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RNA interference therapeutics for amyotrophic lateral sclerosis

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Highlights:

- This review summarizes RNAi therapy for ALS: target genes, molecules, and delivery methods.
- Potent SOD1 silencing in preclinical models, yet limited studies on other ALS genes.
- Major bottlenecks: CNS delivery efficiency, immunogenicity, off-target effects.

Abstract: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, for which gene therapy provides a potential therapeutic strategy. Among various approaches, RNA interference (RNAi) technology has garnered considerable attention. This review summarizes the research progress of RNAi technology for ALS, including the pathogenic genes, effector molecules, and the application of vector delivery systems. Meanwhile, we note that current research has predominantly focused on the superoxide dismutase 1 (*SOD1*) gene, while studies on RNAi strategies targeting other core ALS-causing genes remain relatively scarce. Furthermore, technical challenges persist, including immunogenicity and off-target effects, *etc.* This review concludes that optimizing the specificity of RNAi molecules and delivery systems, expanding targeted genes, and balancing potency with safety are the core directions for future research.

Keywords: RNA interference; amyotrophic lateral sclerosis; motor neuron disease; neurodegeneration

1. Introduction

Globally, the incidence of amyotrophic lateral sclerosis (ALS) is approximately 1–2 per 100,000 people per year, with a median survival time of only 3–5 years from symptom onset to death [1]. Sporadic cases account for 90% of ALS, whereas familial cases represent merely 10% [2]. Typical ALS is primarily characterized by the progressive degeneration of both upper and lower motor neurons (UMN and LMN) [3] resulting in spasticity, clumsy movements, muscle atrophy, decreased tendon reflexes and so on [4]. Other clinical manifestations include cognitive and behavioral dysfunction, where executive function is the most commonly affected cognitive domain and behavioral abnormalities are common neuropsychiatric manifestations [5]. While primary lateral sclerosis (PLS) is characterized predominantly by UMN

degeneration, with extremely slow progression and prolonged survival, progressive muscular atrophy (PMA), mainly involves LMN degeneration, which is more common in males, and approximately 30% of patients develop UMN signs in the later stage [3]. The diagnosis of ALS is mainly based on the El Escorial Criteria, the Revised El Escorial Criteria and the Awaji Criteria, and the introduction of the Gold Coast Criteria has improved the sensitivity of early diagnosis [6,7].

Although more than 80 clinical trials have been conducted in the past few decades, most of them have failed [8–10]. The riluzole was approved for clinical use in 1995 [11]. It exerts neuroprotective effects by blocking glutamatergic transmission in the central nervous system (CNS), yet it can only prolong patients' survival by 2–3 months [12]. Edaravone, a free radical scavenger and antioxidant, protects the nervous system against oxidative stress damage by eliminating free radicals [13]. Approved in 2017, this drug also shows fairly limited clinical efficacy [14]. The reason for the failure is that the early research on the treatment strategy did not target its molecular mechanism.

The discovery of ALS-related genes such as superoxide dismutase 1 (*SOD1*), fused in sarcoma (*FUS*), *etc.* reveals the genetic heterogeneity of ALS and provides molecular targets for precise treatment [15]. The $SOD1^{G93A}$ transgenic mouse model has also become the gold standard for ALS research. Tofersen is the first precise treatment of ALS for *SOD1* mutations. It is an antisense oligonucleotide (ASO) that degrades *SOD1* mRNA through a mechanism mediated by RNase H. Clinical trials show that it can significantly reduce the level of SOD1 protein in cerebrospinal fluid [16]. However, *SOD1* mutations account for less than 5% of all ALS cases, which suggests that our research strategy needs to be universal. Studies on familial ALS-associated pathogenic genes provide a theoretical basis for effective RNA interference (RNAi)-based therapeutic strategies [17]. As shown in Figure 1, the mechanism of RNAi pathway is as follows. Briefly, RNAi is the process in which siRNA induces RNA-induced silencing complex (RISC) to silence mRNA [18,19]. Small interfering RNAs (siRNAs) are generated by the cleavage of double-stranded RNAs by Dicer. It is 21–23 nt long and consists of a passenger strand and a guide strand. The core components of RISC are siRNA and Argonaute2 (AGO2). The guide strand of siRNA directly binds to the target mRNA, and the PIWI domain of AGO2 then exerts its endonuclease activity to silence mRNA.

