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CRISPR/Cas9 gene editing: New hope for Alzheimer's disease therapeutics

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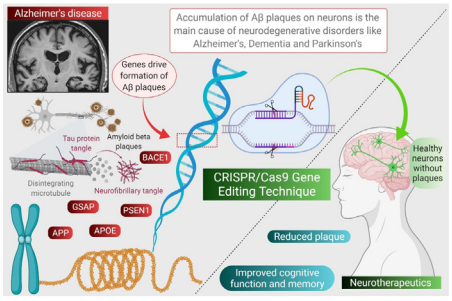
HIGHLIGHTS

- Mutations in APP, PSEN1 and PSEN2 are known factors for AD pathobiology.
- CRISPR/Cas9 genome editing approach hold promises in AD management.
- CRISPR/Cas9 is utilized to help correct anomalous genetic functions.
- Off-target mutations may impair the functionality of edited cells.
- Non-viral vectors show better efficacy and safety than viral vectors.

GRAPHICAL ABSTRACT



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ABSTRACT

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Background: Alzheimer's disease (AD) is an insidious, irreversible, and progressive neurodegenerative health condition manifesting as cognitive deficits and amyloid beta (Aβ) plaques and neurofibrillary tangles. Approximately 50 million individuals are affected by AD, and the number is rapidly increasing globally. This review explores the role of CRISPR/Cas9 gene editing in the management of AD and its clinical manifestations.

Aim of Review: This review aims to provide a deep insight into the recent progress in CRISPR/Cas9-mediated genome editing and its use against neurodegenerative disorders, specifically AD. However, we have referred to its use against parkinsons's disease (PD), Huntington's disease (HD), and other human diseases, as is one of the most promising and emerging technologies for disease treatment.

Key Scientific Concepts of Review: The pathophysiology of AD is known to be linked with gene mutations, that is, presenilin (PSEN) and amyloid beta precursor protein (APP). However, clinical trials focused at the genetic level could not meet the desired efficiency. The CRISPR/Cas9 genome editing tool is one of the most powerful technologies for correcting inconsistent genetic signatures and now extensively used for AD management. It has significant potential for the correction of undesired gene mutations associated with AD. This technology has allowed the development of empirical AD models, therapeutic lines, and diagnostic approaches for better understanding the nervous system, from *in vitro* to *in vivo* models.

Introduction

Alzheimer's disease (AD) is a major worldwide health concern with enormous social and economic impact [1]. The slow decline in cognitive function and irreversible neuronal loss are the primary etiological manifestations of the disease. AD is a chronic neurodegenerative condition in which cognition and memory formation gradually deteriorate due to an irreversible neuron loss. It is characterized by the formation and accumulation of amyloid-beta 42 (Aβ42) and phosphorylated Tau, along with excessive glial cell activation. Further, impaired synaptic function and insufficient neurotrophin signaling are important characteristics of AD. Primary symptoms include memory loss, apathy, depression, and irritability [2]. Despite substantial researches, the etiology, pathophysiology, and mechanisms of both cognitive impairment and synaptic dysfunction are not well characterized. In addition, the available therapeutic options are merely symptomatic and supportive, with side effects such as confusion, dizziness, depression, constipation, and diarrhea [3]. Progress towards effective disease-modifying therapies has proven challenging despite the wealth of knowledge on the molecular underpinnings of AD. For instance, several clinical trials have failed to meet efficacy standards against Aβ production, accumulation, and toxicity. This put questions on the amyloid beta hypothesis and advocates for additional treatment strategies. One of the very important and recently invented strategies, clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins 9 system (CRISPR/Cas9) gene editing, attracted attention for possible benefits in the management and treatment of AD. This emerging technology is relatively straightforward, inexpensive, and precise, which has led to an increased interest in this technique for neurodegenerative diseases (NDDs). This can be utilized as a direct treatment approach or may assist in establishing better animal models that faithfully mimic human NDDs. Though, this technique has shown promise in other NDDs such as Huntington's disease (HD) and Parkinson's disease (PD). But, the potential of this technology in the management of AD has not been thoroughly discussed or documented elsewhere. Therefore, the purpose of this review was to examine the potential utility of CRISPR/Cas9 as a treatment option for AD by targeting specific genes, including those that cause early onset AD, as well as those that are significant risk factors for late-onset AD such as the apolipoprotein E4 (APOE4) gene. This review also discusses various delivery systems that help in the proper and targeted delivery of CRISPR/Cas9 cargo in cells.

Alzheimer's disease at a glance

AD is a ubiquitous form of dementia that affects the health of millions of individuals globally. Despite being known for over a century, numerous questions about its pathophysiology are yet to be answered. The standard clinical characteristics of AD are displayed by diminished cognitive abilities, including memory, recognition, judgment, and problem solving [4-6]. Studies on the AD brain revealed neuropathological changes that represent the hallmarks of the condition, such as intracellular neurofibrillary tangles (NFTs) comprising hyperphosphorylated Tau and accumulation of extracellular Aβ plaques [7-9]. In early onset AD, symptoms are exhibited in individuals aged between 30 and 65 years and is primarily genetic, as observed in > 92% cases reported [6], whereas in late onset AD, symptoms start after 65 years of age. As per a 2019 report, >5.8 million individuals in the United States (USA) alone have been diagnosed with AD, out of which 45% of subjects are reported in the 75–84 years group [10]. The constantly increasing numbers of affected patients leads to projections of approximately 14 million in the USA by 2050 [11].

Mostly, the disease is believed to be due to external factors other than genetic predisposition. The amyloid hypothesis explains Aβ formation and aggregation in the brain. This hypothesis states that amyloid beta precursor protein (APP) undergoes proteolysis due to the concerted activities of α-, β-, and γ-secretases. An increase in β-secretase 1 (BACE1) activity is responsible for AD owing to aggregation of Aβ monomers into oligomers, and consequently the generation and deposition of Aβ plaques. Furthermore, BACE1-cleaved APP leads to the formation of a C99 fragment further cleaved by the γ-secretase to produce Aβ monomers Aβ40 and Aβ42. Interestingly, the α-secretase can also cleave APP at various sites, thus curtailing Aβ monomer generation (Fig. 1**A**) [12].

The amyloid hypothesis explains Aβ formation; similarly, NFT formation in AD brains is revealed by the popular Tau hypothesis. Tau, a well-known microtubule associated protein, plays a pivotal role in the formation and stabilization of the microtubule cytoskeleton [13]. It has been reported that out of six Tau isoforms, 3R and 4R are prominent in adult human axons. Multiple phosphates and kinases have Tau as target. In the AD brain, Tau isoforms 3R and 4R can accumulate in hyperphosphorylated form and cause NFT formation in neuronal tissues, if present in neuronal cell axons and bodies, leading to Tau pathology. As recently suggested, Tau oligomers can be neuropathology-mediating microstructures or potential molecular initiators in AD (Fig. 1**B**)

