Received: 13 November 2021 | Revised: 19 August 2022 | Accepted: 2 February 2023

DOI: 10.1002/med.21937

REVIEW ARTICLE

Medicinal Research Reviews WILEY

Molecular mechanisms of amyotrophic lateral sclerosis as broad therapeutic targets for gene therapy applications utilizing adeno-associated viral vectors

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Abstract

Despite the devastating clinical outcome of the neurodegenerative disease, amyotrophic lateral sclerosis (ALS), its etiology remains mysterious. Approximately 90% of ALS is characterized as sporadic, signifying that the patient has no family history of the disease. The development of an impactful disease modifying therapy across the ALS spectrum has remained out of grasp, largely due to the poorly understood mechanisms of disease onset and progression. Currently, ALS is invariably fatal and rapidly progressive. It is hypothesized that multiple factors can lead to the development of ALS, however, treatments are often focused on targeting specific familial forms of the disease (10% of total cases). There is a strong need to develop disease modifying treatments for ALS that can be effective across the full ALS spectrum of familial and

Abbreviations: AAV, adeno-associated virus; Ad, adenovirus; ALS, amyotrophic lateral sclerosis; ASO, antisense-oligonucleotide; BBB, blood-brain barrier; CSF, cerebral spinal fluid; dsDNA, double-stranded DNA; fALS, familial ALS; FTD, frontotemporal dementia; HDAC6, histone deacetylase 6; HSPG, heparan sulfate binding protein; HSV, herpes simplex virus; ICV, intracerebroventricular; iPSC, induced pluripotent stem cells; IRT, intron-retaining transcript; IT, intrathecal; ITR, inverted terminal repeat; IV, intravenous; LCD, low complexity domain; LLPS, liquid-liquid phase separation; M-CREATE, multiplexed-Cre-recombination-based AAV targeted evolution; NAb, neutralizing antibody; NHP, nonhuman primate; NIR, nuclear import receptor; NLS, nuclear localization signal; rAAV, recombinant AAV; RBP, RNA binding protein; RNP, ribonuclear protein; sALS, sporadic ALS; scAAV, self-complementary AAV; SMA, spinal muscular atrophy; SP, spinal subpial; ssDNA, single-stranded DNA; Ub, ubiquitin; UPS, ubiquitin proteasome system.

sporadic cases. Although the onset of disease varies significantly between patients, there are general disease mechanisms and progressions that can be seen broadly across ALS patients. Therefore, this review explores the targeting of these widespread disease mechanisms as possible areas for therapeutic intervention to treat ALS broadly. In particular, this review will focus on targeting mechanisms of defective protein homeostasis and RNA processing, which are both increasingly recognized as design principles of ALS pathogenesis. Additionally, this review will explore the benefits of gene therapy as an approach to treating ALS, specifically focusing on the use of adeno-associated virus (AAV) as a vector for gene delivery to the CNS and recent advances in the field.

KEYWORDS

adeno-associated virus, amyotrophic lateral sclerosis, gene therapy, proteostasis, RNA binding proteins

1 | INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease, characterized by the progressive death of motor neurons, including both the upper motor neurons in the motor cortex, and the lower motor neurons in the brainstem and spinal cord. The clinical consequences include progressive paralysis and loss of the ability to eat, speak and eventually breathe. ALS is invariably fatal, resulting in death typically within 3–5 years from diagnosis.[1] The global prevalence of ALS is estimated to be six cases per 100,000,[2] with a lifetime risk between one in 350–472.[3] Age has been shown as one of the greatest risk factors for ALS, highlighted as the average age of disease onset is ~55 years and the disease prevalence increases dramatically for those aged 70–79 years (20 per 100,000).[4]

It is now believed that ALS does not exists as one disease phenotype, but rather as part of a continuum of disease, involving frontotemporal dementia (FTD).[5–8] FTD is the second most frequent cause of early-onset dementia,[9] and results in atrophy of both the frontal and temporal lobes. The symptomatic outcome of FTD mainly effects behavioral, cognitive and motor abilities. Although this review will focus on ALS, strong evidence shows that FTD shares clinical, pathological and genetic overlap with ALS,[5–8] and therefore, the work outlined here may also be relevant across the ALS-FTD disease spectrum.

Despite the comprehensive research into ALS disease etiology, many factors surrounding disease onset remain elusive. Only 10% of ALS cases can be linked to a hereditary cause (familial ALS [fALS]), whereas 90% of cases are characterized as "sporadic" (sALS).[10] In fALS, genetic contributions are transmitted dominantly in families.[4] Although there are now over 50 potential ALS related genes, the two major genetic contributors to ALS currently identified are *C9ORF72* and *SOD1* mutations.[10,11] As the molecular defects caused by these pathogenic mutations lead to defined and tracible disease progression, these forementioned genes have become common targets for the development of gene therapeutics, despite only making up ~10% of all ALS cases.[11]

Although the cause(s) of sALS remain largely unknown, there are common pathological signatures shared by most ALS patients. One hallmark of ALS is the development of cytoplasmic inclusions in motor neurons. These

inclusions are misfolded protein aggregates, composed of the otherwise predominantly nuclear RNA binding protein, TDP-43.[12,13] Although it remains unknown whether these inclusions are the cause of motor neuron degeneration or a consequence of the disease process, they are seen in up to 97% of all ALS cases (familial and sporadic).[4] Although these inclusions themselves play an uncertain role in pathogenesis, it has also been shown that reduction of these TDP-43 oligomers within the cytoplasm leads to neuroprotection and rescue of the ALS phenotype,[14–16] highlighting the role of protein dysregulation as a driving force in ALS disease progression. The cause of aberrant protein misfolding, mislocalisation, and aggregation of an RNA binding protein points toward the potential involvement of the RNA metabolism and processing pathways. When developing a therapeutic for an elusive protein folding disease, such as in the case of ALS, targeting both the protein homeostasis and RNA processing pathways may be advantageous to achieve maximal therapeutic benefit.

The current treatment strategies for ALS remain largely inefficient, with no cure available for any form of the disease. Despite over 80 randomized controlled trials, completed and ongoing, that test various treatments for ALS, currently there are only two drugs that have successfully gained FDA approval in the USA: riluzole [17] and edaravone.[18] Unfortunately, both drugs have only been shown to have a modest effect,[18,19] and demonstrate no improvement to motor function. As the effects of both medications are limited and short-term, the focus of treatment is currently symptom management and palliative care, usually including the insertion of a feeding tube into the stomach when swallowing becomes unsafe and breathing support through noninvasive ventilation.[20]

On the horizon, the use of gene therapy for ALS has been showing promise as therapeutics are currently in clinical trials for directly targeting the monogenic causes of ALS associated with mutations within *SOD1* (NCT02623699, NCT04972487)[21] and *C9ORF72* (NCT03626012).[22] Both of these approaches utilize knock down of the associated ALS mutated gene to achieve therapeutic benefit through reduced expression. Of the two, the antisense oligonucleotide (ASO) targeting *SOD1* (termed "toferson") has indeed led to some promising results in an open label extension study (NCT04972487). In recent months, the FDA has now granted priority review of the drug,[23] creating hope that it will become the first approved treatment targeting a genetic cause of ALS.

In addition, a third clinical trial looks at targeting ALS broadly, using ASO technology to knockdown expression of ataxin-2 leading to a suppression of TDP-43 toxicity (NCT4494256).[24] Although the mechanism of how ataxin-2 knockdown leads to improved disease phenotype is not well defined, the treatment markedly increases both lifespan and motor function in ALS transgenic mice.[24] These therapies demonstrate an exciting step forward toward a potential cure for some forms of ALS as well as for the field of gene therapy as a whole. Indeed, the use of gene therapy in treating ALS could have sustained therapeutic effect by directly targeting the genetic defect.[25] Against this background, using gene therapy to treat ALS by targeting hallmark pathologies associated with the disease may be a promising avenue of research moving forward.

Considering the importance of both protein homeostasis and RNA metabolism in the pathogenesis of ALS, a combination, or "polytherapeutic," approach could target more than one pathogenic process seen in ALS and may be key in developing an impactful treatment. This approach may also be important for targeting the individual phases of disease progression. Therefore, this review will focus on the potential targets for ALS gene therapy within the protein homeostasis and RNA processing pathways, with particular emphasis on approaches that target the most frequently affected pathways in both fALS and sALS. We will also explore methods of gene delivery into the CNS with an emphasis on the development and use of adeno-associated virus (AAV)-based vectors.

