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REVIEW ARTICLE

CRISPR/Cas9 system and its applications in nervous system diseases

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Abstract The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is an acquired immune system of many bacteria and archaea, comprising *CRISPR* loci, *Cas* genes, and its associated proteins. This system can recognize exogenous DNA and utilize the Cas9 protein's nuclease activity to break DNA double-strand and to achieve base insertion or deletion by subsequent DNA repair. In recent years, multiple laboratory and clinical studies have revealed the therapeutic role of the CRISPR/Cas9 system in neurological diseases. This article reviews the CRISPR/Cas9-mediated gene editing technology and its potential for clinical application against neurological diseases.

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Introduction

CRISPR/Cas9 system belongs to the third generation of gene editing technology after zinc finger nucleases, transcription activation-like effect nucleases, and other techniques.[1] CRISPR/Cas9 become the focus of current research with its simple and efficient features. This system allows DNA double-strand to break at corresponding targets by the nuclease activity of Cas9 proteins.[2] Multiple gene editing techniques have been developed based on this principle, including gene knock-out, gene knock-in, transcriptional regulation, and base editing.[3–6] The basic principle of the CRISPR/Cas9 system is to identify the target gene by an artificially designed single guide RNA (sgRNA). The sgRNA guides the cleavage of DNA double strands by Cas9 and forms double-strand breaks (DSB) at the target site, after the DNA damage spontaneous repair occurs, during which gene knock-out or knock-in can be achieved.[7]

Nervous system diseases have complex etiology, are relatively difficult to treat, and seriously affect patients' quality of life. WHO predicts that by 2050, neurodegenerative diseases will replace cancer as the leading cause of death in humans.[8] The CRISPR/Cas9 gene-editing technology can effectively treat neurological diseases, as confirmed by recent studies demonstrating the therapeutic value of the CRISPR/Cas9 system in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), glioblastoma (GBM), epilepsy, amyotrophic lateral sclerosis (ALS), *etc.*[9–14]

This review expounds on the mechanism of the CRISPR/Cas9 system and its mediated gene editing technology (Fig. 1). It summarizes its research progress associated with the pathogenesis and treatment of nervous system diseases and will provide new ideas for its prevention and treatment (Table 1).

Structure of CRISPR/Cas9 system

CRISPR gene

The *CRISPR* gene is a repetitive sequence in the prokaryotic genome that is distributed in 40% of sequenced bacteria and 90% of sequenced archaea.[15] *CRISPR* gene sequence mainly comprises leader, repeat, and spacer.[16–18] The leader sequence is located upstream of the *CRISPR* gene, is relatively simple with high AT content, and acts as a promoter. The repeats are a palindromic sequence of about 20—50 bp long. The transcripts of the repeats form hairpin-like structures essential for maintaining RNA structure and functional integrity. The spacer sequence is an exogenous DNA sequence captured by bacteria, making it the product