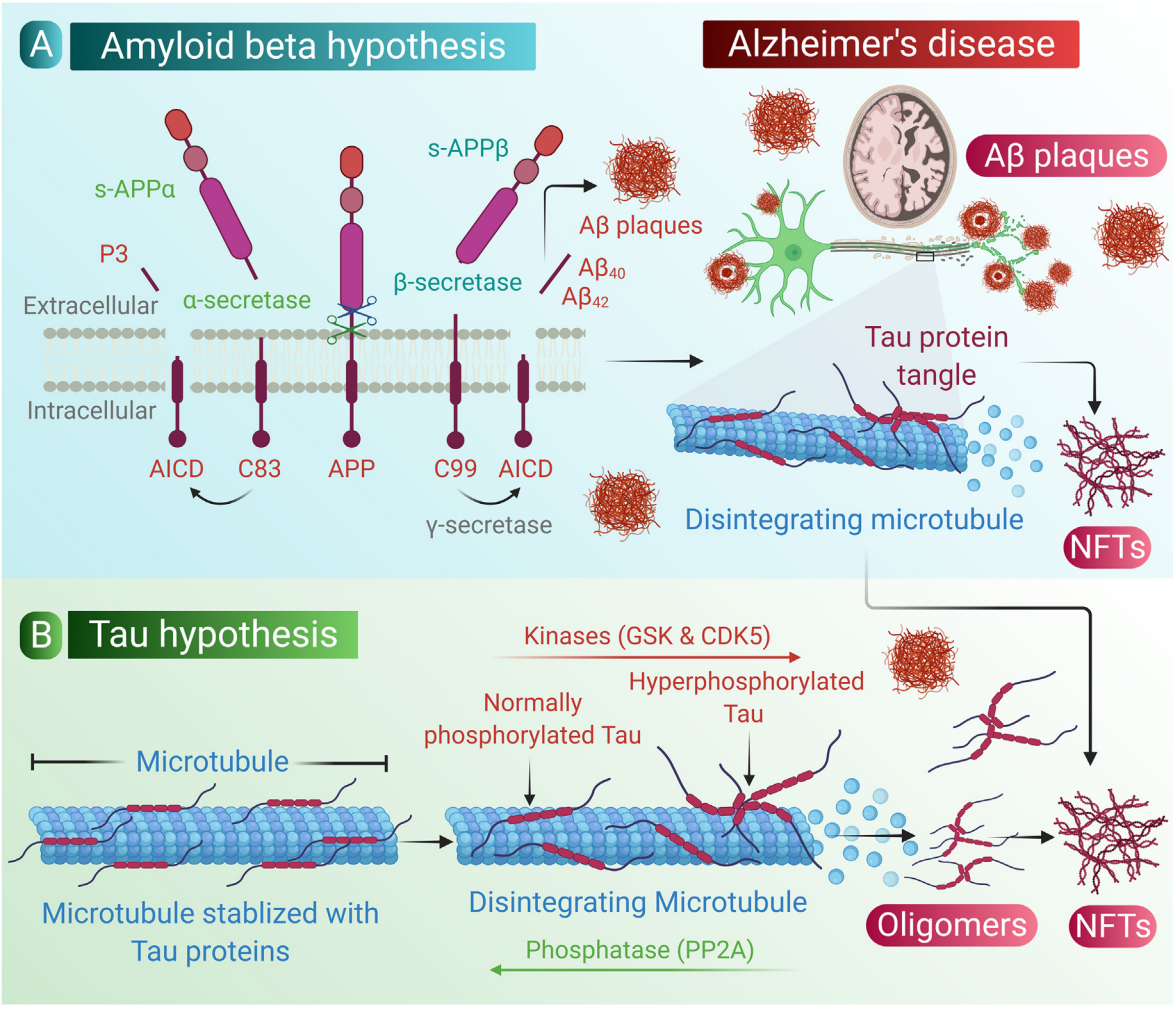

Fig. 1. Schematic showing Amyloid beta and Tau hypothesis that have been suggested to give an explanation for the most common characteristic hallmarks of AD. APP, amyloid precursor protein; AICD, APP intracellular domain; NFTs, neurofibrillary tangles; GSK, glycogen synthase kinase; CDK5, cyclin-dependent kinase 5; PP2A, protein phosphatase 2A.

[14]. Furthermore, a possible molecular link between Aβ deposition and NFT formation has also been reported. The latter has been reported to inhibit neuronal viability, neuroplasticity, and altered microtubule assembly, along with inhibition of mitochondrial transportation along microtubules. Hence, Tau neurotoxicity may be driving an event downstream from Aβ polymerization, and be responsible for the neurotoxicity caused by Tau [15]. However, this hypothesis needs to be experimentally confirmed.

The introduction of the amyloid beta hypothesis provided directions for the development and testing of therapeutic agents for disease modification, which mutually enhance clearance of toxic peptides out of the brain and prevent Aβ formation [16]. Unfortunately, >400 clinical trials conducted over the decade when the last drug for AD was approved have failed. The drug only offered temporary treatment for AD symptoms. A significantly high attrition rate was indicated by an analysis of various clinical trials involving AD between 2002 and 2012, with a mere overall success rate of only 0.4%, with a 99.5% failure rate [11]. Despite the many plausible reasons behind the failure of these clinical trials, a widely-agreed view is that the disease stage was too advanced for any anti-Aβ drugs to impact cognition. Due to the considerable failure on present approaches focused on modifying the disease, other probable treatment methodologies, such as genome editing techniques, are needed. Currently, there are three major prevalent gene editing tools available, including CRISPR/Cas9, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs). Each of these tools has its own merits and demerits [17]. However, the present review mainly elaborates on the possible role of CRISPR/Cas9 in AD due to its low cost, high speed, efficiency, and precision over other genome editing tools.

CRISPR/Cas9: A promising gene editing tool

CRISPR/Cas9 is a recently discovered and promising revolutionary tool for genome editing, which allows to treat diseases with limited or scarce treatment options. This tool was initially identified by Ishino in 1987 [18] (Fig. 2). Since then, several studies have reported that the CRISPR/Cas9 system is an integral part of a bacterium's immune system, which offers protection from undesired integration of mobile genetic elements such as plasmids and viruses. Further, Doudna and Charpentier's pioneering efforts brought CRISPR/Cas9 to laboratory settings to investigate its potential [19]. In-depth studies on CRISPR/Cas9 have been conducted in the recent past, and significantly improved editing efficiency and minimized off-target effects while being extensively used for basic and translational research [19,20].

CRISPR/Cas9 has two main components: the Cas9 enzyme and single-guide RNA (sgRNA). The target DNA sequence is recognized by the sgRNA, wherein various parameters are considered in the design process to improve specificity. In contrast, as an endonuclease, the Cas9 protein acts as a molecular scissor for incision of the DNA double strands (Fig. 3). CRISPR/Cas systems are classified into

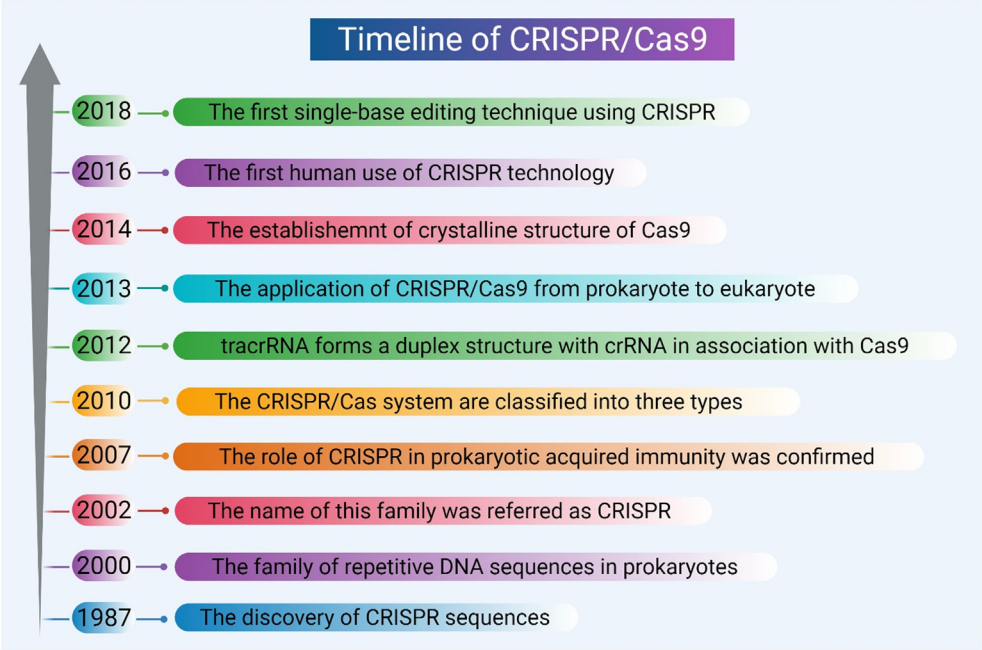

Fig. 2. The CRISPR/Cas9 timeline. crRNA, CRISPR-derived RNA; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins 9 system.