2 | PROTEIN HOMEOSTASIS AND ALS PATHOGENESIS

Protein homeostasis (termed "proteostasis") within a cell underlies almost all cellular mechanisms essential to their health, survival, and overall function. For cells to function correctly, proteins must be at the correct location, concentration and have the required conformation. Cellular dysfunction and death can occur when a cell's tight regulation of these protein processes is compromised. Given its vital role for cell function and survival, the high

complexity of the proteome is not surprising. Unfortunately, our understanding of how cells maintain proteostasis is still very limited, although is now a fast-growing field of science. This is partially driven by the growing evidence of the role of defective proteostasis within the disease progression of numerous neurodegenerative diseases, such as ALS, FTLD, Alzheimer's, and Parkinson's diseases.[26]

Proteostasis dysfunction within these neurodegenerative diseases can clearly be seen through the hallmark "protein aggregates" that form within the CNS of patients affected by many neurodegenerative diseases, including ALS (Figure 1). As stated earlier, in ALS, >97% of patients have cytoplasmic inclusions positive for the presence of the RNA binding protein (RBP), TDP-43.[4] Proper function of the proteasome quality control system should ensure that any excessive or misfolded proteins are effectively cleared. However, for reasons that remain unknown, these insoluble cytoplasmic TDP-43 aggregates are—or eventually become—impossible to effectively be removed from the motor neurons of ALS patients. Although it remains unclear to whether these protein aggregates are the cause of neurodegeneration within ALS, or simply a result of the disease, experimental data provides evidence that the presence of these aggregates (i.e., in oligomeric conformation) is neurotoxic,[27,28] with their induced clearance alleviating toxic effects.[14–16]

It is widely believed that due to the large size and post- mitotic nature of neurons, that their protein clearance machinery is particularly vulnerable. Some motor neurons can produce axon projections exceeding 1 m in length, highlighting the immense area that must maintain proper protein production, degradation, quality control, and efficient protein trafficking. As neurons are mostly nondividing cells, they are also unable to dilute protein aggregates via cell division. Mature neurons are also strikingly resistant to apoptotic stimuli.[29] This highlights the importance of the proteostasis network maintenance systems and how their disruption, potentially exacerbated by aging, may possibly lead to ALS disease development/progression.[30,31] The following sections focus on possible

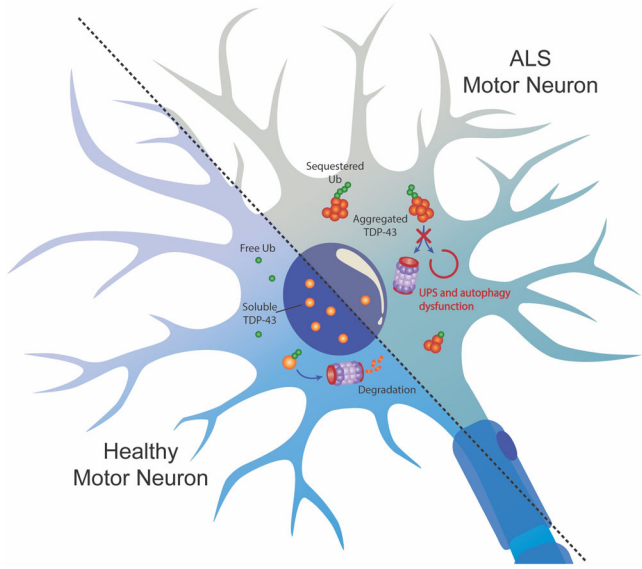


FIGURE 1 Proteostasis dysfunction in amyotrophic lateral sclerosis (ALS) motor neurons. In majority of ALS cases (>97%), the otherwise predominantly nuclear RNA-binding protein, TDP-43, is found to be mislocalised into the cytoplasm where it is prone to aggregate formation. These aggregates are not efficiently removed by the protein degradation pathways: the ubiquitin proteasome system and autophagy. These pathways can be activated in healthy cells via a ubiquitin (Ub) tag. As Ub is sequestered within these TDP-43 aggregates in ALS, there is little free Ub available for other cellular processes. There is evidence to suggest that this combination of dysfunction at multiple points within the proteostasis pathway may contribute to disease progression and motor neuron death in ALS patients. [Color figure can be viewed at wileyonlinelibrary.com]

candidates within the proteostasis network which could be targeted genetically to improve the degradation of aberrant proteins seen in ALS patients to re-establish proteostasis and potentially minimize any potential deleterious effects of protein aggregates.

2.1 | Protein degradation pathways and clearance of TDP-43

The proteome is composed of many thousands of proteins which are required for proper cellular function. Relatively small changes in the production or degradation of these proteins can lead to large error and dysfunction within the proteome. Proteins must be correctly translated and folded to form their functional 3D conformation. Many proteins will not be folded correctly post-translationally and therefore will not be fit for function and will undergo degradation. Furthermore, misfolded and/or nonfunctional proteins can often have adverse effects resulting in both gain-of-toxic-function and loss-of-function properties, particularly if mislocalised, such is the case in ALS.[32,33]

Following this observation, it is clear that the presence of an efficient and functional protein degradation system is essential to the health of the cell. In mammalian cells, proteins can be degraded by one of the two following pathways: The ubiquitin-proteasome system (UPS) or through autophagy mechanisms.[34] Whereas the UPS usually is responsible for degrading small, soluble proteins that have been specifically tagged for degradation, autophagy is responsible for the clearance of larger protein complexes.[35,36] Although this may suggest that therapeutic activation of autophagy is a better fitting target to upregulate clearance of toxic TDP-43 aggregates, the ability of these aggregates to form and progress beyond protein monomers may indicate an initial dysfunction in the UPS (Figure 1). It is also clear that TDP-43 inclusions accumulate following inhibition of both UPS and autophagy.[37,38] Therefore, both protein degradation pathways will be discussed below as possible ALS therapeutic targets.

2.2 | The UPS and autophagy pathway

The UPS is an essential pathway for the degradation of monomeric proteins tagged for proteasomal degradation, responsible for up to 80% of all protein degradation within mammalian cells.[39,40] Once a specific protein is tagged for proteolytic degradation via Ub, the protein is then unfolded into a single chain and cleaved into short peptide sequences by passage through the 26S proteasome. This ATP-mediated process involves a complex enzymatic cascade composed of up to 1000 different proteins and the critical E1—E2—E3 pathway (Ub activation, conjugation and attachment respectively—reviewed in[35,40]). The specificity of the pathway is due to the action of the E3 ligase. There are currently over 600 identified E3 ligases which allow for a wide variety of proteins to be degraded by the UPS through attachment of Ub to the specific substrate of the E3 protein.[41]

Autophagy is a broad term used to define the degradation of material through the lysosome. The most well-defined form of autophagy is macroautophagy. Simply put, macroautophagy (hereby referred to as autophagy) involves the engulfment of complex intracellular materials (such as protein aggregates and damaged organelles) into double-membrane structures termed autophagosomes. These are then able to fuse to the lysosomal structures which allow for degradation.[42,43] In a similar manner to the UPS system, autophagy utilizes Ub as a tag for degradation. However, other substrates can also be recognized for macroautophagic activation in a Ub-independent manner. Although it was initially believed that autophagy was a bulk-removal, nonspecific mechanism induced by the starvation of a cell, based on new evidence we have now begun to truly understand its importance in cellular homeostasis and quality control through the selective degradation of materials such as mitochondria (mitophagy), pathogens (xenophagy), and more pertinently, aggregated proteins (aggrephagy)[44,45] for cell maintenance purposes.

Importantly, multiple ALS causing mutations have been identified within both the UPS pathway (*CCNF*,[46] *FUS*[47]), the autophagy pathway (*TARDBP*,[5] *SQTSM1*,[48] *C9orf72*,[49] *TBK1*[50]) as well as both (*UBQLN2*,[51] *VCP*,[52] *SOD1*[53]), further implicating the role of protein degradation within ALS pathogenesis. However, as ALS aetiopathogenesis remains largely undetermined, this review will focus on the hallmark protein pathologies seen more broadly across sALS/fALS.