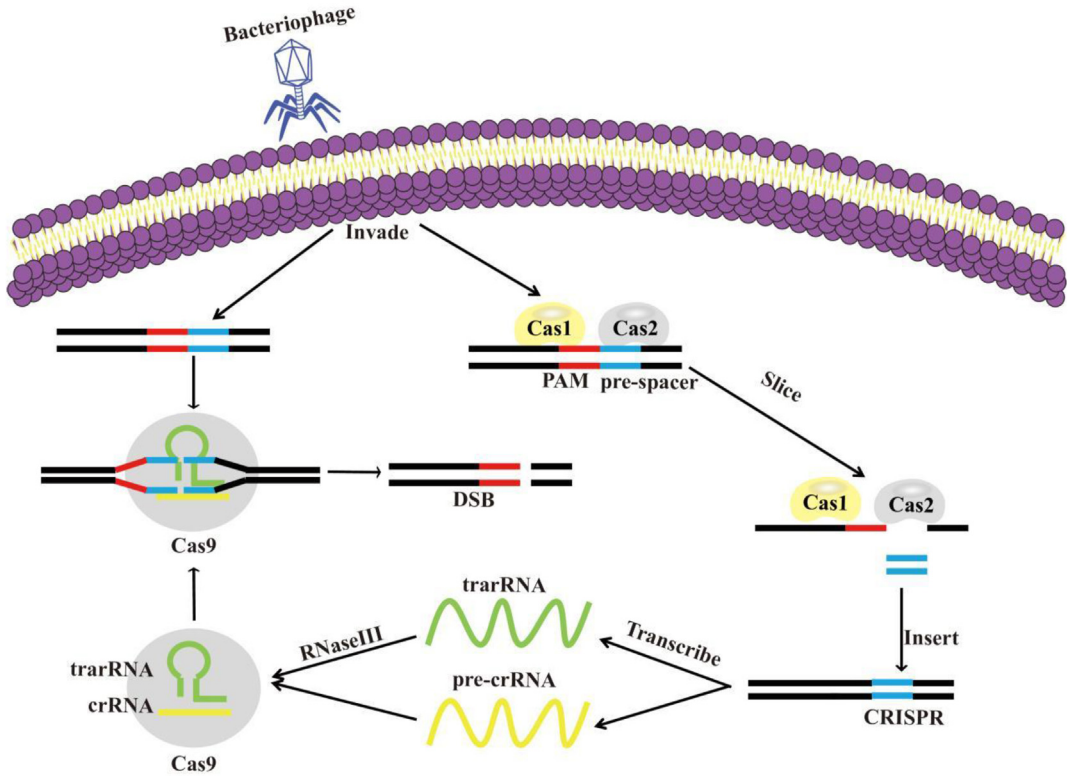

Figure 1 Mechanism of action of the CRISPR/Cas9 system. The process of the CRISPR/Cas9 system can be summarized in three stages: adaptation, expression, and interference. In the adaptation phase, bacteria capture the invading exogenous DNA as a pre-spacer sequence. Then, at the expression stage, bacteria produce crRNA-tracrRNA-Cas9 triplet complex by transcription. Finally, during the interference phase, when exogenous DNA invades again, the bacteria specifically recognize and eliminate the invaders. CRISPR, clustered regularly interspaced short palindromic repeats; Cas1(2,9), CRISPR associated-protein1 (2,9); PAM, protospacer-adjacent motif; pre-spacer, precursor spacer; crRNA, CRISPR RNA; pre-crRNA, pre-CRISPR RNA; tracrRNA, trans-activating crRNA; DSB, double-strand breaks.

Table 1 Applications of the CRISPR/Cas9 system in nervous system diseases.

Pathology	Target gene	Model	Result	Reference
AD	Bace1 gene	5XFAD mouse, APP knock-in (KI) mouse	APP↓, Aβ↓	68
	APP gene	Mouse	Aβ↓	69
	APOE4 gene	Mouse	APOE4 allele→APOE3 allele	56
		iPSCs	Tau hyperphosphorylation↓	73,74
	ATP6V1 A gene	iPSCs	Neuronal activity↓	79
PD	Vps35 D620N	Mouse	Mouse model of PD	87
	α-synuclein gene	Pig	Pig model of PD	88
	Not mentioned	Monkey	Monkey model of PD	90
	DNAJC6	ESCs	Stem cell model of PD	91,92
	Not mentioned	Human pluripotent stem cells	Human brain-like organoids model of PD	93
	SNCA gene	Mouse	SNCA↓, α-synuclein↓	95,96
	VPS59 D620N	Cell	Mitosis↓	99
HD	HHT gene	Pig	Pig model of HD	107
	HHT gene	HD140Q-Ki mice	Early neuropathological changes in the striatum↓	109
GBM	TLE4, IKZF2, EIF5A, TMEM184B	CAR-T cell	Death of CAR-T cells↓	116
	relA/p65, NPLOC4	GBM cell	Death of GBM cells↑	116
	Gene of IFNγR signaling pathway	GBM cell	Drug resistance↑	117
Epilepsy	KCNA1 gene	Mouse	Kv1.1↑, frequency of seizures↓	124
	KCNB1 gene	Mouse	Kv2.1↑, abnormal EEG activity↑	127
	SCN1A gene	Mouse	Nav1.1↑, epileptic symptoms↓	131
ALS	C9orf72	Mouse	Synaptic dysfunction↓	143
	SOD1 gene	SOD1G93A mutant neonatal mice	Quantity of motor neurons↑ Muscle strength↑ Survival rate↑	147,148

of bacterial adaptive immunity. When the bacteria detect these exogenous DNA sequences, the CRISPR/Cas system acts as a part of the immune system.

Cas gene and Cas9 protein

The *Cas* gene is located adjacent to the *CRISPR* gene, but a portion of the *Cas* is also discretely distributed elsewhere in the genome. Cas, the encoded product of the *Cas* gene, is a protein with nuclease activity and is a key component in bacterial autonomous immunity that binds to DNA at its corresponding targets. Based on the different roles of Cas in the immune mechanism, CRISPR/Cas system is divided into two categories. In the first category, Cas (type I, III, and IV) act through a cascade amplification mechanism, which usually produces the Cas complex. In the second category, Cas does not rely on a cascade amplification mechanism, and only a single Cas is involved, including type II and type V. Cas9 belongs to type II.[19]