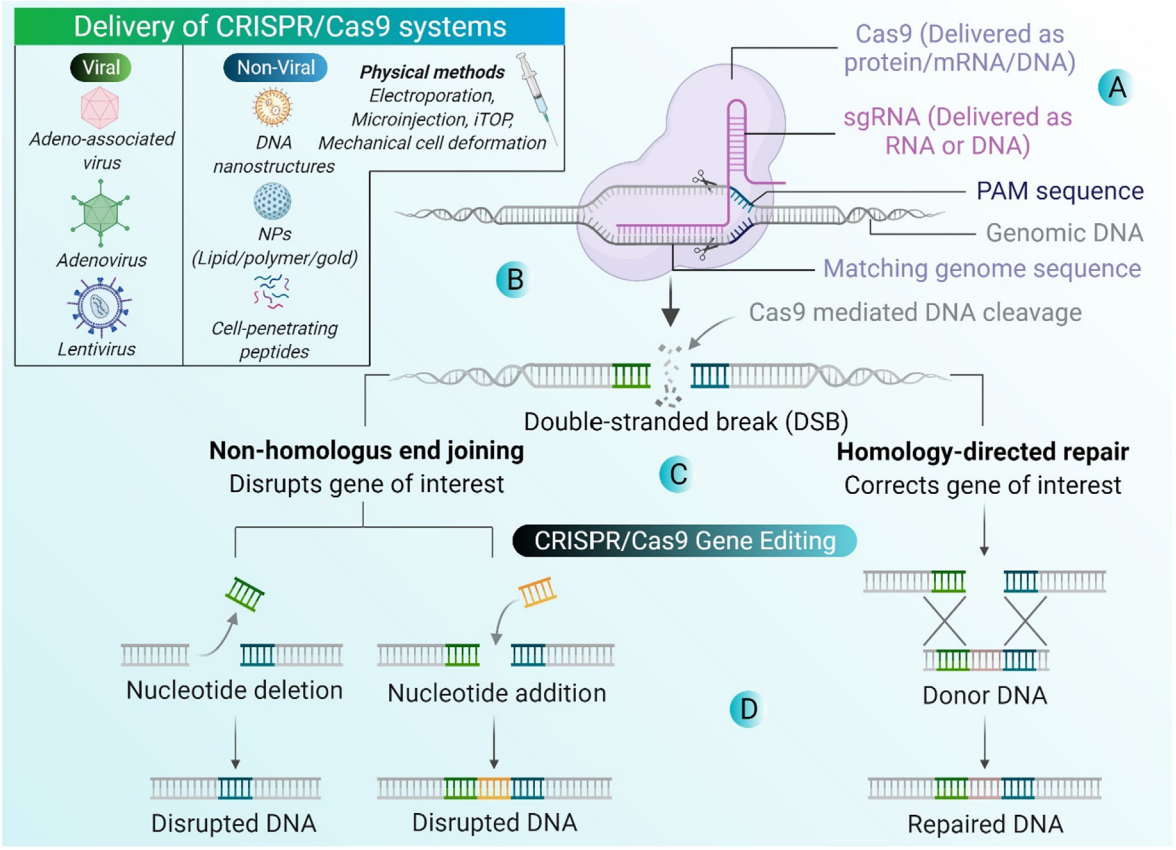

Fig. 3. A schematic cartoon illustrating the steps involved in CRISPR/Cas9 technique. (A) Specially designed sgRNA (guide RNA) which matches with genomic DNA sequence containing mutation, attaches with Cas9 (CRISPR-associated endonuclease), a DNAase capable of inducing a double strand break, thereby, forming Cas9-sgRNA complex. (B) Association of Cas9-sgRNA complex with the target genomic DNA. Cas9 searches for appropriate sequence in target DNA with the help of sgRNA and recognises it with the help of PAMs (protospacer adjacent motifs) sequence, usually 2–6 base pair long, found 3–4 nucleotide downstream from cut site generally serve as a tag. (C) Cas9 mediated DNA cleavage leads to the formation of double strand break (DSB). (D) Formation of DSB leads to the activation of DNA repair mechanism to correct the break by sealing the gap either by Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR).

Table 1
Different strategies that have been utilized by CRISPR/Cas9 tool in genome editing.

Different strategies	Principle	Advantages	Disadvantages	Reference(s)
Plasmid-Borne CRISPR/Cas9 System	Plasmid encoding sgRNA and Cas9 protein with suitable promoters	• Fluorescent protein assisted plasmid to label cells expressing Cas9 enzyme • Good reproducibility, economical and extensive adaptability and customizability because sgRNAs can be cloned from same plasmid and may consists of homology directed repair (HDR) template • Improved stability, particularly in handling and manufacturing in comparison with available strategies • Both Cas9 protein and sgRNA are loaded on the same vector; ensures that both are expressed in the same cell	• DNA and bacterial DNA sequences exists in the plasmid elicits cytotoxicity • Potential for the random insertion of plasmid fragments into the gene • Low transfection effciency of primary cells	[23,24]
Cas9/sgRNA Complex	Delivery of sgRNA complexed with Cas9 protein	• Minimal off -target effects and cell toxicity • The complex is fastest among available strategies are not necessary for transcription or translation to Cas9 protein • Ease and convenience due to Cas9 protein spontaneously forms a complex with sgRNA being oppositely charged	• Purification process of the Cas9 protein and free from contamination of endotoxin is expensive • The intracellular Cas9 delivery is complex and challenging due to large size (about 160 kDa)	[25,26]
Cas9 mRNA and sgRNA	Delivery of sgRNA and Cas9 mRNA	• Lower cytotoxicity, off-target effects reported comparatively plasmid-based system • This method is faster over plasmid approach for the editing of the targeted gene because Cas9 mRNA translation is necessary to generate Cas9 protein	• Instability of RNA	[27]

Class 1 (type I, type III, and type IV) and Class 2 (type II, type V, and type VI). Class 1 contains a variety of Cas proteins that work together, whereas Class 2 utilizes a single Cas protein, which makes it simple and desirable for genome editing [21]. Among Class 2, the type II CRISPR/Cas9 system is one of the most researched and utilized systems in pharmaceutical development. The Cas9 protein generates a double standard break after recognizing the target gene sequence. Subsequently, two distinct pathways can be initiated to repair this break, that is, homology-directed repair (HDR) or non-homologous end joining (NHEJ). NHEJ results in insertion and deletion, leading to premature stop codons and/or DNA frameshifts, eventually resulting in gene inactivation, while the HDR pathway helps replacing the mutated/faulty sequence with the correct one. To initiate HDR, thanks to the assistance of a donor DNA template, the right DNA sequences are incorporated into the desired site [22]. In addition, HDR is limited to the G or S phase, while NHEJ can occur in every cell cycle phase. Generally, the HDR pathway provides a highly reliable DNA repair mechanism, though its efficiency is lower than that of the NHEJ pathway. There are three possible ways to edit the intended gene with the help of the CRISPR/Cas9 system: purified Cas9/sgRNA complexes, plasmid-borne CRISPR/Cas9 system, or a combination of Cas9-mRNA and sgRNA. As summarized in Table 1, each strategy has its own merits and disadvantages.