2.3 | Targeting of protein degradation as a therapeutic for ALS

One of the most striking observations in the ALS-associated protein inclusions is the presence of Ub, implicating dysfunction within either the UPS, autophagy or a combination of these pathways. The presence of Ub suggests that the cell has recognized the aggregates and tagged them for degradation, however, has been unable to complete the process. These hallmark aggregates are also often found containing the autophagy receptor, p62, another pertinent marker for degradation.[48] The presence of these degradation markers in the context of an inability to degrade these aggregates suggests possible dysfunction in the cell's degradation pathways. Therefore, targeting protein degradation is an attractive approach to help clear these aggregates and re-establish proteostasis within the CNS.

To investigate the roles of UPS and autophagy in clearing the pathological aggregates observed in ALS, Scotter et al.[54] investigated aggregate formation of TDP-43 using an in vitro model allowing for mislocalisation of TDP-43 into the cytoplasm. They found that large, insoluble TDP-43 aggregates only formed following inhibition of the UPS, rather than autophagy.[54] However, to remove these aggregates, autophagy was required. It should be noted that autophagy was only able to remove aggregates when at the stage of oligomers/microaggregates, and not when large macroaggregates had already formed. Based on the results of this study, it is thought that a healthy UPS system drives the degradation of soluble TDP-43, however UPS blockage may result in TDP-43 accumulation and crossing the point where UPS, and eventually autophagy, are able to remove them. It is not fully understood why the UPS fails to remove misfolded monomeric TDP-43 proteins before they oligomerise and form aggregate. However, contributing factors may include the saturation of the UPS, and the decline of the UPS efficiency that is well reported as an effect of aging.[31,55] As it is established that ageing is the major risk factor for ALS, could upregulating/strengthening the body's UPS system through genetic therapy possibly lead to decreased propensity of the aggregates to form? Upregulating the UPS system has indeed been shown to be therapeutic within multiple studies of *SOD1*-related ALS models through small-molecule delivery of the drug, Pyrazalone.[56–58] However, the question is how to activate the UPS system using long-lasting genetic approaches and whether this would have therapeutic benefit outside what is seen in the *SOD1*-related model. One possible avenue to explore would involve the genetic overexpression of the key components of the E1-E2-E3 UPS activation cascade, possibly triggering increased activity of the UPS. Of particular interest is the action of specific E3 ligases. As E3 ligases have specific activity to certain substrates, when identifying an E3 ligase to target as a possible therapeutic, it is essential to identify whether it has specific activity against pathogenic TDP-43 variants. Unfortunately, determining the substrate specificity of E3 ligases can be difficult, largely due to the transient/weak interactions of the ligase and substrates which can have rapid disassociation rates.[59,60] While workflows are currently being developed to streamline the characterization process,[60,61] the catalytic activity of many E3 ligases remain to be defined.

Recently identified, the RING finger protein, ZNF179, has been shown to have E3 ligase activity against TDP-43 both in vitro and in vivo.[62] This recent study conducted by Lee et al.,[62] also demonstrated the ability of ZNF179 to modulate the proteome through regulation of key UPS proteins. Lee and colleagues further showed that ZNF179 overexpression leads to increased TDP-43 polyubiquitination and enhanced proteasomal degradation of TDP-43 aggregates. To support the role of ZNF179 in TDP-43 proteinopathy, knockout of ZNF179 in mice resulted in accumulation of insoluble TDP-43 within their transgenic mouse model.[62] These promising results demonstrate a possible therapeutic role of ZNF79 in treating TDP-43 proteinopathy and ALS broadly. Interestingly, this protein

has been found downregulated in mouse models of the neurodegenerative diseases, ALS and Huntington's disease,[62] possibly indicating an important role for ZNF179 in the broad development of neurodegenerative disorders. Importantly, not all E3 ligases which have activity against TDP-43 result in its increased degradation. Despite its ability to directly ubiquitinate TDP-43, overexpression of the E3 ligase "Parkin" did not result in increased proteasomal degradation, and rather, formed a complex together with TDP-43 and histone deacetylase 6 (HDAC6) leading to increased translocation of TDP-43 from the nucleus into the cytosol within their mouse-model overexpressing lentivirus-encoded TDP-43.[63] Despite being an E3 ligase, these results suggest that Parkin may promote the cytosolic aggregation of TDP-43. Overall, gene therapy exploring the delivery of *ZNF179* within ALS models should be further explored as an ALS therapeutic. Further work should also investigate the role of other E3 ligases on TDP-43 ubiquitination and degradation via the UPS.

One possible issue with targeting the UPS is that ALS does not get diagnosed until following symptom onset due to the lack of a well-established, clinically used disease marker. However, ALS is an asynchronous disease, and it is believed that proteinopathy begins focally and spreads out along with disease progression. Therefore, targeting the UPS may hold benefits for protecting areas that have not yet been clinically affected with TDP-43 aggregation. However, there may be other advantages in pursuing an autophagy-related treatment, focusing on the ability of this pathway to remove already formed aggregates in prior affected regions.[45] In a study by Barmarda et al.,[27] pharmacological induction of the autophagy pathway was shown to improve nuclear localization of TDP-43, clearance and improved survival in iPSC-derived neurons and astrocytes in a TDP-43 mutant model of ALS. Interestingly, a separate study on the effects of upregulating autophagy on healthy ageing demonstrated that the overexpression of ATG5 (a key component of autophagosome formation) in *Atg5* transgenic mice activated the autophagic pathway and extended the lifespan of animals by an average of 119 days (a 17.2% increase).[64] These mice not only had a significantly longer lifespan compared to the WT control mice, but also appeared leaner (while maintaining the same diet) and had increased insulin sensitivity and motor function. Furthermore, an earlier study found that loss of autophagy in mice deficient for ATG5 within neuronal cells demonstrated cytoplasmic inclusions and progressive motor disfunction, akin to that observed in neurodegeneration.[65] This highlights a possible direct link to a role of ATG5 in neurodegenerative disease progression. These studies emphasize the advantages to exploring autophagy activating genes, particularly *Atg5*, as a possible target for gene therapy in ALS. These results also demonstrate that targeting the autophagy pathway may have additional benefits to an ageing patient, allowing for possible overall improved health beyond that which may be therapeutic specifically to the disease.

The UPS and autophagy have key differences in their mechanisms of action which may be helpful when developing possible ALS therapies. Therefore, their mechanisms of action complement, rather than compete with, one another. The complementary nature of these pathways raises the question of whether using a combined approach will prove to be more effective than either treatment individually. Indeed, multiple studies have highlighted the importance of both pathways in the degradation of TDP-43 aggregates.[54,66,67] Through analyses of UPS and autophagy inhibition on cultured cells[54] and qualitative assessment of the degradation of TDP-43 aggregates,[23] studies have collectively identified a total of six classes of TDP-43 aggregates based on their propensity to be degraded. The pathway of degradation between these classes is clearly distinguished (i.e., UPS vs. autophagy), highlighting the importance of both pathways in the clearance of aggregates and providing evidence of crosstalk between them. Unfortunately, one of these aggregation classes (insoluble, macroaggregates) could not be cleared by either degradation pathway.[66] This finding indicates that further research is required to find an effective method to achieve aggregate disassembly before clearance via these aforementioned pathways. It is also noteworthy that autophagy does not operate within the nucleoplasm of cells.[68] Thus, for removal of nuclear aggregates, disassembly may be required before UPS mediated degradation.

Overall, ample evidence indicates that upregulation of both the UPS and autophagy pathway may provide therapeutic effect broadly across ALS patients. As the autophagy pathway targets protein aggregates which are already formed, a treatment strengthening the response of both the UPS and the autophagy pathway could possibly

aid in the removal of the cytoplasmic TDP-43 aggregates (through increased autophagic activity), as well as preventing their future formation (through increased in UPS activity). However, an important point to consider before targeting these pathways through gene therapies is the degree of induction. Maintaining an optimal degree protein degradation within the cell requires a very fine balance of the UPS and autophagy systems. Therefore, a valid cause for concern is maintaining the "goldilocks effect" during treatment. Stimulation these pathways needs to be optimized, without over/under stimulation which may lead to adverse effects. Therefore, further research should thoroughly explore the effects of differing levels of UPS/autophagy induction (including potential dual targeting) to ensure both safety and maximal therapeutic benefit.