Mechanism of CRISPR/Cas9 system

Adaptation

The first step is "adaptation", which involves the acquisition of a spacer sequence. Phage or plasmid-derived exogenous DNA is integrated into the phosphate group of the *CRISPR* gene when bacteria are attacked.[20] Therefore, it can be speculated that the position of the phosphate group end to the base end in the spacer sequence is consistent with the temporal order of exogenous DNA invasion. Protospacer adjacent motif (PAM) is a hallmark of the pre-spacer sequence and can be used to distinguish exogenous DNA and *CRISPR* genes.[21] Cas1 and Cas2 are the critical proteins that take part in the adaptation stage. To perform their function, both proteins usually polymerize to form a Cas1-Cas2 complex.[22,23] The Cas1-Cas2 complex cleaves the DNA sequence near PAM by utilizing it as the recognition object. The original spacer sequence is then inserted downstream of the leader sequence, and finally, the DNA completes the spontaneous repair. During the adaptation phase, bacteria generate specific immunity against the exogenous DNA, therefore when it encounters invaders containing the same genetic material again; it specifically identifies and destroys these invaders.

Expression

The leader sequence contains the promoter and spacer sequence integration elements of the CRISPR/Cas9 system. When the intruder is identified, the leader sequence regulates the *CRISPR* gene transcription to synthesize base-complementary pre-CRISPR RNA (pre-crRNA) and trans-activating crRNA (tracrRNA).[24] Pre-crRNA, tracrRNA, and Cas9 constitute a complex. Subsequently, the pre-crRNA is

sliced into smaller units by RNase III, namely CRISPR RNA (crRNA). crRNA corresponds to the corresponding spacer sequence and guides Cas9 to target the DNA cleavage site.[25] crRNA-tracrRNA-Cas9 triplet complex is the basic structural and functional unit of the CRISPR/Cas9 system.[26]

Interference

PAM of exogenous DNA is first recognized by the Cas1-Cas2 complex. Subsequently, crRNA further guides the binding of the crRNA-tracrRNA-Cas9 triple complex to this exogenous DNA according to the principle of base complementary pairing. The crRNA then cooperates with a matching complement (protospacer) on an invading DNA to form the R-loop.[27] In the R-loop structure, crRNA binds to one strand by complementary base pairing, and the other single strand maintains a free state. At this time, Cas9 exerts its endonuclease activity, generates a target cleavage site 3 nucleotides upstream of PAM, and forms a DSB, thereby eliminating the exogenous after a series of complex actions.[7] The six domains of Cas9 have different mechanisms and functions. The NHN domain and the RuvC domain are responsible for targeted DNA cleavage. The NHN domain cuts the complementary DNA strands, regulated by single metal ions, and the RuvC domain cuts the non-complementary DNA strands, regulated by bimetallic ions.[7,28]

Gene editing technology based on CRISPR/Cas9 system

The sgRNA is a synthetic T-shaped RNA that guides Cas9 to bind the target DNA and can achieve editing for different genes instead of the crRNA.[29,30] The basic mechanism of the CRISPR/Cas9 system involves guiding Cas9 to cleave DNA double strands after sgRNA recognizes the target gene and forms DSB at the corresponding site. After DNA damage, gene knock-out or knock-in occurs in the process of spontaneous repair, ultimately achieving gene editing.

Knock-out

Non-homologous end joining (NHEJ) is the most efficient and common cellular DNA repair mechanism.[31] NHEJ repairs DSB by directly connecting the broken ends and performing minimal DNA end treatment.[32] Upon NHEJ activation, bases are randomly inserted or deleted at the DNA ends, causing a complete genetic code disruption downstream of the DSB and stopping the target gene expression.[32] Although the frequency of NHEJ occurrence is relatively high, its low accuracy often causes non-target editing, which greatly limits its application. To achieve precise target gene knockout, mutations in the NHN or the RuvC domains can be artificially produced. This allows Cas9 to act on only one DNA strand. To achieve DSB, two different sgRNA are designed to target DNA double strands, which significantly improves gene knockout specificity. It has been shown that the size of the Cas9-mediated deletion and its frequency are negatively correlated.[33] For Cas9-induced DSBs, first, the PAM proximal end of DSB is released by Cas9, while its distal end remains attached to Cas9 for a long period.[34,35] Ultimately, the PAM proximal DSB end is exposed for repair treatment, while the distal end is not repaired temporarily due to Cas9 binding. Cas9 can be removed from the distal end of PAM by RNA polymerase, but the specific mechanism is not clear.[36]