Promising role of CRISPR/Cas9 in AD

Genetic mutations account for approximately 1% of familial cases of AD; thus, genome editing with CRISPR/Cas9 may be useful in familial AD (FAD) at large with minimal or negligible benefits in sporadic AD (SAD). However, considering the involvement of dysregulated Aβ metabolism in FAD and SAD, limiting Aβ production may offer a therapeutic approach independent of the onset, whether familial or sporadic (Fig. 4). Table 2 presents a brief summary of studies that demonstrated the benefits of CRISPR/Cas9 technology as an experimental therapeutic approach in FAD and SAD.

Prospect in early-onset AD models

Treatment with CRISPR/Cas9 might not be appropriate because the onset and progression of most AD cases are sporadic and involve unknown triggers. Actually, only in a few AD cases (<1%), there are real or known mutations in associated genes, that lead to APP production facilitating APP processing to generate Aβ. However, mutations constitute only a small fraction of AD cases, but elicit increased Aβ generation [31]. Similarly, mutations in the presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes also cause early AD onset [32,33], as they result in accelerated Aβ1-42 production, possibly by altering the APP cleavage site [34]. In most cases, these mutations are usually evident in < 60 years of age and hence categorized as early onset AD. The CRISPR/Cas9 technique can significantly correct these autosomal dominant mutations. Recent studies also support the potential of this gene editing system, which has been reported to correct similar types of mutations. For instance, the CRISPR/Cas9 system was utilized in basal forebrain cholinergic induced pluripotent stem cells (iPSC)-derived neurons from a PSEN2N141I mutated individual for the correction autosomal dominant mutations [35], resulting in correction and stabilization of the Aβ42/40 ratio. Furthermore, the PSEN2 mutation corrected by this editing system also reversed electrophysiological deficits. Previous studies wherein CRISPR/Cas9 was utilized to fix PSEN gene mutations in FAD using iPSCs derived from the patient further supported these results [36,37]. Another study reported that this system helps knocking out Swedish APP mutations in patient-derived fibroblasts finding a 60% Aβ reduction [38]. The Swedish mutation is known to promptly adjoin the β-secretase site in APP [39]. The researchers also interrupted this mutation in Tg2576 mice, which displayed a manifold APP Swedish mutation. To accomplish this, DNA encoding both guide RNAs and Cas9 in AAV vectors was injected into the hippocampus of transgenic mice. Following injections, disruptions such as single-base pair insertions in the APP Swedish gene were observed. However, we need to understand whether such manipulations can improve the behavior and pathology deficits in Tg2576 mice. Notably, when CRISPR/Cas9 was directly injected into the hippocampus, it resulted in only 2% of transgenes disrupted on the injection site [38]. This can be attributed to the fact that Tg2576 mice have approximately 100 transgene copies per neuron, and therefore the injected CRISPR/Cas9 levels appear insufficient to correct the Swedish mutation. While this analysis promises tantalizing developments, an overall more systematic study of the targeted hippocampal cells is required to recognize and improve the editing efficiency and translate the effects *in vivo*.

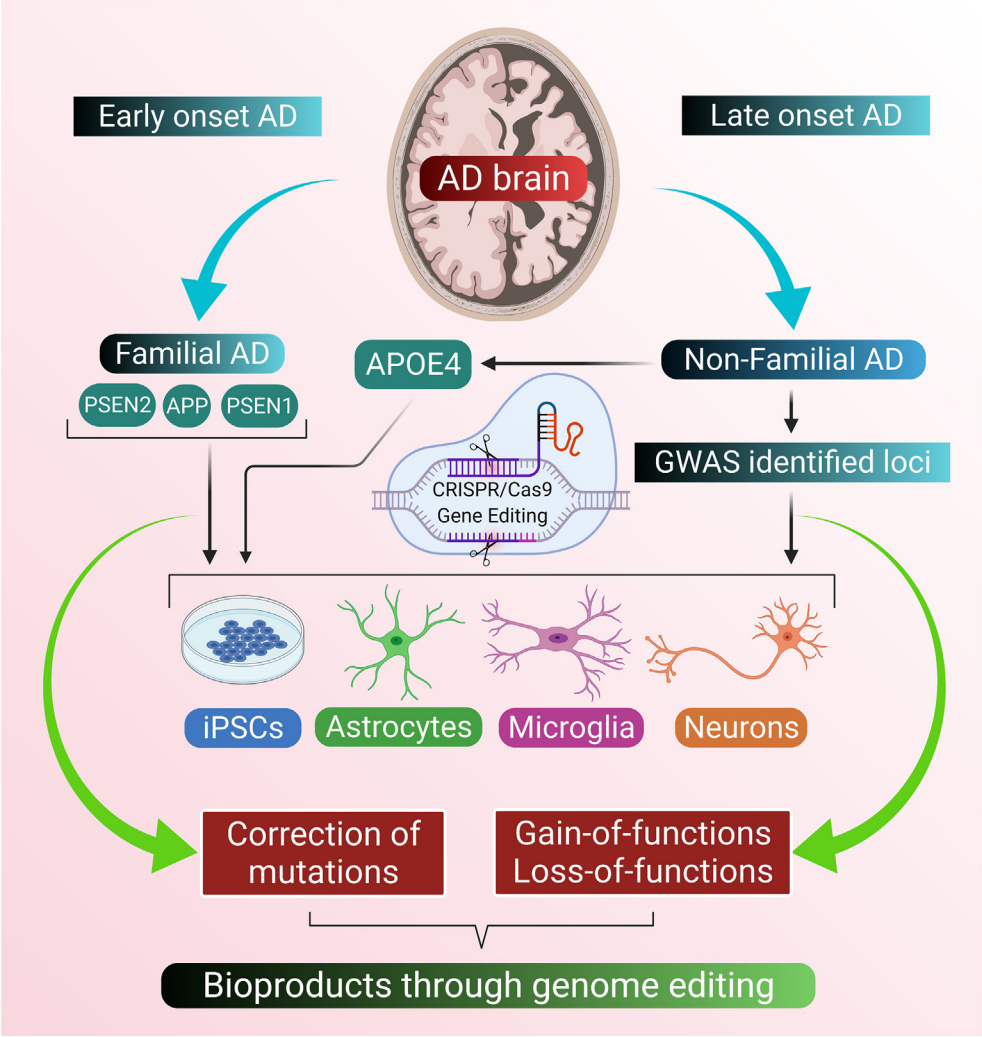

Fig. 4. Illustration showing the possible CRISPR/Cas9 mediated gene editing approach in AD. GWAS, genome-wide association studies.

Table 2
Summary of various reported studies on AD treatment involving CRISPR/Cas9 technique.