3 | RNA HOMEOSTASIS AND ALS PATHOGENESIS

RNA homeostasis (termed "ribostasis") is equally fundamental as proteostasis. Aberrant RNA metabolism can cause toxicity in a number of ways, some of which link back to proteostasis. Looking back at the central dogma of molecular biology, RNA is indeed the "messenger" between DNA and the protein production machinery, providing an initial entry point to the suspicion of RNA's involvement when investigating the pathogenicity of protein folding diseases, such as ALS. However, the lifecycle of RNA is not a simple one, and many regulatory RNA binding proteins (RBPs) play an essential role in the transport, posttranscriptional editing, translation and degradation of RNAs.[69] Beyond this, both RNA and RBPs can form membraneless organelles together (termed ribonuclear protein [RNP] granules), which are essential in both RNA metabolism and gene regulation.[70] The function of these RNP granules can vary depending on the RNA and RBPs they contain. Defined RNPs are becoming the focus of recent research, including structures such as stress granules and paraspeckles, both of which have been implicated in ALS pathogenesis.[71–74] The role of stress granules in particular has been widely debated as key in the etiology of ALS-related aggregation. However, as it has been shown that the pathogenic cascade of ALS can occur independently of stress granules,[75–77] they will not be explored further in this review as a therapeutic avenue (although have been reviewed elsewhere[73,74]).

Without the tight regulation and maintenance of RNA pathways via RBPs and RNP granules, cellular homeostasis cannot be sustained (Figure 2). Importantly, multiple strong genetic links to ALS have been associated with the RNA metabolism pathways,[78] including *C9ORF72*,[79,80] *SOD1*,[81,82] *TDP-43*,[83,84] and *FUS*.[85–87] These mutations among others have revealed crucial roles for RNA production, editing and localization for the onset of ALS, demonstrating convincing evidence that deregulated RNA metabolism plays a broader role in the onset of ALS. The following sections focus on the importance of RBPs and RNP granule formation in ALS and their targeting as a possible broad therapeutic to restore proper RNA metabolism and therefore, proteostasis in patients of ALS.

3.1 | RBPs

The key role of RBPs is to maintain RNA metabolism and the transcriptome.[88] Indeed, RBPs are the watchkeepers of RNA processing, achieving tight regulation through binding to multiple RNA targets and consequently influencing their downstream processing and translation.[69,89] Therefore, it is clear that any aberrant changes to RBPs can result in cascading adverse effects through the protein production and maintenance pathways. This is particularly true for neuronal cells which require stringent regulation due to their large size and post-mitotic state.

The first link of RBPs to ALS pathogenesis was established with the discovery of TDP-43 positive aggregates within the cytoplasm of ALS patients.[5] TDP-43 itself is a key RBP, implicated in RNA processing, including splicing, transport, stability and suppression.[83,84] Over 6000 mRNA targets (~30% of the transcriptome) have been shown to have binding sites for TDP-43.[90] Furthermore, it was also shown that mutations in the gene encoding TDP-43, *TARDBP*, itself were a cause of fALS.[91,92] Although over 97% of ALS cases exhibit TDP-43 proteinopathy at

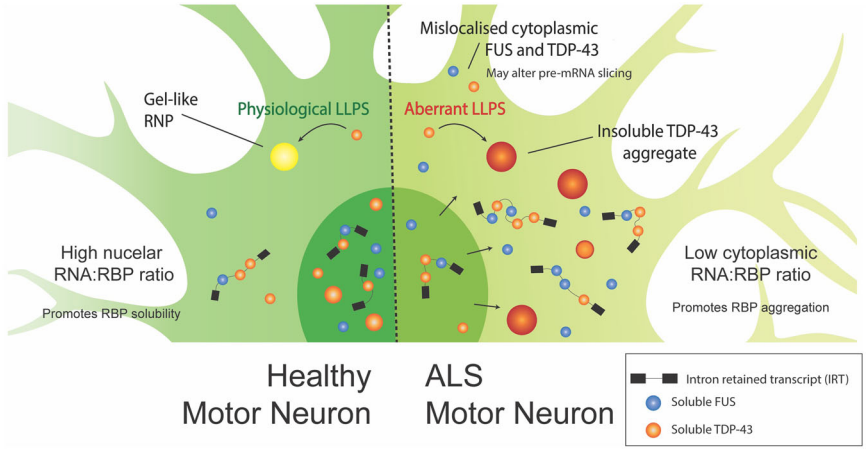


FIGURE 2 RNA metabolism dysfunction in ALS motor neurons. TDP-43, the predominantly nuclear RNA binding protein (RBP), is mislocalised to the cytoplasm in majority of ALS cases. Once in the cytoplasm, TDP-43 is prone to aggregation. Evidence suggests that this aggregation may be caused by aberrant liquid-liquid phase separation (LLPS), altering the state of TDP-43 from soluble to insoluble. As RNA is an antagonist of TDP-43 LLPS and encourages the soluble state of TDP-43, evidence suggests that the low RNA concentration within the cytoplasm (compared to TDP-43's predominant location within the nucleus) may lead to its lowered solubility in the cytoplasm and promote it's aggregation. Another hallmark of ALS which contributes to RNA dysregulation is increased aberrant intron-retaining transcripts (IRTs). The intron retaining portion of these IRTs are prone to binding RBPs (such as TDP-43 and FUS) and are exported into the cytoplasm, thereby transporting the RBPs outside of their predominant location within the nucleus. As the RBPs have key roles in regulating RNA metabolism within the nucleus, their removal from their physiological location may contribute to disease progression through aberrant pre-mRNA splicing. [Color figure can be viewed at wileyonlinelibrary.com]

histological examination, only 1%–5% of ALS patients presented with the *TARBDP* mutation.[93,94] These findings highlight that alternative dysregulated mechanisms must be present outside mutations within the *TARBDP* gene itself that negatively affect TDP-43 protein localization and function, and lead to disease progression within majority of ALS patients. Due to the essential role of TDP-43 for RNA processing, extensive research has focused on the role of this pathway in ALS pathogenesis. It was found that the gene encoding the RBP, fused in sarcoma (FUS), is an additional key genetic cause of ALS.[47] Similarly, to TDP-43, FUS plays an important role in RNA metabolism, orchestrating events involved in transcriptional regulation, splicing, subcellular localization and translation.[95] Patients with mutations in the *FUS* gene do not contain the hallmark TDP-43 protein within their disease aggregates, but rather the FUS protein itself. Similarly, ALS patients with *SOD1* mutations, present with SOD1 containing-aggregates, rather than TDP-43.[96] It should be noted that although SOD1 is not a canonical RBP, studies have demonstrated a role for it in RNA metabolism.[81,82] However, as it has been suggested that the mechanism of *SOD1* ALS disease progression may be distinct from other forms of ALS,[96,97] it will not be explored further within this review (although this topic has been reviewed elsewhere[1,11]). Importantly, multiple other RBPs have now been shown to be implicated in ALS pathogenesis (reviewed in[69,98]). Therefore, evidence suggests that restoring proper function of RBPs in ALS patients may help to maintain proper protein interactions and RNA metabolism.

3.2 | RNP granules and Liquid-liquid phase separation (LLPS)

Another recent focus within the ALS field is the implication of RNP granules in the onset of ALS. These membraneless organelles play an essential role in the temporal/spatial organization of a cell and can regulate

protein translation specifically within organized cellular compartments.[70] They usually manifest transiently under certain cellular conditions/requirements (i.e., the formation of stress granules under adverse conditions). Interestingly, both paraspeckles and stress granules seem to be expressed aberrantly in models of ALS,[71,74,99] allowing studies into role of RNP granules in disease pathology. The following sections will focus explore the role of RNP granules, their formation and maintenance in ALS disease etiology and progression.