In 2018, patisiran became the first Food and Drug Administration (FDA)-approved RNAi drug for the treatment of hereditary transthyroxine protein amyloid degeneration [20,21]. Givosiran and lumasiran were also approved later. These drugs illustrate the safety and effectiveness of RNAi therapy, and also provide important references for their application in neurodegenerative diseases. RNAi shows unique advantages in ALS treatment. RNAi can achieve high gene silencing [22], long duration [23], and can be designed for any known sequence of genes [24], which is of great significance for the treatment of a variety of subtypes. Despite the many advantages, the biggest obstacle to RNAi is the CNS delivery problem. ALS needs to deliver therapeutic molecules to motor neurons in the motor cortex of the brain and the anterior horn of the spinal cord. Due to the protection of the blood-brain barrier, traditional intravenous injection can hardly allow siRNA to reach the target cell [20,21]. These suggest the importance of developing a new delivery system.

This review aims to conclude the current situation, challenges and future direction of the application of RNAi technology in the treatment of ALS. We will sort out the main ALS pathogenic genes, analyze the advantages and disadvantages of different RNAi molecular types, explore the technological progress of CNS delivery systems, and evaluate the challenges faced by RNAi therapy. We hold the view that

RNAi therapy exhibits substantial application potential in the treatment of ALS. The core of ALS research lies in precision targeting, which involves three interconnected steps: designing specific molecules that target ALS-causative genes, delivering these molecules to lesion sites through vector systems, and efficiently silencing the expression of pathogenic genes to further inhibit the progressive degeneration of neurons. In the future, to achieve more effective clinical translation, it is essential to optimize the coherence of these three steps. Despite the numerous challenges, with the advancement of technologies and understanding of disease mechanisms, ALS will ultimately evolve from an incurable disorder into a manageable condition.