Delivery system	Targeted genes	FAD or SAD	Cell lines	Animals tested	Reference(s)
Lentiviral CRISPR/Cas9 system	*APP* at the extreme C-terminus	FAD	-HEK293 -neuro2a cells	Injections administered in the dentate gyrus of *C57BL/6* mice	[28]
R7L10 peptide, a component of nanocomplexes complexed with Cas9-sgRNA ribonucleoprotein	BACE1	FAD	-Mice embryos derived primary neurons -Human induced pluripotent human stem cells and human embryonic stem cells -GFP^{+} HEK293T cells	Injections into the hippocampus:- *−5xFAD* transgenic mice co-expressing 5 FAD mutations - *APP* knock-in transgenic mice	[25]
Recombinant adeno-associated virus (rAAV) mediated delivery of CRISPR/Cas9	*KM670/671NL* APP (APP^{swe}) mutation	FAD	-APP^{swe} fibroblasts in Human -Tg2576 mice embryos derived primary neuronal cells	Injection into hippocampus of *Tg2576* mice	[23]
CRISPR/Cas9 delivered via lentivirus	*APOE E4*	SAD	-Mouse astrocytic cells expressing the human APOE3 or APOE4 gene	NA	[29]
Cytidine deaminase enzyme conjugated CRISPR/Cas9 plasmids	*APOE E4* (converting it into *APOE3r*)	SAD	-HEK293T cells -Immortalized mouse astrocytes containing the APOE E4 isoform of the APOE gene	NA	[30]

Prospective in sporadic AD models

These findings paved the way for evaluation of CRISPR/Cas9 gene editing in subjects with early AD onset, but what about SAD, which by far, represents the vast majority of cases in the USA? To check the efficacy of this gene editing technology in SAD, Sun et al. demonstrated editing of endogenous APP at the extreme C-terminus to mitigate β cleavage and Aβ generation [27]. In doing so, they inhibited further interactions with BACE1 within endosomes, thus avoiding the most important cleavage

event in Aβ generation [40]. Findings in human iPSC-neurons, cultured neurons, cell lines, and mouse brain showed that this limits the physical contact of APP and BACE1 and therefore mitigates Aβ generation [28]. The APOE4 allele is another significant risk factor contributing to late-onset AD [41]. APOE exists in three isoforms: APOE2, APOE3, and APOE4. All of them vary only by substitution of a single amino acid leading to the replacement of cysteine-arginine at positions 112 and 158 [42]. And different isoforms have different properties, for instance, the possibility of developing AD is reduced by up to 40% by one copy of the E2 allele, which is the rarest form of APOE. APOE3, one of the common alleles, does not appear to affect the risk of AD, while APOE4 present in approximately 10–15% of the people, reduces the age of AD onset, and enhances the risk of AD [43]. Risk, which can increase by 2–3-fold if individuals carry a single copy of E4 (E3/E4), whereas double copies of E4 (E4/E4) may enhance it by approximately 10–15 times. Actually, 65–80% of all patients diagnosed with AD have $\geq$ 1 APOE4 allele [43,44]. Although several APOE4 adverse effects appear linked with Aβ accumulation, a recent study suggests that Tau phosphorylation in neurons derived from human iPSCs may be promoted by APOE4, independent of Aβ [45,46]. In this study, gene editing by zinc-finger nuclease to converts APOE4 to APOE3, prevented APOE4-linked pathology in their model system [46]. Hence, CRISPR/Cas9 could also act as a potential editing tool to transform APOE4 to APOE2 or E3. Notably, a study points towards important APOE4 structural features, which differentiate it from APOE2 to E3, and involves a domain interaction facilitated through a salt bridge between amino acids Arg-61 and Glu-255 [47]. Thus, altering one of these amino acids with CRISPR/Cas9 can effectively neutralize the risk related to the APOE4 allele. Further, Table 3 addresses the therapeutic role of CRISPR/Cas9 in correcting specific gene sequences.

CRISPR/Cas9 delivery system: A possible way to target AD

In the management of AD, CRISPR/Cas9 genome-editing holds promise. Although efficient and safe delivery systems are still lacking, it is a vast task that requires translation of this technological approach into real therapeutic applications. To date, viral and non-viral methodologies for the delivery of the CRISPR/Cas9 systems are available.

Viral vectors for CRISPR/Cas9

The use of viral vectors for delivering CRISPR/Cas9 is a classical method in experimental models, including cell lines and animals, and one of the most dynamic systems for the targeted delivery of plasmid-based CRISPR/Cas9. Moreover, they may incorporate mutations that have significant adverse effects. Adeno-associated virus (AAV) are a frequently used vector because of its high infectivity, low immunogenicity, and low integration into the human genome [24,25]. With > 200 variants, the AAV genome comprises single-stranded DNA [26]. One study reported the use of two separate AAV vectors packaging APPsw-specific gRNA and Cas9 targeting the AD-causing KM670/671NL APP mutation. The viruses were tested *in vitro* in primary neuronal cells from Tg2576 mouse embryos and *in vivo* via intrahippocampal injection in Tg2576 mice. This treatment reduced Aβ generation by approximately 60% in human fibroblasts [38]. Due to the low packing capability of the 4.7 kb of AAV, co-injections of two viruses might be required. However, this would further complicate the procedure as both can not concurrently infect the same cell. Lentivirus incorporate long DNA inserts comprising 8–10 kb but with lower brain disseminating efficiency [27]. But, contrary to AAV, lentiviruses cannot be so easily produced in large quantities, and can be incorporated into the human genome, thereby provoking immune reactions [25]. However, studies have demonstrated the possible use of lentivirus to target three genes, namely, APOE4, APP, and caspase-6, in SAD and familial AD [28-30].

Non-viral vectors for CRISPR/Cas9

Non-viral vectors have been found promising for the targeted delivery of CRISPR/Cas9, attributed to better cost effectiveness, relative ease, feasibility, and flexibility. Consequently, they are extremely suitable for application in AD. It is very easy to form nanocomplexes by combining positively charged CRISPR/Cas9 peptides with negatively charged nucleic acid cargo. Comparatively, they are less immunogenic than viral vectors; they can also assist in numerous applications, as they are compatible with ligands. Although delivering nanocomplexes into the brain is challenging, as they are unable to properly cross the blood brain barrier (BBB) via systemic administration, and the reticuloendothelial system (RES) also actively removes them from the blood. Hence, intracerebroventricular and intrathecal injections were used as standard. However, in direct injection methods, multiple injections are needed to ensure proper distribution across the brain, thereby restricting their application. Park et al. used a Cas9–sgRNA ribonucleoprotein specifically targeting BACE1 complexed with nanocomplexes made of R7L10 peptide [49], and reported successful targeting, reducing BACE1 expression without any substantial off-target mutation *in vivo*. In addition to CRISPR/Cas9 delivery, several other vehicles can transport short interfering RNAs (siRNAs) for targeting AD across the BBB. Recent reports have used polymeric nanocomplexes of Poly(mannitol-polyethyleneimine) (PMT) carrier amended with rabies infection glycoprotein (RVG) [27]. The polymer was designed to form a complex with siRNA to target the BACE1 gene. The nanocomplexes were intended to possess enhanced transmission ability owing to the presence of the RVG ligand, which ameliorates permeation across the BBB and focuses on nerve cells. Polyethylene glycol (PEG) reduces transfection efficiency by creating a positively charged protection that prevents linking to cell membranes. It was proposed that this issue could be resolved using the RVG ligand, thereby ameliorating the cellular uptake of the nanocomplexes. The decrease in Aβ1-42 cortical levels further validated the silencing ability of nanocomplexes. However, considerable therapeutic potential loss may result from unknown body distribution, therefore, the suitability of such a delivery approach should be investigated in different AD models.