When taking a closer look at the structure of both FUS and TDP-43, they both contain a region termed the low-complexity domain (LCD).[100] As the name suggests, LCDs are regions of low sequence complexity, often containing a limited number of repetitive amino acids sequences which are inherently highly charged. Interestingly, many RBPs involved in RNP granule formation contain an LCD as a conserved structural component.[100] What is the function of the LCD and how is it implicated in ALS pathogenesis? LCDs are essential in mediating protein-RNA, RNA-RNA and protein-protein interactions. Due to the structural nature of LCDs, they allow for weak binding to other complementary LCDs and proteins with interaction domains. This explains the ability of membraneless organelles to remain together in defined and working structures without the presence of a membrane to enclose them.[101] This process is further explained with the process of LLPS. This complex biochemical process can be more simply likened to the ability of water to form distinct structures depending on the environment (gas, liquid, solid). Similarly, in LLPS, protein complexes are able to do the same, establishing dynamic and distinct forms (i.e., liquid or gel-like) depending on the cellular environment.[70,101,102] LLPS therefore allows the spontaneous formation of gel-like droplets or "condensates" for intracellular compartmentalization and organization. These droplets can then co-exist within a pool of dilute-phase, liquid-like, soluble proteins within the cytoplasm/nucleoplasm (in a similar manner to how oil can exist in water). Although the specific pathways required for phase-transition remain elusive, it is now known that this process is how certain RNP granules can be formed dynamically under specific conditions, such as stress granules (reviewed in[70]). Therefore, evidence suggests that LLPS and the presence of these LCDs play important physiological roles within healthy cells.[70,101] In the field of neurodegeneration, however, it is hypothesized that aberrant LLPS may lead to conditions where the RNPs move beyond "gel-like" structures to form insoluble inclusions which can no longer be disassembled (Figure 2). Indeed, the presence of an LCD in RBPs is critical for LLPS, however provides an aggregation-prone quality to these proteins. Supporting this, evidence demonstrates that mutations within the TDP-43 LCD modify LLPS and can augment its aggregation propensity.[100,103] Therefore, developing a deeper understanding of how LLPS is maintained and its mechanisms of action within healthy and diseased individuals may lead to the identification of new targets for treatment, which are further explored below.

3.3 | Targeting RNA processing as a therapeutic for ALS

In recent years a flux of research has explored the importance of functional LLPS in physiological RNA metabolism. LLPS is important within this pathway by regulating RBPs and RNP granules which interact directly with RNA and proteins to regulate their expression and function. Therefore, developing methods to normalize LLPS dynamics will allow suitable functioning of downstream RNA metabolic processes. To explore the importance of LLPS in the context of TDP-43 and ALS pathology, Mann et al.[76] created a light-inducible system to model TDP-43 proteinopathy. Using this model, they studied the mechanisms governing pathogenic phase-separation in an inducible manner. Their results indicated that phase separation of TDP-43 was dependent on the LCD region. Specifically, bait oligonucleotide binding to the RNA recognition motif (RRM) of TDP-43 abolished light induced phase separation, likely through prevention of LCD:LCD interactions in human neurons.[76] In the context of disease, this finding may suggest that within TDP-43's nuclear environment, the high abundance of RNA may inhibit its phase transition into insoluble aggregates, however, within the cytoplasm, the lower RNA:RBP ratio may conceivably increase the propensity of TDP-43 to aggregate (Figure 2). Based on this result, the authors hypothesized that RNA-binding to the RRM directly regulates the ability of TDP-43 to form aggregates.

In support of the use of bait oligonucleotides in maintaining RBP solubility, study by Maharana et al.,[104] explored the LLPS dynamics of FUS. Using an in vitro FUS phase separation assay, Maharana et al.[104] found that small amounts of RNA promoted the formation of condensates. However, increasing concentrations of RNA resulted in smaller, and eventually completely diffuse condensates. The subsequent addition of RNase A resulted in the FUS condensates to reappear, confirming that the changes in LLPS within their model were due to the altered concentration of RNA.[104] Both of these studies provide strong evidence for a direct relationship between RNA binding and LLPS of RBPs, with a high RNA abundance inhibiting phase separation, and a low RNA concentration promoting it. To further this line of research, another study utilizing human iPSC-derived motor neurons and cultured mouse cortical neurons found that simply increasing the concentration of TDP-43 within the cytoplasm without the addition of any further stressors resulted in eventual nuclear depletion of TDP-43 and subsequent cell-death over a 6-week period.[75] It is noteworthy that nuclear "leakiness" is a reported consequence of ageing within neuronal cells.[105]

It should be noted that the characteristics and valency of the interacting RNAs have important implications for LLPS. Short RNAs with low valency dissociate RNP condensates,[76,104] whereas long RNAs with high valency enhance LLPS[106,107] and sequestration.[108] These results suggest that a variety of conditions may lead to aberrant TDP-43 and FUS phase separation and eventual aggregation. Perhaps this may explain the elusive etiology of ALS and how distinct mutations can lead to the same disease pathology. However, this can also be encouraging from a therapeutic perspective, as targeting LLPS broadly may lead to therapeutic benefits for a range of ALS patients.

One approach to ameliorate aberrant LLPS was explored in the aforementioned study by Mann et al.[76] The authors found that the presence of "bait oligonucleotides" inhibited phase separation of TDP-43 and FUS within their light-inducible model,[76] demonstrating a similar effect to increasing the cytoplasmic RNA concentration within Maharana's study.[104] These bait oligonucleotides were designed to specifically mimic a natural RNA binding partner of TDP-43, competitively binding with its LCD so that it was unable to bind to other TDP-43 proteins and thus to oligomerise. A similar approach has also proved successful in limiting aberrant LLPS through the delivery of certain RNAs, particularly *NEAT1*, which is known to be a strong binding partner of FUS and able to solubilize FUS condensates.[104] These results provide an exciting avenue for possible ALS therapeutics by limiting LLPS of key ALS-related RBPs. However, these studies require further validation in other models before the clinical setting. To our knowledge, no in vivo model has yet explored this effect. Further work is also required to explore the ability of RNA binding to disassemble aggregates which have already formed. If successful, gene therapy approaches for delivering these bait oligonucleotides or specific RNAs may be an interesting therapeutic option to inhibit/reverse the effects of dysregulation within ALS RNA processing/LLPS and consequently proteostasis.

Although the above studies provide some insight into the cytoplasmic aggregation of RBPs in ALS, it remains elusive as to why these RBPs are mislocalised. A recent study by Tyzack et al.,[109] set out to investigate FUS protein localization within ALS, utilizing three ALS models: patient-derived iPSC, a transgenic VCP mutant mouse model and sALS post-mortem samples. Interestingly, they found that increased mislocalisation of soluble FUS from the nucleus to the cytoplasm was a common feature across these models, including within sALS post-mortem tissue. These data suggest that the mislocalisation of soluble FUS is a hallmark of the disease, which escaped earlier recognition as it remained within a soluble state rather than forming part of the observed TDP-43 positive inclusions. Interestingly, mislocalisation of soluble FUS into the cytoplasm was found to occur at early stages within iPSC and mouse models, possibly indicating an early role for unaggregated FUS in ALS pathogenesis/progression.[109] It follows that targeting mislocalisation of FUS may be a viable therapeutic target. To understand the mechanism behind this mislocalisation of FUS, public iCLIP data was used to identify RNA binding targets to the FUS protein.[109] It was found that FUS binds to an aberrant intron retaining transcript of another RBP called *splicing factor proline and glutamine rich (SFPQ)*. This group had previously shown that SFPQ protein binds to the aberrantly retained intron 9 on the SFPQ transcript as a hallmark of ALS.[110] This was further supported by a recent study, finding this same result within sALS human post-mortem tissue.[111] Overall, these studies provide a model whereby both FUS and SFPQ proteins bind to the aberrantly retained intron 9 in *SFPQ* transcripts, which essentially transport the proteins out of the nucleus and into

the cytoplasm, contributing to their mislocalisation (Figure 2). Overall, the nuclear loss and aberrant localization of RBPs (particularly FUS, *SFPQ*, and TDP-43) may alter pre-mRNA splicing and RNA processing within motor neurons, thereby contributing to ALS pathogenesis.