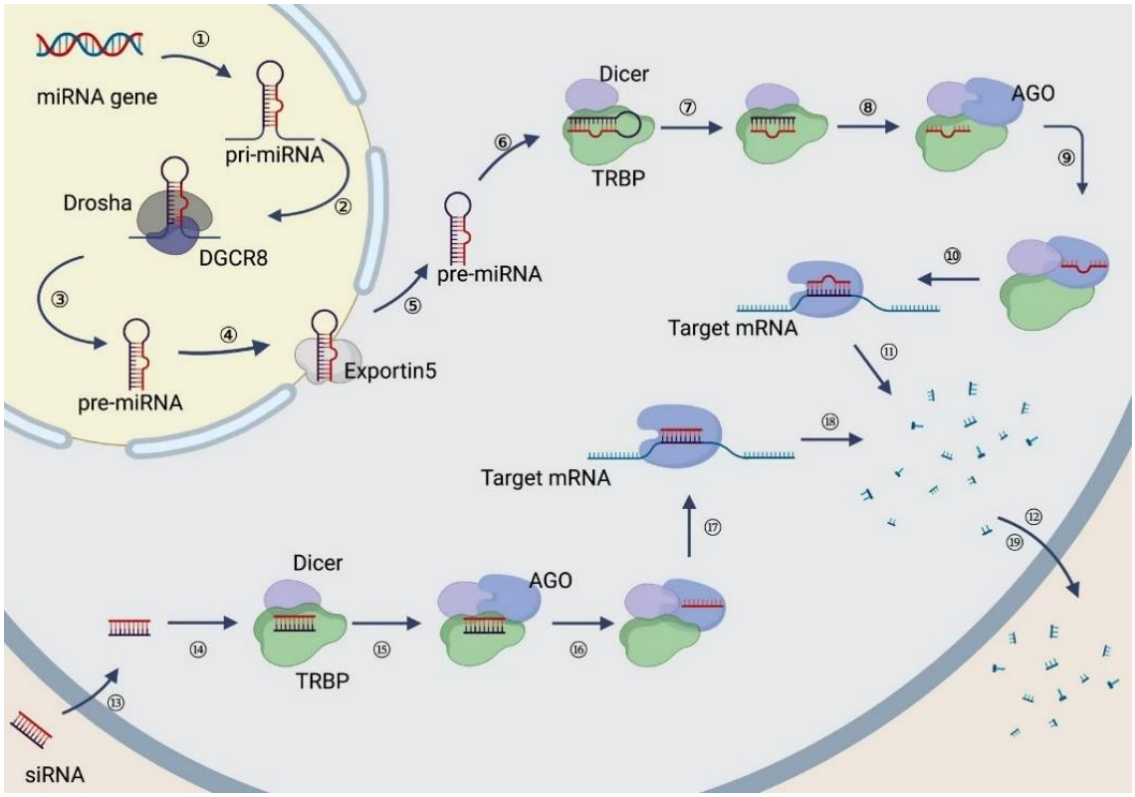

Figure 1. Mechanism of RNAi pathway. ① Mammalian microRNA (miRNA) genes are transcribed into primary miRNAs (pri-miRNAs) in the nucleus. ②③ Pri-miRNAs are cleaved into precursor miRNAs (pre-miRNAs) by the Drosha-DGCR8 complex. ④ Exportin 5 transports pre-miRNAs to the cytoplasm. ⑤ Pre-miRNAs dissociate from Exportin 5. ⑥ Pre-miRNAs bind to Dicer and trans-activation response RNA-binding protein (TRBP). ⑦ The terminal loop structures of pre-miRNAs are cleaved by Dicer to form mature miRNAs. ⑧ Dicer mediates the binding of mature miRNAs to AGO proteins. ⑨ The guide strands of miRNAs bind to AGO proteins while the passenger strands are discarded. ⑩⑪ The guide strands of miRNAs complement target mRNAs and induce their degradation, whereas they inhibit the translation of target mRNAs or trigger their deadenylation if complementarity is incomplete. ⑫ The degraded target mRNA is excreted out of the cell. ⑬ siRNAs enter the cytoplasm via endocytosis and bind to Dicer and TRBP. ⑭ In contrast, short hairpin RNAs (shRNAs) endogenous to the cytoplasm bind to Dicer and TRBP before being cleaved into siRNAs by Dicer. ⑮ Dicer mediates the binding of siRNAs to AGO proteins to form RISCs. ⑯ The guide strands of siRNAs bind to AGO proteins while the passenger strands are discarded. ⑰⑱ The guide strands of siRNAs usually achieve full complementarity to target mRNAs and induce their degradation. ⑲ The degraded target mRNA is excreted out of the cell.

2. Target genes

Generally, ALS is divided into sporadic amyotrophic lateral sclerosis (sALS) and familial amyotrophic lateral sclerosis (fALS) forms [25]. As shown in Table 1, the most prevalent disease-associated genes in ALS include *SOD1*, TAR DNA-binding protein 43 (TDP-43) and its encoding gene TARDBP (TDP-43/*TARDBP*), *FUS*, and *C9orf72*, which collectively contribute to 60% of fALS and 10% of sALS cases [26]. A critical factor in the application of RNAi therapy is the identification of appropriate targets.

2.1. SOD1

The *SOD1* gene comprises 5 exons and encodes a protein consisting of 153 amino acids. The encoded SOD1 protein is a homodimer, with each subunit containing one copper ion-binding site and one zinc ion-binding site; these metal ions are critical for the enzyme's catalytic activity [27]. Mutations in the *SOD1* gene have been identified as the underlying cause of approximately 20% of fALS cases [28]. To date, more than 200 *SOD1* mutations have been identified, including missense mutations, nonsense mutations, insertions, and deletions [29]. Different *SOD1* mutations give rise to heterogeneous clinical phenotypes, including rapidly progressive, slowly progressive subtypes and so on.

Mutant SOD1 protein forms insoluble aggregates through misfolding, a core pathological feature of *SOD1*-related ALS, and the gain-of-function of these mutation-induced toxic aggregates is precisely the critical factor driving ALS pathogenesis [27]. Numerous studies have confirmed this. For instance, transgenic mice expressing human mutant *SOD1* exhibit the major pathological and clinical features of ALS, such as SOD1 protein aggregation and progressive motor neuron loss [30,31]. Motor neurons differentiated from induced pluripotent stem cells derived from SOD1 mutation carriers exhibit ALS-associated pathological features, such as synaptic gene dysfunction, and transcriptome analyses have been performed to reveal the early molecular alterations caused by *SOD1* mutations [32]. Similarly, Garau *et al.* [33] performed RNA expression profiling on lymphoblastoid cell lines derived from ALS patients carrying *SOD1* mutations, and identified that these patients exhibited a distinct gene expression pattern involving multiple pathways associated with neurodegenerative diseases.

2.2. TDP-43/TARDBP

The TDP-43 gene/*TARDBP* harbors 6 exons and encodes TDP-43, which consists of 414 amino acids [34]. TDP-43 is synthesized in the cytoplasm, translocated to the nucleus to regulate gene expression, and primarily modulates RNA function [35]. To date, more than 50 pathogenic mutations have been identified in the TDP-43 gene, most of which are concentrated in its C-terminal low-complexity domain [36].

The association between the TDP-43 gene and ALS was discovered in 2006, when Neumann *et al.* [37] reported that TDP-43 is the major component of ubiquitin-positive inclusions in the brain tissue of patients with sALS. Almost simultaneously, multiple research groups identified pathogenic mutations in the TDP-43 gene in patients with fALS. TDP-43 dysfunction is a core pathological mechanism of ALS. In vitro and cellular studies have shown that TDP-43 mutations impair TDP-43's ability to bind to specific RNA sequences, and the nuclear clearance and cytoplasmic aggregation of TDP-43

protein are pathological hallmarks of most ALS patients [30,38]. Research by Romano *et al.* [39] found that allele-specific silencing has therapeutic potential for fALS caused by the p.G376D mutation in the TDP-43 gene, and this finding also highlights the potential of TDP-43 as a target gene for ALS. Pottinger *et al.* [39,40] conducted a rare variant analysis in a cohort of 6970 ALS patients, and the results revealed a significant association between the TDP-43 region and ALS (odds ratio [OR] = 10.08, $p = 3.62 \times 10^{-16}$).