The following systems are promising for applications in AD. DNA nanoclews could deliver the Cas9–sgRNA complex. The traditional assembly of DNA nanostructures based on base-pairing is complex and time-consuming. Sun et al. reported that DNA nanoclews are confined nanosized DNA moieties that comprise polyethylenimine for applying a positive charge for endosomal seepage and improved cellular uptake [60]. Nanoclews bearing the sgRNA-Cas9 complex targeting enhanced green fluorescent protein (EGFP) were injected locally into tumor-bearing mice. After ten days of treatment, this resulted in a 25% reduction in EGFP expression [60]. However, nanoclews might induce an immunogenic response, which needs to be studied further. Importantly, polymeric nanoparticles and lipid nanoparticles also exhibit potential as CRISPR/Cas9 delivery tools. These nanoparticles have been largely utilized for carrying gene editing tools in hepatitis, cancer, and several viral infections [61,62]. However, their potential use in AD requires further investigation. In addition, Wang et al. used AuNPs for investigation [63]. The CXCR4 gene targeted with CRISPR-Gold achieved HDR efficiency about 3–4% in many types of human cells. Furthermore, local infusion of CRISPR Gold into

the gastrocnemius and tibialis frontal muscles of mdx mice resulted in correction of the mutated dystrophin gene, causing inborn Duchenne muscular dystrophy [63]. However, after CRISPR Gold injection, no meaningful changes in the inflammatory cytokine profile were found, indicating low toxicity. Recently, attention has been drawn to the therapeutic delivery of CRISPR/Cas9 through microvesicles. Typically, a "producer" cell line is transfected with a microvesicle-prompting protein (RAB proteins), sgRNA, and Cas9 proteins [64]. The cells produce microvesicles consisting of Cas9–sgRNA complexes, which are shed into a medium subsequently sterilized and used to deliver gene editing tools to the target cells.

CRISPR/Cas9 technique: A boon in the bag for neurodegenerative diseases

In the previous section, we extensively elaborated on the possible role of CRISPR/Cas9 in the treatment of AD. However, it is also important to address the implications of this technology in other NDDs and for the management and treatment of brain abnormalities. Recently, this technology has been implemented in the treatment of several neurodegenerative disorders, including PD and HD, as discussed in the following sections.

Parkinson's disease (PD)

PD is an age-related, progressive, multifactorial and one of the most common neurodegenerative condition, manifested by motor and non-motor motor indications. Proper treatment for PD is still lacking because the pathological mechanism and signaling axis responsible for disease progression are not fully understood. However, CRISPR/Cas9 might be helpful in the identification of pathways and proteins associated with PD pathogenesis (Table 4). Additionally, this technology may also help in the identification of complex interactions between human genetics and environmental factors leading to PD [65]. Single nucleotide polymorphisms (SNP) variants in the SCNA gene (α-synuclein) were analyzed by Soldner et al. using this system, and they reported the presence of a non-coding distal enhancer element common variant responsible for the enhanced SCNA expression [66]. This tool also helps scientists in PD research to produce isogenic cell lines for PD modeling and hence could contribute to analyzing PD phenotypes. In this regard, Arias-Fuenzalida et al. applied fluorescent markers and the CRISPR/Cas9 system to obtain biallelic genome-edited cell populations. They called this approach FACS-assisted CRISPR/Cas9 editing (FACE). The FACE method helps in deriving a set of isogenic cell lines with mutated α-synuclein associated with PD [67]. On the other hand, mutations in leucine-rich repeat kinase 2 (LRRK2) are the most common genetic cause of sporadic and familial PD, which causes toxicity in dopaminergic neurons. The CRISPR/Cas9 tool was used to edit mutated LRRK2 and reduced neurite complexity in dopaminergic neurons and the incidence of both sporadic and familial PD [68].

CRISPR has also recently been recognized as a promising system to assist in the understanding of the interplay between PD genes and the identification of new apoptotic cascades directly or indirectly associated with PD. For instance, a Parkin regulator, THAP11, was recently validated in CRISPR/Cas9 knockout research in various cell types, to find previously undiscovered regulatory crosstalk/networks [69]. This system has also been employed to study the neuroinflammatory mechanisms associated with PD. For instance, protein kinase Cδ (PKCδ) signaling linked with Mn-induced apoptotic cell death in PD involves PKCδ activation. CRISPR/Cas9-mediated PKCδ downregulation in dopaminergic neurons (DA) neurons, considerably hindered the DNA fragmentation induced by Mn. In addition, this system can also help understand the PKCδ downstream pathway driving apoptosis [70].

The role of CRISPR/Cas9 was also reported by Gordon et al. in the elimination of Prokineticin-2 (PK2). In PD, altered PK2 has been observed to enhance neuronal susceptibility to neurotoxic-stimulated cell death following neuroinflammatory mechanisms. They also reported higher expression of PK2 in PD post-mortem brains, and served as a protective compensatory response against neurodegeneration in cell culture and PD animal models [71].

Glia maturation factor (GMF), a neuroinflammatory and microglia-associated protein is abundantly expressed in the brain, with increased expression in the substantia nigra (SN) of PD brains [72]. Selvakumar et al. applied this system to investigate the impact of GMF editing in microglial cells under oxidative stress conditions and Nrf2/HO-1-dependent ferritin activation. They noticed that GMF knockout in microglial cells weakened oxidative stress by curtailing reactive oxygen species (ROS) production and lowering calcium flux. Moreover, the absence of GMF reduced Nrf2 nuclear translocation, thus preventing microglia activation

Table 3
Overview of reported clinical trials on AD therapeutics using CRISPR/Cas9 technique.

Mutations may fixed with CRISPR/Cas9	Targeted genes for CRISPR/Cas9	Clinical outcomes	Reference(s)
PSEN2^{N141I} mutation	*PSEN2*	Decreased Aβ42/40 ratio	[48]
Manipulation in Aβ-linked pathologies	*BACE1*	Considerable downregulation of Aβ42 plaque aggregation in mice	[49]
NHEJ-mediated exon removal	*MAPT*	Production of new Tau knockout strain (tauΔex1) in mice	[50]
HDR-mediated mutation	*PSEN1M1*	Disease models generated by CRISPR	[51]
HDR-mediated mutation	*APPS*	Disease models generated by CRISPR	[51]
Reciprocal manipulation of the amyloid pathway	*APP*	Attenuation of β-cleavage and Aβ production	[28]
N141I	*PSEN2*	Normalization of enhanced levels of Aβ42/40 via CRISPR/Cas to correct mutation in PSEN2^{N141I}	[35]
Met146Val	*PSEN1*	Establishment of homozygous and heterozygous mutations	[52]
Conversion of APOE E4 to APOE E3	*APOE*	Arg158 converted to Cys158 in 58–75%	[53]
Mutations in L52P, T48P and K53N	*APP*	A model to investigate outcomes of APP mutations in cleavage of γ-secretase and Notch signaling	[54]
Deletion of Swedish mutation	*APP*	Reduction in *ex vivo* and *in vivo* production of Aβ peptide	[38]
Glia maturation factor (GMF)	*GMF*	Reduction in GMF and p38 MAPK	[55]
3′-UTR amyloid precursor protein (APP)	*APP*	Reduction in APP and Aβ	[56]
γ-Secretase activating protein (GSAP)	*GSAP*	Reduction in GSAP, γ-Secretase activity and Aβ	[57]
β-secretase 1 (BACE1) and Tyrosine hydroxylase (Th)	*BACE and Th*	Reduction in BACE1, Th1 and Aβ	[58]
APOE-E3/E4 APOE	*APOE*	Reduction in APOE-E3/E4 (high risk for AD), hyper-phosphorylation of Tau protein and amyloid deposition and upregulation in APOE-E2/E2 (low risk for AD) and turning APOE4 to APOE3 is increased	[59]

Table 4
Overview of reported clinical trials on PD and HD therapeutics using CRISPR/Cas9 technique.