Knock-in

Homology-directed repair (HDR) is another DNA repair mechanism, which results in gene insertion. Unlike NHEJ, cells perform HDR using repair templates. DNA reconstruction occurs at the DSB site based on the repair template. HDR is a high-fidelity repair pathway, and repair templates often include genes that require the insertion of homologous sequences at the insertion site. Homologous sequence matching is a primary condition for HDR.[31,37,38] Repair templates can either be endogenous DNA or artificially introduced exogenous genetic material such as plasmids.[39] The incidence of HDR is much lower than NHEJ, but it has higher accuracy. Compared with NHEJ, HDR is restricted to the S/G2 phase of the cell cycle,[40] which provides a theoretical basis for improving HDR efficiency. NHEJ inhibitors or HDR enhancers can be used to improve HDR efficiency, and the rationale for inhibitors or enhancers is to limit cell division to the S/G2 phase with increased HD, allowing the cells to undergo DNA repair by HDR rather than NHEJ.[41,42] Furthermore, that chemical modification has been indicated to increase HDR incidence.[43,44]

Transcriptional regulation

Due to the poor specificity, Cas9 is more prone to off-target effects, increasing the possibility of non-target mutations[45]; to overcome this, dead Cas9 (dCas9) mutates the two domains NHN and RuvC to lose DNA cleavage activity,[46] while retaining the ability to fuse to DNA mediated by sgRNA. The dCas9 can fuse to the transcriptional activation domain (ADS), including VP64, P65, and RTA, to stimulate transcriptional activation.[47–49] Additionally, dCas9 inhibits transcription and gene expression by fusing to the promoter region or the transcriptional repressor Krab-dCas9.[50] Thus, dCas9 differs from Cas9 as dCas9 stimulated transcriptional activation or repression is transient, reversible, and does not cause a permanent alteration or DNA genomic damage. Recently, researchers have constructed a dimer complex in which dCas9 fuses to FokI (dCas9-FokI) and achieve transcriptional regulation mediated by RNA; this has minimum off-target effects.[51]

Base editing

Single-base pair mutations are the leading causes of many inherited and acquired diseases.[52] Gene editing both by HDR and NHEJ pathways has less specificity and efficiency, and once the mutations occur, they can cause great damage to the human body.[53] Moreover, due to the specificity of HDR and NHEJ mechanisms, their mediated gene editing can only be applied to dividing cells, thus limiting the scope of disease treatment.[54] Recently, a new Cas9-mediated base editing tool was introduced, which is of two types, cytosine base editing molecule (CBES) and adenine base

editing molecule (ABES).[52,55] The base editing tool consists of two key components: dCas9 which binds to DNA and enzymes that target base changes. CBES tool combines rat-derived cytosine deaminase apolipoprotein B mRNA editing enzyme catalytic subunit 1 (APOBEC1) with dCas9 for gene editing,[56] whereas ABES combines deoxyadenosine deaminase with dCas9 for base editing.[57] CBES and ABES can achieve four changes (A-G, G-A, C-T, and T-C) to achieve a single base editing.[56,58] The efficiency of ABES in human cells is about 50%, while that of CBES remains unknown.[57]

Epigenome editing

The conventional CRISPR/Cas9 system can produce an irreversible DNA sequence alteration. Moreover, the over-reliance on endogenous DNA repair mechanisms also increases the complexity and uncontrollability of artificial operations to a large extent. Recently, an epigenetic editor called CRISPRoff was reported. CRISPRoff methylates specific DNA fragments as guided by sgRNA. These methylated genes are then silenced or shut down, thereby epigenetic gene silencing is achieved.[59] CRISPRoff does not alter the DNA sequence and is not limited by endogenous DNA repair mechanisms. Furthermore, CRISPRoff-stimulating methylation is reversible as it can be removed by an epigenetic editor called CRISPRon. It is worth noting that CRISPRoff can silence genes lacking CpG islands (CGIs), which were previously considered essential for DNA methylation.

CAPTURE

CRISPR affinity purification *in situ* of regulatory elements (CAPTURE) is a derivative technology of the CRISPR/Cas9 system. CAPTURE comprises specific sgRNA, biotin-labeled dCas9 (FB-dCas9), and biotin ligase BirA. Briefly, CAPTURE uses sgRNA to guide FB-dCas9 to the target DNA sequence element and then separates and purifies proteins, DNA, RNA, and other macromolecules at the corresponding sites by high-affinity streptomycin.[60] It provides a new method for studying the function and specific regulatory factors of DNA non-coding regions. At present, CAPTURE has played a huge role in the regulation mechanism of the snRNA gene, mouse embryonic stem cells, and other fields.[61,62] It can also provide new paths to discover novel drug targets.