Further exploring the role of intron-retention within ALS pathogenesis may be an interesting therapeutic avenue. Indeed, intron retention may result in RNA destablization and reduced protein synthesis—both known features in ALS pathogenesis.[112–114] Intron retention also plays an important role in regulating the transcriptome through RNA detention within the nucleus,[115,116] although it's role within the cytoplasm (as compared to nuclear intron-retaining transcripts) remains relatively unexplored. One possible method of inhibiting the aberrant intron retained *SFPQ* transcript may be through an antisense-oligonucleotide (ASO) treatment. ASOs are short, single stranded nucleotide sequences that are synthetically made to bind to specific RNAs.[117] Through this binding, ASOs are able to modify RNA processing and protein expression through distinct mechanisms. The benefit of this method for targeting *SFPQ* is that the ASO may be designed specifically to bind to the retained-intron proportion of *SFPQ*, thereby preventing RBPs such as FUS and SFPQ from binding to the aberrantly retained intronic portion explored above while retaining the function of correctly spliced *SFPQ*.[118] Although it is unlikely that this is the only mechanism of FUS mislocalisation in ALS pathogenesis, it is potentially an important contributing factor.[110] Building on the above study, Tyzack et al.[119] identified an additional pool of >100 intron-retained transcripts within human ALS-mutant iPSCs that demonstrated increased cytoplasmic localization. Interestingly, the RBPs found to bind with the most affinity and specificity to the aberrant cytoplasmic intron-retaining transcripts were TDP-43, FUS and SFPQ. Overall, these results suggest that ALS may increase aberrant intron-retaining transcripts within the cytoplasm which contain regions of high affinity to known ALS-related RBPs (FUS, SFPQ, TDP-43), possibly promoting their incorrect localization in the cytoplasm and potentially creating an environment of increased aggregation propensity (Figure 2).

Another interesting avenue to re-establish RNA metabolism within ALS patients is to increase the import of RBPs back into the nucleus. This method may restore the regular function of these RBPs within the nucleus, possibly reducing aberrant LLPS and their aggregation within the cytoplasm. One method to achieve this was explored in a recent study by Guo et al.[120] In this study, the authors show that the increased expression of chaperone nuclear import receptors (NIRs) was able to restore the localization and function of RBPs back within the nucleus, as well as prevent and reverse the formation of disease-related aggregates within the cytoplasm. These results demonstrate the remarkable ability to reverse aberrant LLPS and aggregate formation. These NIRs work through binding to the nuclear localization signal (NLS) present within the RBPs. As different RBPs contain different NLSs, they found that the upregulation of the NIRs, Impα and Kapβ1, were effective against aberrant TDP-43 LLPS, and Kapβ2 was effective against a range of other ALS-associated RBPs (e.g., FUS, and hnRNPA1). This approach represents an exciting avenue to explore further as a possible gene therapy for ALS, particularly as it may be therapeutic in not only disaggregating already formed FUS and TDP-43 aggregates (which may be important in later stages of ALS pathogenesis), but additionally re-instating the nuclear function of these essential RBPs. However, what remains unexplored is the impact on the localization of the remaining endogenous proteins when these NIRs are overexpressed. Although an exciting avenue as a possible therapeutic, further studies should focus on the effects of NIR overexpression on the transcriptome and proteome.

The degradation of excess *TARDBP* transcript has been highlighted in recent years as a possible therapeutic target for ALS. TDP-43 is tightly auto-regulated, with nonsense mediated decay (NMD) thought to play a role in transcript degradation. Overexpression of the essential NMD component, up-frameshift protein 1 (UPF1), has been demonstrated by multiple studies to show therapeutic effect across varies models of ALS. In a study by Barmada et al., overexpression of UPF1 improved neuronal survival in both familial and sporadic models of ALS.[121] The therapeutic potential of UPF1 was also observed in a rat model of TDP-43 induced motor paralysis. AAV-mediated delivery of *UPF1* resulted in improved motor scores and maintained forelimb function compared to the quadriplegia observed in rats receiving empty vectors.[122] Of note, the therapeutic intervention was highly dose dependant, with a low/medium dose demonstrating enhanced survival, while a high dose (~10-fold excess of endogenous UPF1),

resulted in enhanced toxicity.[121] Although it is encouraging that only a small dose may be required to result in therapeutic effect, it also highlights the importance of controlling dose to avoid adverse effects. There is clear therapeutic potential shown by UPF1 delivery in an array of models, warranting further studies to explore UPF1 gene therapy to treat ALS.

As explored above, multiple methods have currently been investigated to decrease aberrant LLPS of RBPs and re-instate the physiological function of RBPs within the nucleus. Successfully achieving these results in a clinical setting is key in correcting the altered RNA metabolism in ALS pathology. Therefore, further exploration into genetic treatments targeting RNA metabolism is well justified. In parallel, therapeutic approaches to restore proteostasis (explored in the section above) may prove to be important in ALS.

4 | CLINICAL/PRECLINICAL METHODS OF GENE DELIVERY TO THE CNS

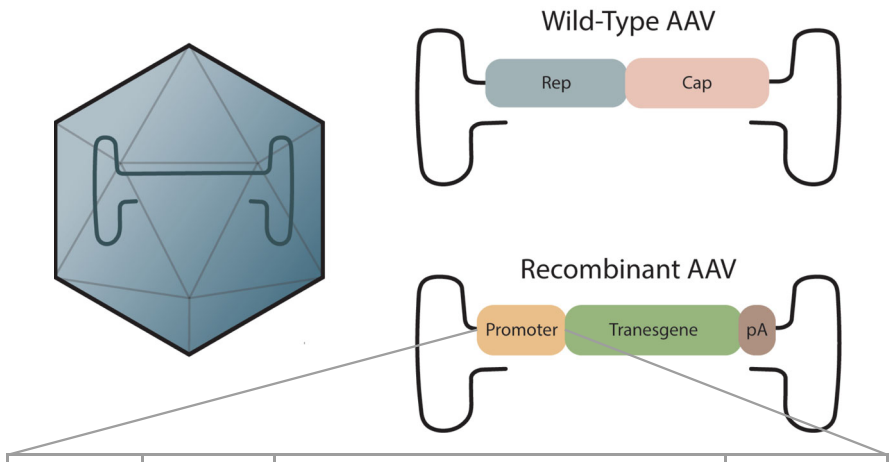
4.1 | AAV as a gene delivery vehicle

The delivery of a therapeutic payload in form of a nucleic acid to target cells for the purpose of clinical benefit is referred to as gene therapy. This fast-growing field has gained a lot of attention in recent years due to its proven ability to correct disease at the genetic level. The success of gene therapy can be seen with the recent market approved treatments for the neuromuscular degenerative disease, spinal muscular atrophy (SMA). Type-1 SMA is a lethal child-onset monogenic disease with no previous therapy.[123] Patients with this disorder contain mutations within the survival motor neuron 1 (*SMN1*) gene,[124] resulting in progressive degeneration of motor neurons.[125] The recently approved gene therapy for SMA utilizes the AAV-mediated delivery of a healthy, WT version of *SMN1* to the CNS following systemic vector administration to restore the function of the defective gene within patients.[126]

One of the biggest current limitations in the field of gene therapy is the development of vehicles which can transport the therapeutic cassette to the cell type of interest in a safe, efficient and specific manner. These vehicles, often referred to as "vectors," can have either viral or nonviral origins. Although the study of nonviral, biomaterial vectors have attractive features (such as low immunogenicity, safety and ease of production[127,128]), the use of viral vectors, particularly AAV, has been shown to be increasingly effective within the past 20 years, particularly for targeting the CNS.[129]

AAVs are small (~4.7 kb), single stranded DNA (ssDNA) viruses which exist ubiquitously within human and nonhuman primate (NHP) populations. At the present time, 12 naturally occurring AAV serotypes have been isolated either from human or NHP origin (AAV1-12). Each serotype holds unique properties and surface antigens which make them distinguishable. Various studies have shown that antibodies against AAV serotypes are present globally with up to 80% prevalence.[130] Despite its widespread nature, AAV has not been associated with any human disease. AAV is a dependovirus which is unable to replicate without the presence of a helper virus, such as adenovirus (Ad) or herpes simplex virus.