Target genes	Mechanisms	Main findings	*In vitro* or *in vivo*	Disease	Reference (s)
SNCA	CRISPR-mediated SNCA deletion in human embryonic stem cells (hESCs)	Reduction in SNCA alleles	hESCs		[82]
-Parkin -SNCA	CRISPR/Cas9-based gene targeting in α-Syn preformed fibrils (PFF cells)	The use of gene-targeted somatic cells as a donor for somatic cell nuclear transfer (SCNT) to generate gene-targeted animals with single and identical mutations	PFF cells		[83]
SNCA	Fluorescent markers derived biallelic genome-editing	Examine a set of isogenic lines comprising PD-linked α-Syn in mutation	*In vitro*	PD	[84]
LRRK2	The Cre-LoxP recombination system has been utilized to alter the LRRK2-G2019S mutation in the human induced pluripotent stem cells (hiPSCs) to produce isogenetic controls	Production of an edited footprint-free LRRK2-G2019S isogenic hiPSCs	hiPSCs		[68]
-DJ-1 -Parkin -PINK1	Co-injection of multiplexing sgRNAs and Cas9 mRNA into *in vivo* derived pronuclear embryos	Reduction in DJ-1, Parkin, and PINK1	*In vivo*		[85]
The polyglutamine repeat in the huntingtin gene (HTT)	CRISPR/SpCas9 technique	Non-allele specific CRISPR/Cas9 mediated permanent elimination of polyQ domain of mHTT to reduce neuronal toxicity in the adult brain	Mouse striatum		[86]
Single nucleotide polymorphism (SNP) sites	Allele specific CRISPR/Cas9	Specific CRISPR/Cas9 alleles mediated permanent inactivation of Huntington's mutation allele	Fibroblasts cells	HD	[87]
Transient receptor potential canonical 1 (TRPC1)	CRISPR/Cas9 system	CRISPR/Cas9 mediated TRPC1 Inhibition may serve as a neuroprotective tactic in the treatment of disease	YAC128 HD mice/ HEK293T cell line		[88]
The SNPs at upstream and downstream (Intron1) of HTT exon-1	CRISPR/SpCas9 technique	SNPs are identified that either accountable for causing or destroying PAM motifs critical for CRISPR-selective editing of one allele versus the other in cells from HD patients and in a transgenic HD model harboring human allele	Fibroblast and HEK293 cell		[77]
5′-DNA at the uORF region/exon1-intron region of the mHTT	CRISPR/Cas9 mediated silencing of the mHTT gene *in vitro*	Reduction in mHTT production	Plasmids used in MSCs extracted from the YAC128 mice bone marrow, which carries the transgene for HD		[89]

and reducing the expression of pro-inflammatory molecules in the brain [73].

Both monogenic recessive and dominant-negative disorder-induced mutations could be corrected or inactivated by the CRISPR/Cas9 system. In DA neurons of PD animal models, CRISPR/Cas9 has been reported to inactivate and deplete mutated genes that express mutant SNCA [74]. For instance, Chen et al. used this system to delete the SNCA gene in hESCs and slowed the progress of this pathological outcome associated with SNCA [75]. However, more clinical research is still required to validate these results and find the proper treatment of PD involving this technology.

Huntington's disease (HD)

HD is an autosomal dominant trinucleotide repeat neurodegenerative disorder caused by the expansion of CAG repeats in the Huntington (HTT) gene, which encodes a mutant HTT protein (mHTT). Expansion of the CAG trinucleotide segment consists of glutamine residue repeats (PolyQ), which makes the mutant HTT protein longer than normal. CRISPR/Cas9 can selectively suppress mHTT expression through direct interaction with the DNA. Furthermore, the production of mHTT can be reduced by suppressing its endogenous expression in the striatum of mHTT-expressing mice [76]. In another study, SNPs with either causative or destructive effects on PAM motifs were critical in the selection of one allele for CRISPR editing vs. the other to enhance the efficiency of Cas9 nuclease and apply CRISPR strategy for therapeutic purposes of HD [77]. The presence of the mHTT gene leads to sensitization of type 1 inisitol 1,4,5-triphosphate receptor (InsP3R1) and, consequently, calcium outflow from the ER and a compensatory elevation in neuronal store-operated calcium (nSOC) entry, ultimately resulting in synaptic damage of striatal MSNs in an HD animal model [78]. A transient receptor potential canonical 1 (TRPC1) knockout (which is one of the nSOC components) using CRISPR/Cas9 resulted in improved motor performance and salvage of MSN spines *in vivo* and *in vitro* [79]. Further studies proposed for the regulation of HTT protein synthesis, the 5 untranslated region (UTR) plays a major role as it contains an uORF that encodes 12 amino acid long potential polypeptide that controls downstream ORF translation. Hence, the expression of Huntingtin mRNA is negatively affected by the presence of uORFs [80]. Likewise, previous studies have proposed that disruption of the uORF in the 5UTR of mRNA using CRISPR/Cas9 may lead to decreased translational products of mutant huntingtin gene in MSCs derived from HD mouse models. Kolli et al. studied mHTT inhibition with two types