Applications of CRISP/Cas9 system in nervous system diseases

Alzheimer's disease

One of the most common neurodegenerative diseases is AD, characterized by senile plaques [abnormal accumulation of extracellular amyloid-β (Aβ)] and neurofibrillary tangles (abnormal hyper-phosphorylation of intracellular tau protein).[63] AD is also associated with extensive neuronal loss, hippocampal pyramidal cell granule vacuolar degeneration, and other pathological changes. No effective radical cure nor an efficient strategy to reverse AD progression exists. Traditional drug therapy is relatively effective over a long period. However, as the disease progresses, the effect of drug treatment decreases significantly. CRISPR/Cas9 system provides a new door for identifying novel ideas and methods for treating AD.

The metabolic abnormality of Aβ is manifested by the imbalance between the production and elimination of Aβ, subsequently causing its abnormal accumulation.[64] Aβ is produced by amyloid precursor protein (APP) catalyzed by β-secretase 1 (Bace1) and γ-secretase, where Bace1 plays a key role. Therefore, any link of this metabolic pathway can reduce the content of Aβ, thereby reducing the pathological damage of AD.[65] The CRISPR/Cas9 system has been used to model AD in a variety of cells *in vitro*,[66,67] however, targeted editing of highly differentiated cells such as neurons has been difficult, making *in vivo* AD studies difficult. Recently, Park et al used CRISPR/Cas9 nano-complex (Cas9-sgRNA supplemented with two amphiphilic R7L10 peptides in different proportions) to introduce sgRNA containing *Bace1* gene targeting sites into five familial Alzheimer's disease (5XFAD) and APP knock-in AD mouse models, respectively. A significant reduction was noted in Bace1 and Aβ. In addition, treatment mice performed better in the water maze test compared with control mice, indicating a shorter escape time to find the platform.[68] These results indicate that CRISPR/Cas9 nano-complexes can effectively reduce the pathological manifestations and cognitive impairment caused by AD. As the upstream protein of Aβ, the expression of APP directly affects Aβ content. Knocking out *APP* allele by CRISPR/Cas9 can also reduce Aβ expression.[69]

Age is the most dangerous pathogenic factor of AD. Based on age and onset, AD is divided into early-onset AD (EOAD) and late-onset AD (LOAD, by 65 years old), where LOAD is more common.[70] The Apolipoprotein E (*APOE*) gene is an important risk factor for LOAD. *APOE* has three subtypes: *APOE2*, *APOE3*, and *APOE4*. The *APOE4* allele greatly increases the risk of carriers, whereas *APOE2* has a protective effect.[71,72] Using CRISPR/Cas9, Komor et al successfully transformed *APOE4* into the *APOE3* allele in mouse astrocytes by generating a C-T transformation at codon 158 via base editing techniques.[56] Lin et al established induced pluripotent stem cells (iPSCs) comprising homozygous *APOE4* alleles using CRISPR/Cas9 and demonstrated that the conversion of *APOE4* to *APOE3* can greatly improve AD-related pathological features.[73] Based on this mechanism, studies have shown that the conversion of *APOE4* to *APOE3* can also reduce tau hyper-phosphorylation to some extent.[74]

Most current AD therapeutic studies are focused on AD-related proteins and genes such as APP and Aβ.[75–78] Wang et al conducted a large data comprehensive network analysis of multiple LOAD samples and found that neuronal gene subnetwork dysregulation was the main pathogenic factor of LOAD and identified ATP6V1 A as a key regulator. Wang et al targeted *ATP6V1A* genes by using dCas9 and found that its expression was reduced while neuronal activity was inhibited. This network modeling strategy provides a novel perspective on AD treatment.[79]

Parkinson's disease

Tremor paralysis or PD is a common neurodegenerative disease of the middle-aged and elderly and is ranked the second most common neurodegenerative disease globally.[80] The

main clinical manifestations of PD are static tremors, bradykinesia, myotonia, and postural imbalance.[81] The major pathological changes associated with PD include the degenerative loss of the dopaminergic neurons in the substantia nigra and the emergence of eosinophilic inclusions, namely the Lewy body.[82] Approximately 10% of all PD patients are familial and 90% are sporadic. Genetic susceptibility is the main reason in the familial type of PD, where the *SNCA* and *LRRK2* gene mutations are autosomal dominant, and *Parkin*, *PINK1*, and *DJ-1* gene mutations are autosomal recessive.[83,84] The cause of sporadic AD is undetermined, it is presumed to be related to oxidative stress and other factors.[85]