2.3. FUS

FUS is widely expressed in both the cytoplasm and the nucleus [41]. The *FUS* gene is located on chromosome 16, contains 15 exons, and encodes a 526-amino-acid FUS protein [42]. Since its discovery in 2009, more than 50 pathogenic mutations have been identified in the *FUS* gene, which are mainly concentrated in the C-terminal region but also distributed in other exons [43].

Expression of human *FUS* in Drosophila leads to motor deficits and neurodegeneration [44], while transgenic mice expressing mutant human *FUS* exhibit motor dysfunction, among other phenotypes [31]. These findings indicate that *FUS* may have a causal relationship with ALS. Therapies targeting *FUS* serve as more direct evidence. Shneider *et al.* [45] found that the targeted regulation of *FUS* gene expression exhibits safety and preliminary efficacy in patients with *FUS*-mutant ALS. Similarly, Korobeynikov *et al.* [46] demonstrated that the therapeutic strategy of FUS gene silencing mediated by antisense oligonucleotides exhibits efficacy and feasibility in ALS-related models.

2.4. C9orf72

The *C9orf72* gene has a complex structure, contains 11 exons, and can generate multiple transcript variants via alternative splicing, encoding a protein whose function has not been fully elucidated [38,47]. The GGGGCC (G4C2) hexanucleotide repeat expansion in the *C9orf72* gene represents the most common genetic cause of ALS [48]. In the general population, the repeat number of the G4C2 fragment ranges from 2 to 23, whereas in patients, the pathogenic expansion of this fragment is characterized by more than 30 repeats [49].

The association between the G4C2 repeat expansion in the *C9orf72* gene and ALS was discovered in 2011. Bush *et al.*'s study confirmed this: the specific clearance of pathogenic G4C2 repeat expansions alleviates ALS-associated symptoms [50]. The clinical manifestations of *C9orf72*-associated ALS include various neurological and motor deficits, which can be recapitulated in Drosophila expressing repeat-expanded G4C2 fragments of the *C9orf72* gene [44]. ASO-based therapies targeting *C9orf72* repeat RNAs have been shown to reduce RNA foci, decrease dipeptide repeat protein levels, and ameliorate phenotypes in cellular and animal models in preclinical studies. Collectively, these therapeutic investigations provide direct evidence for the pathogenic role of *C9orf72* repeat expansion in ALS.

Table 1. Target genes.

Target Gene	Proportion/%		Number of Variants	RNAi Applicability	Clinical Drugs
	fALS	sALS			
SOD1	12–20 [51]	1–2 [52]	> 200 [29]	Applicable [53,54]	Tofersen, FDA-approved [55]
FUS	3–4 [56,57]	3–5 [56]	> 50 [43]	Applicable [46]	ASO Jacifusen (ION363), phase I/III [46]
TDP-43/ *TARDBP*	1–3 [56]	1–3 [56]	> 50 [36]	Applicable [58]	DNL-343, phase I [59]
C9orf72	45–50 [47]	5–10 [49]	> 30 [49]	Specialized RNAi strategies required [60]	BIIB078, Phase I [61]; WVE004, Preclinical study [62]

3. Therapeutic molecules

Once the targeting sequence is identified, the therapeutic molecules can then exert their effects.

3.1. siRNA

The double-stranded siRNAs generated by Dicer enzyme consist of two single strands of approximately 19–25 nucleotides in length, and mediate gene silencing via RISC-driven sequence-specific mRNA degradation. The guide strand of siRNAs forms perfect complementary pairing with the 3' untranslated region or coding region of target mRNAs, followed by cleavage of the target mRNA by Ago proteins within the RISC complex, resulting in its degradation [63,64].

In the application of siRNA for ALS therapy, the major challenges faced by siRNA include CNS delivery barriers, nuclease degradation, and off-target effects [65]. Weiss *et al.* [64] developed a chemically stabilized di-valent siRNA scaffold, which achieved long-acting efficacy. This novel construct can be directly injected and delivered to the *SOD1*^{G93A} ALS mouse model, extending survival to over 250 days with a single injection and further prolonging it to more than 300 days following repeated administrations. Subsequently, their follow-up studies demonstrated that the di-siRNA outperforms the antisense oligonucleotide tofersen in terms of survival extension, providing robust preclinical evidence for siRNA-based therapy for ALS [64]. Russo *et al.* [58] developed polymeric nanocarriers for delivering TDP-43-targeting siRNA for neuronal cell therapy in 2024, which effectively reduced TDP-43 protein levels. This represents a simple and clinically translatable siRNA approach, leveraging biocompatible and biodegradable carriers to provide a novel strategy for crossing the BBB. Research on siRNAs targeting *C9orf72* and *FUS* is relatively limited, and this highlights important directions for future studies: optimizing the specificity and stability of siRNA molecules, controlling off-target effects, and providing therapeutic strategies for *C9orf72/FUS*-associated ALS subtypes.