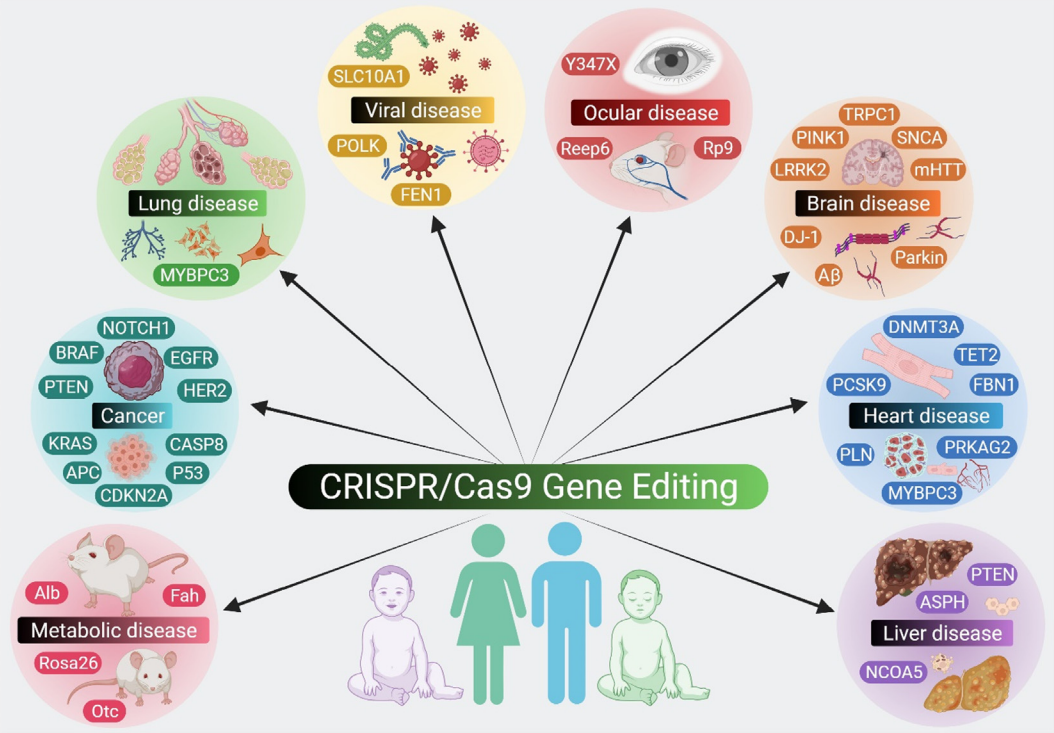

Fig. 5. The diverse applications of CRISPR/Cas9 technique in human diseases. Here in this schematic, we have highlighted the disease associated various genes and proteins which may be one of the possible target for this gene editing strategy. MYBPC3, myosin binding protein C3; BRAF, B-Raf proto-oncogene, serine/threonine kinase; PTEN, phosphatase and tensin homolog; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; CASP8, caspase-8; CDKN2A, cyclin-dependent kinase inhibitor 2A; SLC10A1, solute carrier family 10 member 1; TRPC1, canonical transient receptor potential; PINK1, PTEN-induced putative kinase 1; LRRK2, leucine rich repeat kinase 2; SNCA, α-synuclein; mHTT, mutant huntingtin protein; DJ-1, PARK7; DNMT3A, DNA methyltransferase 3a; FBN1, fibrillin-1; PCSK9, proprotein convertase subtilisin/kexin type 9; PLN, phospholamban; PRKAG2, kinase AMP-activated noncatalytic subunit-2; ASPH, aspartate beta-hydroxylase; KRAS, kirsten rat sarcoma viral oncogene homolog; APC, adenomatous polyposis coli; p53, tumor suppressor gene; FEN1, flap endonuclease 1; TET2, epigenetic modifier enzyme; NCOA5, nuclear receptor coactivator 5; Y347X, nonsense point mutation; Reep6, receptor expression-enhancing protein 6; Rp9, pre-mRNA splicing factor; Alb, albumin; Fah, fumarylacetoacetate hydrolase; Otc, Ornithine transcarbamylase; POLK, DNA Polymerase Kappa.

of CRISPR/Cas9 system, one that cuts the DNA at exon1 – intron boundary, and the other in the untranslated uORF region, and found that CRISPR/Cas9 mediated mHTT silencing affects the mHTT translational process and reduces mHTT production in bone-marrow-derived mesenchymal stromal cells (BM-MSCs) [81]. Further, Table 4 addresses the therapeutic potential of CRISPR/Cas9 in NDDs such as PD and HD.

Concluding remarks, major challenges and future perspectives

As a gene-editing tool, CRISPR/Cas9 holds promise by correcting specific gene sequences and having significant potential for the treatment of AD and other human diseases [90-96] (Fig. 5). In SAD and FAD, an altered Aβ metabolism is commonly observed, irrespective of genetic factors. Thus, CRISPR/Cas9 technology could correct increased Aβ production or mutations in APP, PSEN-1, and PSEN-2, as mutations in these genes are a causative factor of FAD.

There are numerous challenges related to the efficiency of AD management following CRISPR/Cas9 brain delivery via non-viral vectors. Preferably, the vectors should be steady and able to efficiently carry the load to the desired site. When the targeted cells are approached by the vectors, they should be internalized to avoid lysosomal degradation and target the nucleus.

It is essential to consider the large size of CRISPR/Cas9 for future designs and application. Instead of plasmid-assisted delivery approaches, the Cas9-sgRNA complex is preferred because of its smaller size. Moreover, owing to the circulating proteases and nucleases, the constituents of the developed formulations are vulnerable to degradation. Although widely employed to lessen the recognition of these systems by RES, PEGylation can generate specific PEG-antibodies and reduce cellular uptake, leading to immunogenic responses [97]. For *in vivo* applications, non-viral vectors are preferred, but improving the diverse formulation features limits real-life applications.

The systemic route is widely studied because of its *in vivo* feasibility, specifically in the case of AD patients, depite the issues regarding delivery vector's stability and targetability. Hence, intracerebroventricular and intrathecal injections are usually adopted for administration. Stereotaxic microinjection surgery has been used for gene delivery in the brains of experimental animals with PD [98]. However, for AD, this procedure can be challenging because of the widespread nature of Aβ pathology. Another promising approach is the intranasal route as it bypasses the BBB. However, more clinical studies are needed on the nasal delivery of CRISPR/Cas9 based therapeutics.

To ensure the safety of CRISPR/Cas9 therapeutics, further research is essential, as genome editing is irreversible. Furthermore, studies investigating possible off-targets and long-term effects are still insufficient, and ethical considerations are needed before any application in humans. Importantly, the CRISPR/Cas9 tool affects somatic rather than germline cells. Hence, gene editing would not be transferred to subsequent generations and only manifest in the individuals undergoing treatment [23].

Although CRISPR/Cas9 involves double-stranded DNA breaks, the recent prime editing can rectify gene mutations without double-strand breaks. Instead, prime editing involves an impaired Cas9 following catalytic processes bonded to a reverse transcriptase, with guidance provided through a prime-editing

guide RNA (pegRNA). Thus, directing the system to the DNA site targeted for the required correction [64]. Further studies are required to establish off-target and potential of this new technology.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Shanu Bhardwaj: Writing - review & editing, Formal analysis. **Kavindra Kumar Kesari:** Writing - review & editing, Formal analysis. **Mahesh Rachamalla:** Writing - review & editing, Formal analysis. **Shalini Mani:** Writing - review & editing, Formal analysis. **Ghulam Md. Ashraf:** Writing - review & editing, Formal analysis. **Saurabh Kumar Jha:** Writing - review & editing, Formal analysis. **Pravir Kumar:** Writing - review & editing, Formal analysis. **Rashmi K. Ambasta:** Writing - review & editing, Formal analysis. **Harish Dureja:** Writing - review & editing, Formal analysis. **Hari Prasad Devkota:** Writing - review & editing, Formal analysis. **Gaurav Gupta:** Writing - review & editing, Formal analysis. **Dinesh Kumar Chellappan:** Writing - review & editing, Formal analysis. **Sachin Kumar Singh:** Writing - review & editing, Formal analysis. **Kamal Dua:** Writing - review & editing, Formal analysis. **Janne Ruokolainen:** Writing - review & editing, Formal analysis. **Mohammad Amjad Kamal:** Writing - review & editing, Formal analysis. **Shreesh Ojha:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Formal analysis. **Niraj Kumar Jha:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contributions

NKJ and SO conceptualized the study and hypotheses. SB, KKK, MR, and SM performed literature search. NKJ draw the schemes and drafted the artwork. DKC, SKS, KD, JR, MAK drafted the tables. NKJ, and other authors contributed significantly in editing the manuscript. GMA, SKJ, PK, RKA, HD, HPD and GG significantly contributed during revision. All authors read, edited and approved the manuscript.

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Consent for publication

All authors have read the final version of the manuscript and have given their consent for publication.

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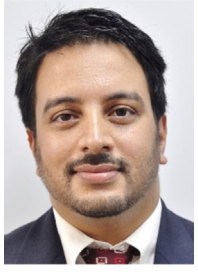

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