The construction of the PD model has always been complex and difficult. The construction of traditional animal models has defects such as long cycles, low success rates, and low specificity.[86] The CRISPR/Cas9 system, with its precise targeted editing function, provides conditions for animal modeling. Ishizu et al used the CRISPR/Cas9 technique to knock in the *Vps35 D620N* allele in mice that exhibited age-related substantia nigra-striatum neurodegeneration and had positive features similar to PD.[87] Zhu et al successfully created the PD pig model by combining CRISPR/Cas9 with somatic cell nuclear transfer (SCNT).[88] *PINK1* gene mutations are the main cause of early-onset PD.[89] However, neither the current *PINK1* knock-out mouse model nor the pig model has fully demonstrated the typical PD-associated pathological changes. Chen et al injected sgRNA and Cas9-D10A nicking enzyme into zygotes of cynomolgus monkeys to establish a typical PD neurodegenerated primate model.[90] Cell models also help study the pathogenic mechanisms and molecular pathological alterations in PD. Wulansari et al established *DNAJC6* gene mutation in human embryonic stem cells and observed the key pathological features of PD including dopaminergic neuronal degeneration and abnormal aggregation of α-synuclein91. Dolatabadi et al also constructed similar cellular models.[92] Furthermore, organoid models have been reported. Jo et al used human pluripotent stem cells and CRISPR/Cas9 genetic engineering to construct human brain-like organoids in a 3D culture system, producing human PD-specific Louis bodies in organoids.[93] Organoids provide a new idea for the construction of *in vitro* PD models.

SNCA gene mutation, specifically *SNCA* hypermethylation, critically participates in the pathogenesis of PD.[94] Guhathakurta et al revealed that histone H3 lysine 4 trimethylation (H3K4me3) was recruited in the *SNCA* promoter of PD patients. The team used dCas9-mediated demethylase to specifically block *SNCA* promoter expression, thereby down-regulating α-synuclein.[95] Gene knockout can also mitigate PD damage. Targeted knockout at A53T mutation sites on *SNCA* by CRISPR/Cas9 technique significantly reduced *SNCA* expression both *in vitro* and *in vivo*.[96]

Oxidative stress and mitochondrial damage have also been implicated as major factors in PD pathogenesis.[97] PTEN-induced putative kinase 1 (PINK1) protein is important for the maintenance of normal morphology and mitochondrial function. The reduction or deletion of PINK1 disrupts the redox balance and promotes PD development.[98] Ma et al constructed a PD cell model with the D620N mutation in *VPS59*, which ultimately affected PINK1-mediated mitosis,[99] suggesting that the study of oxidative stress mechanism may be a breakthrough in the treatment of sporadic PD.

In addition to studying the bio-molecular mechanisms of PD, the CRISPR/Cas9 system can also be used for screening the therapeutic drugs against PD. Basu et al inserted a luciferase gene into *SNCA* by CRISPR/Cas9 to measure α-synuclein expression in living cells.[100] This technology can achieve high-throughput drug screening, which greatly improves the efficiency of drug development and testing for PD or other neurodegenerative disorders.

Huntington's disease

Huntington's disease, also known as Huntington's chorea, is an inherited autosomal dominant disorder of the central nervous system. It is caused by an abnormal expansion of the CAG repeat of the Huntington's protein (HTT) gene on chromosome 4, resulting in a toxic gain of function.[101,102] The severity of the clinical symptoms is proportional to the CAG copy number. No effective treatment for HD exists. Based on the characteristics of HD single gene inheritance, the CRISPR/Cas9 system is expected to become a promising treatment method.[103]

Animal models allow the study of pathogenesis and pathological changes associated with HD, therefore, rodents such as mice are commonly used as disease models.[104,105] However, although these models can express mutant HHT, it lacks the neurodegeneration typical of HD and fails to comprehensively mimic the manifestation of human HD development at both molecular and behavioral phenotype levels.[104,106] Yan et al have successfully developed the world's first HD knock-in pig model via CRISPR/Cas9 and inserted the CAG repeat sequence into the pig *HHT* gene. This model revealed typical pathological manifestations of striatal medium spiny neuron degeneration and dance-like involuntary movement in the behavioral phenotype. More importantly, these characteristics exhibit age-related similarities to humans and can be stably transmitted through the reproductive system.[107] The study provides a basis for animal models of other neurological diseases.

CAG repeats knockout in the *HHT* gene using CRISPR/Cas9 reduced *HHT* expression.[108,109] Notably, previous studies suggest that the *HHT* gene knockout by CRISPR/Cas9 causes mice death (not in adults) from acute pancreatitis.[110] In a later study, Yang et al knocked out the *HHT* gene in HD140Q-Ki mice, reducing early neuropathological changes in the mouse striatum.[109]

Most current HD treatments mainly target the disease remission after onset. However, 50% of the striatal volume disappears before the symptoms even appear.[111] Therefore, early detection and treatment before the onset are very critical. Liu et al used an inflow-based vascular-space-occupancy (IVASO) MRI to map the time-varying model of cerebral blood volume (CBVa) in small arteries of zQ175 mice. HTT in zQ175 mice was mutated and progressed slowly. As the disease progressed, CBVa gradually increased. This study revealed that mice treated with HTT sgRNA and Cas9 lacked striatal atrophy and behavioral phenotype alterations compared with the control group.[112] Therefore, the CBVa model can be used as a sensitive noninvasive indicator for early intervention in HD.