3.2. miRNA

miRNAs are a class of endogenous non-coding RNAs approximately 22 nucleotides in length. Processed by Drosha and Dicer enzymes, mature miRNAs bind to the Ago protein subfamily and mediate gene silencing by forming imperfect complementary pairing with the 3' untranslated region of target mRNAs, thereby inhibiting translation or promoting mRNA degradation [66,67]. A distinctive feature of miRNA

technology lies in its multi-targeted regulation, where a single miRNA can target multiple genes, thus exhibiting significant potential in disease diagnosis and treatment [68].

Researchers primarily adopt the artificial miRNA strategy for ALS therapy, which is delivered via AAV vectors. Selection of miRNAs is of great significance in RNAi-based gene therapy. Lau *et al.* [22] demonstrated that miR-155-based vectors achieved more potent SOD1 knockdown but exhibited a higher risk of CNS toxicity. In contrast, miR-30a-based vectors, though less potent, significantly delayed ALS-like phenotypes, extended survival, and improved safety in *SOD1*^{G93A} mice. This also provides important guiding principles for miRNA design, emphasizing the need to balance potency and safety.

3.3. shRNA

shRNA is an artificially designed RNA molecule that contains a stem-loop structure. It is processed into functional siRNA by Dicer enzyme in cells, and subsequently mediates the degradation of mRNA through the RISC [23]. Similar to miRNAs, shRNAs are typically delivered via viral vectors such as AAV, enabling sustained expression in host cells and achieving long-term, stable gene silencing effects.

However, shRNAs also face several challenges, including overexpression-associated toxicity, and the risk of immune responses. Numerous studies have demonstrated the efficacy of AAV-mediated shRNA [23,69]. Subpial injection can maintain therapeutic effects throughout the entire lifespan of mice, which is of great significance for ALS and other chronic progressive diseases. Despite the remarkable success of shRNA in targeting *SOD1*, research focusing on other major ALS-causing genes remains relatively limited, which represents a promising direction for future investigations.

4. Delivery method

Precise delivery of therapeutic molecules to target neurons is a core challenge in the treatment of ALS, while crossing the blood-brain barrier (BBB) is an even more critical bottleneck in this delivery process. To address these issues, research on delivery systems has been continuously advancing. Currently, lipid nanoparticles (LNPs), adeno-associated virus (AAV) vectors, engineered exosomes, and chemically modified RNA are all important research directions in this field, each exhibiting unique advantages alongside corresponding technical challenges. When it comes to clinical feasibility, AAV and ASO present distinct advantages in short-term feasibility, whereas engineered exosomes hold greater promise for achieving long- and medium-term clinical viability.

4.1. LNPs

Over four decades ago, research on lipid polymorphism by Pieter Cullis and his team paved the way for the development of modern LNP-RNA therapeutics [70]. LNPs represent the most widely used delivery system in RNAi therapeutics, and LNP formulations are composed of four types of lipids, including ionizable lipids, cholesterol, helper lipids, and cyclic acetylated lipids [71,72].

Owing to the constraints imposed by the BBB, the efficiency of LNPs in delivering therapeutics to the central nervous system remains low, and most relevant research has focused on peripheral delivery. For example, Amogh Vaidya *et al.* designed siRNA-selective Organ-targeting LNPs, achieving efficient and long-lasting silencing effects in the kidneys, lungs, and spleen [73,74]. While LNPs have remarkable efficacy in delivering therapeutics to peripheral organs such as the liver, crossing the BBB remains a major

challenge. However, a study by Zhang *et al.* [75] published in Nature Communications reported a novel type of LNP, which achieved mRNA delivery to extrahepatic organs via optimization of lipid composition and thus provided new insights for LNP-mediated delivery to the CNS. Similarly, in 2023, Melamed discovered that LNPs deliver mRNA to pancreatic β-cells via a transfer mechanism mediated by peritoneal macrophage exosomes, which suggests that LNPs may cross the BBB through analogous mechanisms [76].

