contributing to understanding diseases and improving human health.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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