Glioblastoma

Glioblastoma is a neuroepithelial tissue-derived neurodegenerative disorder. It is the most common, aggressive, and least-prognostic malignancy of the central nervous system.[113] Currently, there are no effective treatments for GBM. Surgery combined with chemoradiotherapy is usually the therapeutic strategy applied, which only prolongs the median survival time of GBM by < 3 months. Chimeric antigen receptor T cell therapy (CAR-T) is a method that allows T cells to express the tumor chimeric antigen receptor (CAR) through gene editing technology to specifically identify and kill tumor cells.[114] CAR-T is effective for the treatment of hematological malignancies but is relatively insensitive for solid tumors.[115] Therefore, it is important to find therapeutic strategies for these tumors. Wang et al established a new high-throughput screening platform for high-throughput CRISPR gene knockout screening in CAR-T and GBM cells, respectively. The team identified four sites *TLE4*, *IKZF2*, *EIF5A*, and *TMEM184B* in CAR-T cells, and two *relA*/*p65* and *NPLOC4* in GBM cells. Knocking out these genes increased the GBM cell killing and reduced the death of CAR-T cells *in vitro* and *in vivo*.[116] Furthermore, reducing GBM resistance to cell therapy is also one of the CAR-T strategies. Larson et al used CRISPR for genome-wide screening of GBM and found that gene deletion in the IFNγR signaling pathway increases drug resistance in GBM, which is associated with reduced adhesion of GBM cells to CAR-T cells.[117]

CRISPR/Cas9 is also used for targeted drug therapy of GBM. In the past, CRISPR/Cas9 therapy for tumors had low specificity, low editing efficiency, and drug resistance; however, Rosenblum et al reported a method for targeted delivery using lipid nanoparticles (LNP) coated with CRISPR/Cas9 (CRISPR-LNP). *In vivo*, intracerebral CRISPR-LNP injection in GBM mice reduced tumor volume by 50% and significantly improved mouse survival.[118]

With high efficiency for targeting tumor cells and extremely low drug resistance, CRISPR/Cas9 gene editing technology allow new avenues for GBM treatment, its proper use can spare patients the pain caused by radiotherapy and chemotherapy. Moreover, it provides new possibilities for treating malignant pan-tumors.

Epilepsy

Epilepsy is caused by the abnormal synchronized discharge of brain neurons; it is a clinical syndrome rather than an independent disease. The etiology of epilepsy is very complex, it can be caused by gene mutations or occur secondary to various neurological diseases, such as stroke, brain trauma, brain tumors, intracranial infections, *etc*.[119,120] Treatment for epilepsy remains to be established. Drug therapy is not effective because of its high drug resistance; however, gene therapy may be a powerful new tool for its treatment.

Ion channels maintain normal neuronal structure and function.[121] Mutation in genes encoding ion channels leading their abnormality, an important cause of epileptic seizures. Therefore, studying ion channel-related genes can be a key to overcoming epilepsy.[122] Exogenous overexpression of *KCNA1* reduces neuronal excitability,[123] however, its effect on neurons remains unknown. Colasante et al combined a dCas9 targeting the promoter region of *KCNA1* (encoding Kv1.1) and a sgRNA to construct the CRISPRa system (*KCNA1*-dCas9A).[124] In the mice temporal lobe epilepsy model, *KCNA1*-dCas9A up-regulated Kv1.1 expression by activating the *KCNA1* gene and reduced neuronal excitability and seizure frequency. More importantly, *KCNA1*-dCas9A also improved cognitive deficits in mice.[124] However, the consistency of the temporal lobe epilepsy model with other types of models in terms of gene expression patterns remains unconfirmed.[125,126] *KCNB1* (encoding Kv2.1) is the causative gene of developmental and epileptic encephalopathies (DEE). Unlike *KCNA1*, mutations in *KCNB1* can trigger epilepsy. Hawkins et al used CRISPR/Cas9 to knock out *KCNB1* in mice and observed typical DEE manifestations such as abnormal EEG activity and hyperactivity.[127] The model can be used for drug evaluation and screening to solve the problem of drug-resistant DEE.