The clinical translation of LNPs for ALS therapy faces substantial hurdles. Compared to peripheral organs like the liver, BBB penetration efficiency is significantly impaired, limiting use in CNS disorders [75,77]. Although studies have achieved sustained silencing effects with LNPs, repeated administration is currently necessary, which invariably increases the risk of immunogenicity [73,78]. Nevertheless, despite these numerous challenges, LNP-based therapeutics offer considerable advantages. For instance, LNPs are classified as non-viral vectors and exhibit superior safety profiles; they can deliver a broad spectrum of nucleic acids; and their manufacturing processes are mature, facilitating large-scale production [70,79]. Currently, LNPs are confined to local injection, and future research may explore their intrathecal administration to overcome the limitations imposed by the BBB.

4.2. AAV

AAV viral particles are icosahedral structures composed of 60 capsid proteins, with a diameter of approximately 25 nm, and can package a single-stranded DNA genome of roughly 4.7 kb [80]. One reason why AAV has become the preferred vector for gene therapy is its relatively low immunogenicity [81].

Different AAV vectors exhibit varying delivery efficiencies. For example, AAV6 vectors cannot cross the BBB, whereas AAV9 exhibits favorable therapeutic efficacy [54]. Thanks to its BBB-crossing ability and broad CNS neuronal transduction capacity, AAV9 is the preferred serotype for neurological disorder gene therapy, with extensive research conducted thereon [82,83]. Stoica *et al.* [84] reported that AAV9 vectors delivering an artificial miRNA targeting the SOD1 exerted beneficial effects on disease progression and survival in ALS mouse models, such as extending median survival and delaying hindlimb paralysis. Similar studies have been conducted with other AAV vectors; for instance, AAV5-mediated delivery of siRNAs targeting the *C9orf72* repeat expansion has successfully reduced the levels of toxic RNA aggregates and dipeptide repeat proteins [85]. Despite the robust capabilities exhibited by AAV9, the AAV-PHP.B variant, developed by Deverman *et al.* via a Cre-dependent selection strategy, demonstrated 40-fold higher cerebral transduction efficiency than AAV9 in mouse models. The insertion of a heptapeptide sequence into the VP1 capsid protein of AAV-PHP.B significantly enhanced its capacity to cross the BBB and transduce neurons in the central nervous system [86].

Compared with LNPs, AAV exerts a more durable effect, and a single AAV injection can achieve sustained gene expression lasting from several months to several years [84,87]. AAV is also capable of delivering RNAi, CRISPR, functional gene supplementation and other modalities, which provides a reference for the treatment of different ALS subtypes [85,88,89]. Since AAV is a viral vector, its immunogenicity cannot be ignored. The host's pre-existing immunity or anamnestic response to wild-type AAV viruses, as well as the specific immunity against the transgene product carried by the vector, both interfere with the therapeutic effect [90]. Future AAV research should focus on developing novel immune evasion strategies and vector-transgene optimization technologies to overcome immunological barriers and ensure the long-term efficacy and safety of gene therapy.

4.3. Engineered exosomes

Exosomes are extracellular vesicles (EVs) secreted by cells with a diameter of 30–150 nm, playing a crucial role in intercellular communication. Due to their natural biocompatibility, low immunogenicity, and ability to cross biological barriers, exosomes have received widespread attention. The membrane of exosomes is enriched in marker proteins such as tetraspanins (CD9, CD63, CD81), integrins, and major histocompatibility complex (MHC) molecules, while their interior contains proteins, lipids, mRNAs, microRNAs, and other non-coding RNAs [91]. Given the limited yield of exosomes, which makes it difficult to meet clinical application needs, researchers have specifically developed various strategies to enhance their drug delivery capacity. For instance, conjugating targeting ligands to their surface, loading therapeutic nucleic acids or drugs into their interior, or genetically editing donor cells to enable the exosomes they secrete to naturally carry therapeutic molecules [92–94].

Engineered exosomes represent an emerging field in ALS treatment, with several innovative studies reported. A study by Guo *et al.* [95] designed a CMV promoter-directed synthetic construct that enables the *in vivo* self-assembly of small extracellular vesicles (sEVs) surface-displaying the rabies virus glycoprotein (RVG) targeting ligand and encapsulating *SOD1*-siRNA. These sEVs can target the SOD1 gene and ameliorate symptoms such as weight loss, motor dysfunction, and muscle atrophy in $SOD1^{G93A}$ transgenic ALS mice. Wu *et al.* [96] extended the *in vivo* self-assembled exosome technology to the treatment of TDP-43-related neurodegenerative diseases. They also employed the *in vivo* self-assembled technology to design RVG-tagged sEVs encapsulating TDP-43-specific siRNA, which reduced the cytoplasmic aggregation of pathological TDP-43 protein, decreased the level of phosphorylated TDP-43, and improved motor function and behavioral performance in TDP-43 pathological mouse models.