The genome of AAV is quite simple and consists of only two open reading frames containing the genes responsible for viral replication and capsid assembly (*rep* and *cap*, respectively). These genes are flanked by inverted terminal repeats (ITRs) at both the 3' and 5' region. For use as a vector (recombinant AAV [rAAV]), the *rep* and *cap* gene are removed to make room for the therapeutic expression cassette (Figure 3). The viral-mediated delivery of this therapeutic cassette into target cells is referred to as "transduction." The only remaining elements of the WT AAV within rAAV are the flanking ITRs which serve as origins of replication and packaging signals.[131] This allows for ~4.4 kb of packing space inside the "gutted" AAV. The removal of the majority of the WT AAV genome from rAAV additionally contributes to its safety. When packaging rAAV, the *rep* and *cap* genes are provided to the packaging cells *in trans* on an exogenous plasmid construct(s) along with the key adenoviral genes required for efficient AAV replication and packaging.



Promoter	Specificity	Details	References
CMV	Ubiquitous	• Cytomegalovirus (CMV) immediate early enhancer and promoter • 800bp • Strong promoter • Prone to silencing and altered expression in certain cell-types (including CNS)	123–126
CBA	Ubiquitous	• Hybrid CMV enhancer/ chicken ß-actin (CBA) • 1,600bp • Strong promoter	127,128
SV40	Ubiquitous	• Simian virus 40 promoter • 627bp	129,130
Cbh	Ubiquitous	• Hybrid CBA and minute virus of mice (MVM) intron with the CBA promoter • 800bp • Strong promoter	131,132
MeP	Neurons	• MeCP2 promoter • 229bp • Weak neuronal expression.	131
PGK	Cortical neurons and oligodendrocytes	• Mouse phosphoglycerate kinase 1 promoter. • 511bp • Strong promoter	133,134
GFAP	Astrocytes	• Glial fibrillary acidic protein • 681-2,200bp	135,136
gfaABC1D	Astrocytes	• Truncated GFAP promoter • 683bp	137,138
hSyn	Neurons	• Human synapsin 1 promoter • 495bp • Strong promoter	139

FIGURE 3 (See caption on next page)

Over the past 20 years, use of rAAV has gained overwhelming attention, and AAVs are being widely evaluated in a large number of clinical studies, including delivery applications to the CNS (explored in a recent review[132]). This attention is well warranted as their use holds many advantages over other vector types, such as: (1) low pathogenicity—rAAVs are based on a nonpathogenic WT AAV; (2) ability to transduce both dividing and nondividing cells, which is essential when targeting post-mitotic cells within the CNS; (3) nonintegrating nature—AAVs integrate at only a low frequency into the host genome (0.1%–3%)[133] and exist largely as exogenous episomes with the cells, thus lowering the risk of insertional mutagenesis leading to genotoxicity; (4) persistent transgene expression—AAVs can drive long-term transgene expression in nondividing cells (such as neurons); (5) simple genome—allowing for robust genetic manipulation and bioengineering; (6) ability to manufacture in large quantities, including clinical manufacturing; (7) ability to target various cell types by taking advantage of unique tropism of existing natural serotypes and bioengineered variants.

Despite these attractive features, a number of limitations remain to be addressed to ensure maximum clinical impact, such as the small payload size that can be delivered using rAAVs and the high prevalence of neutralizing antibodies in patient population. Furthermore, as the AAV genome is single stranded (ssDNA), once within the cell the complementary strand must be synthesized before transgene expression can occur. This step can be rate limiting, particularly for applications which warrant high kinetics of expression. While self-complementary AAVs (scAAV) containing a double-stranded genome (dsDNA) have been developed, the faster kinetics of expression have come at a price of vector size, as the scAAV can only package ~2.2 kb of dsDNA. The packaging of scAAVs is also often less efficient than their single-stranded counterparts, which could have significant consequences on clinical manufacturing and thus translational development. However, this section will focus on the use of AAVs for gene delivery to the CNS and current advances within the field.

4.2 | Use of AAV for gene delivery to the CNS

The difficulty of genetic delivery to the CNS largely stems from the presence of the blood-brain barrier (BBB). Indeed, this protective layer which stringently selects only specific molecules for entry into the CNS, evolved to ensure that pathogens/toxic materials, including viruses, are not able to access this indispensable organ. In 2009, an exciting development was made with the discovery that AAV serotype 9 (AAV9) had the ability to cross the human BBB and transduce neurons and astrocytes when injected intravenously.[134] Although this was a significant advancement for the field, a key issue quickly arose: although AAV9 demonstrates clear ability to enter and transduce cells in the CNS, it also has strong tropism for human liver. Therefore, when injected intravenously for use in clinical application, the vector doses required to achieve sufficient delivery and transgene expression within the CNS can often lead to liver toxicity and potentially increased risk of tumorigenesis.[135–137] Unfortunately, liver toxicity has been recently reported in the patients treated with AAV9-SMN1 and the long-term effects related to

FIGURE 3 Graphic representation of the genome of wild-type (WT) adeno-associated virus (AAV) and recombinant AAV vector (rAAV) and a summary of commonly used promoters for targeting the central nervous system. The genome of WT AAV is relatively simple, composed of a *rep* and *cap* genes encoding proteins responsible for viral replication and capsid assembly, respectively. These genes are flanked by inverted terminal repeats which function as origins of replication and packaging signals. For use as gene therapy vectors, the *rep* and *cap* genes are removed, converting the virus into a replication-incompetent vector and freeing ~4.4 kb space for the transgene cassette, including transgene and regulatory elements such as a promoter and a poly-A tail. The choice of promoter in the construct can have a strong impact on the expression of the transgene and the cell types in which expression will occur. Promoters additionally vary in size and thus driven by limited packaging capacity of AAV, the choice of the promoter may be limited by the size of the therapeutic transgene that is to be delivered/expressed. [Color figure can be viewed at wileyonlinelibrary.com]

potential genotoxicity in the CNS as well as liver and other off-target tissues remain to be studied. Similar adverse events have been observed in a recent clinical study that utilized AAV8 serotype for the treatment of a neuromuscular disease, X-linked myotubular myopathy.[138] The high titers of AAV8 used ultimately led to the death of three participants to date due to progressive liver failure and eventual sepsis.[138] This indicates that liver toxicity reported in patients treated with the AAV9-SMN1 was not a unique consequence of using AAV9 but rather was a more general phenomenon when using high doses of AAV vectors. Therefore, ongoing research around AAV vectors for CNS targeting aims to: (1) increase vector ability to cross the BBB; (2) improve specificity to target cells within the CNS while detargeting vectors from the liver; (3) evaluate alternative routes of vector delivery to the CNS to increase specificity and lower liver transduction (while simultaneously decreasing the therapeutic dose, increasing the safety and lowering the overall cost).

4.3 | Crossing the BBB

Immense efforts have been put into the development of novel bioengineered AAVs with the ability to cross the BBB, however, only recently have effective AAV variants come to light. This is partially due to the optimization of methods used to perform AAV bioengineering to develop vectors with novel desired properties. Currently within the field, two main methods are often employed for AAV development: rational design and directed evolution. These methods are directed toward editing of the AAV *cap* gene, since capsid proteins encoded by this gene are the main determinants of vector tropism. Rational design requires prior understanding of the relation between capsid structure and function and employs methods such as site-directed mutagenesis to make targeted changes of *cap* gene to alter function of the capsid. Rational design has the benefit of making limited and direct changes which result in clear mechanistic alterations. Unfortunately, the knowledge of which regions should be modified to result in the desired outcome is often lacking. In contrast, directed evolution can overcome this lack of mechanistic knowledge as it does not require prior understanding of the intimate relationship between capsid sequence, structure and function. Directed evolution works by creating a large pool of random AAV variations, known as the "AAV library," using methods such as capsid shuffling, error prone PCR or peptide insertion. This "AAV library" is then selected by applying a controlled selective pressure in a preclinical model. Although this method can be high throughput and has been shown to lead to the identification of novel highly functional variants, the exact mechanism correlating a new selected variant with the observed function is often lacking. Importantly, although a novel variant developed via AAV bioengineering may work well in the preclinical model used, there are no guarantees that it will work in a clinical application. An excellent example of this in the story of the AAV-PHP.eB.[139] The research team created a library of AAV capsids and applied positive selective pressure for selection of AAV capsids that transduced both neurons and glial cells within their C57BL/6 J mouse model, which led to the identification of a CNS tropic capsid, PHP.B.[140] Using PHP.B as a base to build an AAV library led to the identification of PHP.eB which had even higher transduction efficiency in the same mouse model.[139] However, when further testing was done using NHPs, disappointingly low transduction efficiencies were reported within the CNS.[141,142] Interestingly, the high transduction efficiency seen with PHP.B and PHP.eB also seemed to be mouse strain specific, with limited efficiency seen in the BALB/cJ mouse strain compared to the C57BL/6 J strain used for selection.[141] Subsequent research into the observed inter- and intra-species differences identified the cellular receptor AAV used to cross the BBB, LY6A[143,144]—a receptor which is not present within NHPs, humans, nor certain mouse strains.