Dravet syndrome (DS) is a hereditary epileptic encephalopathy. It is commonly triggered by a fever.[128,129] Although DS is generally defined as a disease independent of epilepsy, the two are usually discussed together because of their similar pathogenesis and clinical manifestations. DS is also closely related to ion channels. The heterozygous deletion mutation of the *SCN1A* gene (encoding Nav1.1) is the main cause of DS.[130] Colasante et al applied *SCN1A*-dCas9A to *SCN1A* mutant mice and revealed that *SCN1A* stimulation increased Nav1.1 levels and the excitability of intermediate inhibitory neurons. The epileptic symptoms of *SCN1A*-activated mice were also significantly reduced compared with the control group,[131] suggesting a strategy to rescue DS and other fever-induced seizures.

Zebrafish are excellent animal models for studying gene function but are rarely utilized for human neurological diseases. Griffin et al established 40 zebrafish lines with childhood epilepsy via CRISPR/Cas9 and identified eight single-gene phenotypes after electrophysiological screening.[132] However, this model cannot summarize the typical human epilepsy phenotype, which may be related to multiple epilepsy etiological mechanisms, such as multiple gene mutations, environmental factors, *etc*.[133,134]

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is characterized by the progressive loss of motor neurons in the central nervous system,[135] it is also the most common motor neuron disease characterized by progressive muscle weakness that eventually leads to respiratory failure and death.[135] Multiple genetic loci are associated with ALS, such as the *C9orf72*, *SOD1*, *TARDBP,* and *FUS* genes.[136–139] The CRISPR/Cas9 system is effective against these gene loci *in vitro* and *in vivo*.

A hexanucleotide repeat expansion of the *C9orf72* sequence is the most critical and common cause of ALS, specifically the G4C2 repeat. Mutations cause a dipeptide protein clearance disorder, and excessive accumulation, which in turn produces neurotoxicity.[140] A study showed that when IPSCs were inhibited by CRISPR/Cas9 in *C9orf72* patients, dipeptide levels reduced dramatically.[141] At the transcriptional level, CRISPR/Cas9 knockout of G4C2 repeats reduces RNA transcription and nuclear RNA foci.[142] It was

revealed that reducing the G4C2 repeat sequence can protect neurons. *C9orf72* mutation causes synaptic neuronal dysfunction. In a recent study, Perkins et al used CRISPR/Cas9 to correct the *C9orf72* mutation and successfully rescued synaptic dysfunction,[143] providing new insights into the early mechanisms of ALS and new options for drug targets.

The *SOD1* gene, located on chromosome 21, is the first and the most studied ALS-related gene discovered.[144] The copper-zinc superoxide dismutase encoded by the *SOD1* gene is very important for maintaining normal redox balance in cells. The mutation of the *SOD1* gene can lead to mitochondrial dysfunction, oxidative stress, abnormal protein accumulation, cell inflammation, *etc.*, and then promote ALS incidence and development.[145,146] In a previous study, *Staphylococcus aureus*-derived Cas9 (SaCas9) and sgRNA targeting the *SOD1* gene were injected via the facial vein in SOD1G93A mutant neonatal mice mediated by AVV9. The results revealed that mutant SOD1 significantly reduced, the number of motor neurons increased significantly, and the motor function was effectively protected.[147] Based on this study, Duan et al injected a high dose (3.3 times the normal dose) of SaCas9 to observe the phenotype of SaCas9-modified mice and found that mice had increased muscle strength, reduced muscle atrophy, and significantly improved survival.[148] The above two studies demonstrate the potential of SaCas9 to correct ALS both in neuropathology and in clinical phenotype. However, the small carrying capacity of AAV limits the SaCas9's clinical application. Lim et al invented a method to intrathecally inject AVV and CBES encoding split introns into SOD1G93A mice and observed reduced muscle atrophy and denervation.[149] The CRISPR base editor largely solves the problem of AVV's limited carrying capacity, making it possible to use CRISPR/Cas9 for the clinical treatment of ALS.

Conclusions

As researchers delve deeper into the CRISPR/Cas9 system, its potential is being tapped continuously. The advent of the CRISPR/Cas9 system has greatly facilitated the study of neurological diseases. The application of CRISPR/Cas9 gene editing technology has allowed us to achieve more precise modification of pathogenic genes. It can also be used to establish cellular or animal models that closely resemble human disease phenotypes. At the neuropathological level, the application of CRISPR/Cas9 more clearly reveals the molecular mechanisms of the disease. Its application also provides new ideas for the treatment of other types of diseases, such as AIDS, malignant tumors, *etc*. However, there are still some shortcomings in this technology, such as the off-target effect, the specific sgRNA mechanism, and the immunity of the human body toward Cas9. The biggest concern is the potential ethical issues posed by CRISPR/Cas9. If the technology is misapplied to the human reproductive system, it can have unimaginable consequences.

Author contributions

All authors made a significant contribution to this review in the conception, study design, execution, acquisition of data, analysis, and interpretation; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Conflict of interests

The authors report no conflict of interests in this work.

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