These two studies demonstrate the unique advantages of engineered exosomes in ALS treatment. Non-invasive delivery via intravenous injection enables CNS targeting, avoiding the risks and inconveniences associated with intrathecal or intracerebroventricular injection. RVG peptide modification achieves efficient neuron-specific targeting and reduces off-target effects. The *in vivo* self-assembled (IVSA) platform is compatible with various siRNA sequences and targets, exhibiting broad application potential. Combination with AAV vectors allows for long-term, stable exosome production, reducing the need for repeated administration. However, despite these numerous advantages, the mass production methods of exosomes, the loading efficiency of macromolecules such as siRNA [93], and the long-term safety and immunogenicity of the IVSA system still require further investigation. In a word, research on engineered exosomes remains in the early age, and challenges such as scale-up, batch variability, and long-term immune surveillance still need to be resolved.

4.4. Chemically modified RNA

In the field of ALS treatment, chemically modified RNA is mainly represented by ASO technology, which has achieved significant clinical breakthroughs. Chemical modifications of RNA include 2’-O-methyl, 2’-O-methoxyethyl, fluoro, locked nucleic acids, phosphorodiamidate morpholino oligomers, and others, among which 2’-O-methylation of the ribose 2’-hydroxyl group and phosphorothioate modification of the phosphodiester bond are the most commonly used types in ASOs [97,98]. These modifications significantly enhance the nuclease resistance, serum stability, and cellular uptake efficiency of ASOs, while reducing their immunogenicity and off-target effects [99].

Kauffman *et al.* [100] systematically compared the *in vivo* efficacy of unmodified mRNA and pseudouridine-modified mRNA in LNP delivery systems. The results demonstrated that pseudouridine-modified mRNA exhibited approximately 10-fold higher protein expression levels in the liver and spleen than unmodified mRNA, with a longer duration of expression. Furthermore, the levels of proinflammatory cytokines induced by the modified mRNA were significantly reduced. The metabolic stability of chemically modified siRNAs is also crucial for their potency and duration of action [101]. GalNAc-siRNA achieves long-acting gene silencing for several weeks following liver targeting, exhibiting excellent pharmacodynamic durability [102].

Chemically modified RNA, especially ASOs, has demonstrated numerous advantages in the treatment of ALS. Among these, the ASO targeting *SOD1*, namely tofersen, has been approved by the FDA, which constitutes a significant milestone in ALS gene therapy. A review published by Ito [9] summarized the results of the Phase I/II clinical trials of tofersen, indicating that intrathecal injection of the drug led to an approximately 36% reduction in cerebrospinal fluid SOD1 protein levels in patients with *SOD1*-mutant ALS and was well-tolerated. Furthermore, Miller *et al.* [16] published data from the Phase III clinical trial of tofersen for the treatment of *SOD1*-ALS in The New England Journal of Medicine. The results showed that patients who received intrathecal injection exhibited not only a decrease in cerebrospinal fluid (CSF) SOD1 levels but also a significant reduction in neurofilament light chain (NfL) levels, which suggests that tofersen may exert a neuroprotective effect.

The success of tofersen has demonstrated the feasibility and efficacy of ASOs targeting ALS-causing genes. However, ASO drugs are associated with high production and long-term treatment costs, and the currently approved tofersen is only applicable to patients with *SOD1* mutations [103,104]. Repeated intrathecal injections also impose inconvenience and risks on patients [16]. The long half-life of ASOs in the CNS indicates that they cannot be rapidly cleared, and prolonged retention may increase non-specific binding and off-target effects. Furthermore, ASOs may induce potential neurotoxicity in the CNS, and some ASOs lead to last-onset neurotoxicity days or weeks after dosing, which is related to their long-term retention in the CNS [105]. It is anticipated that future efforts will expand the target spectrum to treat more ALS subtypes, while reducing treatment costs and developing combination therapies.

5. Challenges

5.1. Limitations of approved medicine

Currently, the only drugs approved by the U.S. FDA for the treatment of ALS are edaravone, riluzole [106], and tofersen [103]. Although tofersen is the only approved drug for *SOD1*-mutant ALS, edaravone has short-term beneficial effects in ALS treatment [107], and riluzole is currently the only treatment proven to extend the lifespan of ALS patients, all three agents still have side effects including asthenia, mobility decreased, limb discomfort, *etc.* [108]. Combining RNAi-based therapy with existing treatments is worth considering. RNAi-based therapeutic targets pathogenic pathways at the genetic level, while medicine like riluzole exerts neuroprotective effects on pathological processes [12]. This combined strategy may not only matches the complex pathological characteristics of ALS but also compensates for the insufficient efficacy of single-agent administration.

5.2. Precision medicine

ALS is a heterogeneous disease with numerous biomarkers, and there is no single specific biomarker that meets the requirements for drug development [109,110]. This highlights the importance of precision medicine for ALS drug development and disease treatment [111]. The discovery of biomarkers helps refine diagnostic criteria, facilitate patient stratification and develop novel therapeutic approaches [112]. For example, Federico Verde *et al.* [113] identified serum NfL as a diagnostic biomarker for ALS; Ruth Chia *et al.* [114] studied 33 differentially expressed proteins in the plasma of ALS patients and established a highly accurate model for predicting ALS based on these proteins. Genetic testing for ALS-associated genes such as *SOD1* and *C9orf72* helps identify patient subgroups and formulate targeted treatment plans. For sporadic ALS, the lack of specific genetic markers results in difficulties in disease stratification, and the problem of disease heterogeneity remains intractable [115].

5.3. Delivery efficiency and target effect

The BBB protects parts of the CNS, but it also blocks the entry of drugs for treating neurological disorders into the brain, which undoubtedly impairs the efficiency of drug delivery and targeted therapy [116,117]. However, several approaches are being explored to enhance delivery efficiency. For example, Wei Cheng *et al.* [118] optimized the AAV vector, and the engineered AAV-neuron-derived neurotrophic factor (AAV-NDNF) exhibits remarkable protective effects on spinal motor neurons, although it has a relatively modest impact on cortical motor neurons.

6. Conclusion

After more than two decades of development, RNAi therapeutics have exhibited promising potential for high specificity and long-term efficacy in the treatment of ALS. Yet this therapeutic strategy still confronts numerous challenges, among which efficient delivery to the central nervous system stands out as a major hurdle. Furthermore, the pathogenic mechanisms underlying ALS have not been fully elucidated to date, with only a limited number of these mechanisms having been subjected to in-depth investigation. RNAi-based therapies for ALS therefore still have a long way to go, and the research progress and challenges summarized herein also provide valuable insights for the exploration of therapeutic strategies targeting other neurological disorders.

ALS exhibits overlap with diseases such as frontotemporal dementia (FTD) centered on *C9orf72* mutation and abnormal TDP-43 aggregation [119]. Approximately 50% of patients exhibit cognitive or behavioral abnormalities, and 15% are complicated by FTD, forming the ALS-FTD continuum [3]. These seems to be a new direction worthy of exploration. These shared pathological mechanisms suggest that RNAi-based therapeutics may enable coordinated intervention and treatment across multiple diseases. However, challenges such as difficulties in target selection, insufficient delivery precision and potential brain damage cannot be ignored. What's more, SG-associated protein ataxin-2 (ATXN2) is abnormally localized in spinal cord neurons of ALS patients, and intermediate-length polyQ expansions (27–33 glutamines) in ATXN2 were significantly associated with ALS [120]. There is evidence that administration of ASOs targeting ATXN2 in TDP-43 transgenic mice increases the

survival rate [121,122]. This suggests that we can further explore more novel targets similar to ATXN2 by focusing on relevant pathways.

Taken together, current RNAi-based strategies for ALS face a triad of challenges: target validity in a genetically heterogeneous disease, efficient and cell-type-specific delivery across the blood-brain barrier, and the long-term safety of sustained gene silencing. Progress in any single dimension is unlikely to translate into meaningful clinical benefit unless coordinated advances are achieved in all three. We therefore propose that future ALS RNAi therapeutics should be developed within an integrated framework that combines genetic stratification, spatially resolved delivery, and adaptive dosing strategies.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used DeepSeek, a generative AI tool, only to improve language and readability. The authors take full responsibility for the content of the manuscript.

Authors' contribution

Conceptualization, J.G.; investigation, Q.Z. and X.L.; resources, J.G.; writing—original draft preparation, Q.Z.; writing—review and editing, Q.Z., J.G. and X.L.; visualization, Q.Z. and X.L.; supervision, J.G.; project administration, J.G.; All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations

Abbreviation	Full description
AAV	Adeno-associated virus
AGO	Argonaute
ALS	Amyotrophic lateral sclerosis
ASO	Antisense oligonucleotides
ATXN2	Ataxin-2
BBB	Blood-brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
EV	Extracellular vesicle
fALS	Familial amyotrophic lateral sclerosis
FDA	Food and Drug Administration
FTD	Frontotemporal dementia
IVSA	*In vivo* self-assembled
LMN	Lower motor neurons
LNP	Lipid nanoparticle
miRNA	MicroRNA
NDNF	Neuron-derived neurotrophic factor
NfL	Neurofilament light chain
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RVG	Rabies virus glycoprotein
sALS	Sporadic amyotrophic lateral sclerosis

sEV	Small extracellular vesicle
SG	Stress granule
shRNA	Short hairpin RNAs
siRNA	Small interfering RNA
TDP-43	TAR DNA-binding protein 43
TRBP	RNA-binding protein
UMN	Upper motor neurons

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