These findings clearly demonstrate the importance of the preclinical model used for vector development and evaluation. However, they also demonstrate the efficiency and specificity of the method as well. Therefore, further research is needed to develop novel and improved biologically- and clinically- predictive in vitro and in vivo models of human CNS and the BBB. The clear difficulty here is finding a model which closely mimics humans in terms of BBB and CNS structure. Brain organoids derived from human iPSCs may be used to select for capsids which have

high tropism to the cell types present in this model, however, cannot select for a capsid which can cross the BBB. To overcome this, recent work has also looked at mouse models which contain humanized brain organoids integrated within the mouse CNS.[145] While highly promising, it is important to keep in mind that the structure of the mouse BBB contains key differences from human, as highlighted above with the LY6A receptor example and the effect it had on the function of AAV-PHP.eB.[141,142] The best option moving forward would be to use NHPs directly for selection. However, even this method contains issues surrounding cost, feasibility and ethical considerations. Beyond these concerns, there is still no guarantee that a capsid which is able to cross the BBB and transduce the CNS efficiently within NHPs will translate directly within humans. To solve this enigma, further research is required into methods of directed evolution, as well as rational design and continued search for naturally occurring AAV variants within humans.

4.4 | Specific targeting of the CNS

As AAVs are naturally occurring viruses, they have evolved to infect cell-types broadly to gain an evolutionary advantage.[146] Although this may be preferred for the survival of the virus, it could be a limitation when using AAVs for cell-type specific transgene delivery. This is particularly an issue when targeting the CNS. As high titers of vectors are usually required to obtain sufficient transduction within the CNS following intravenous (IV) injection for AAV9, low vector specificity can often lead to dangerously high vector levels within off-target peripheral organs, such as the liver. This has been observed in a recent study exploring the adverse effects of high dose IV AAV injection in piglets and NHPs.[135] The vector used in that study was an AAV9 variant (AAVhu68) and the dose applied was similar to that used within the SMA clinical trial (2×10^{14} genome copies/kg).[135] Unfortunately, both the NHPs and piglets demonstrated evidence of toxicity, with liver toxicity observed in NHPs and sensory neuron degeneration present in both species.[135] Similar results were observed in another study exploring AAV9 and PHP.B transduction efficiency within different tissue types in NHPs.[141] The results of this study demonstrated vector copy numbers up to 3 logs higher within liver and spleen when compared to brain and spinal cord, eventually leading to acute toxicity in the NHPs.[141] Notably, these adverse events were not seen in lower doses delivered by IV injection, highlighting the importance of both vector specificity as well as its efficiency to reduce IV-delivered AAV doses required for clinical benefits.

To overcome this issue, a recent study has evolved a novel vector (AAV.CAP-B10) which has been specifically de-targeted from the liver while still maintaining its tropism to the CNS in both mice and NHP.[147] To achieve this, the Gradinaru lab employed both positive and negative selective pressures, rather than only positive selection methods used to evolve PHP.eB. This method termed "Multiplexed-Cre-recombination-based AAV targeted evolution" (M-CREATE) works through taking advantage of Cre-Lox recombination dynamics.[147,148] To apply positive selection pressure, transgenic mice are created which express Cre recombinase within the cellular population of interest. AAV libraries can then be generated which encode a Lox site within their AAV genome. Using this approach, it can be identified which AAVs are able to successfully transduce target cells as their genomes will be flipped due to the action of the Cre-Lox system. Only the Lox-flipped DNA are then recovered leading to specific PCR amplification of only the successful AAV capsids. By analyzing organs separately, this method allows for the calculation of an AAV variant's relative infection efficiency specifically across cell-types. Therefore, post-hoc analysis could be utilized for negative selection, removing variants which displayed high off-target effect, such as high liver tropism. The success of this method is highlighted with the recent development of the novel capsid, AAV.CAP-B10. Based on the current data, AAV. CAP-B10 is the first AAV variant to retain the ability to cross the BBB and transduce primary neurons with high efficiency (~350 fold greater than AAV9), while being strongly detargeted from the liver in the preclinical animal models used.[147] This new vector has also shown to have desired tropism in NHPs (albeit, less efficient than its ability in mice), demonstrating a ~six-fold increase in RNA brain expression, and a ~five-fold decrease in RNA liver expression when compared to AAV9.[147]

As vector efficiency has been shown to differ even between mouse strains,[141] further evaluation of AAV.CAP-B10's efficacies and safety is needed before it's use in clinical application can be alluded. It should also be noted that the use of specific promoters which drive transgene expression only within certain cell-types is another method which can circumvent the issue of using nonspecific AAVs for gene therapy (summarized in Figure 3).

4.5 | Methods of injection for widespread CNS transduction

Minimally invasive methods of vector delivery, such as IV or intramuscular injection (IM), are preferable for clinical application. However, a simple alternative when trying to bypass the BBB, is to use a more direct method of delivery. This includes direct injection into the brain/spinal cord and injection into the cerebral spinal fluid (CSF)—such as intrathecal (IT) and intracerebroventricular (ICV) injection. However, the invasive nature of these methods warrants concern. Although potentially less clinically applicable in a wider context, the benefits of a more direct method of gene delivery include increased ability to target desired cells with a lower vector dose, lower off-target tissue transduction and decreased neutralization by pre-existing neutralizing antibodies (NAbs). These advantages may out weight the associated risks depending on the desired application. This is the case in the context of lethal diseases, such as ALS, when the risk associated with no treatment or ineffective vector delivery is considered. As the low efficiency capsid, AAV9, is the only AAV capsid to have been used intravenously for CNS targeting in clinical applications, alternative methods of injections should be explored to improve gene delivery outcomes.

Although invasive and requiring specialized surgery, direct injection into the CNS can result in high vector expression and low off-target effects while reducing the total vector dose required. A recent study found that a single injection of AAV9 directly into the spinal subpial (SP) region of adult mice, pigs and NHPs resulted in transduction of neuronal and glial cells broadly throughout the cervical spinal cord and motor centers of the brain.[149] Compared to direct parenchymal injections which must be done with "blind" needle penetration, this study has utilized a for-purpose-designed specialized device for injection into the SP region while maintaining full visual control. Remarkably, no complications related to the surgical procedure were reported using this novel injection device, highlighting the increased safety of their injection method compared to traditional parenchymal injections.[149] Unfortunately, this study did not explore the impact of SP injection on transduction of peripheral organs, an important consideration before use in clinical applications.

When performing direct injection, another important consideration is the ability of the vector to spread from the site of injection throughout the CNS. For examples, AAV2 is known to have only limited spread from the injection site,[150] whereas AAV9 has been shown to have a much wider distribution of transduction.[151] This phenomenon can be thought of as how "sticky" the vector is, with less "sticky" vectors having the ability to transduce cells more widely from the initial site of injection. The "stickiness" of a vector is largely attributed to how well it can bind to the membrane-associated heparan sulfate proteoglycan (HSPG). Indeed, recent studies have collectively found that HSPG binding can be detrimental to the spread of a vector, and its reduction can largely increase a vectors transduction efficiency in vivo within the liver,[152] retina,[153] and brain.[150,154,155] Therefore, when exploring the use of ICV or SP injections directly within the brain for widespread transduction, a vector with low HSPG binding is preferred to increase its ability to further distribute and transduce widely throughout the CNS.[153,155] Indeed, the importance of HSPG for the efficiency for AAV tissue transduction is of growing interest in the field of vectorology with powerful implications for future capsid development.

Alternatively, methods of injection into the CSF via IT and ICV injections have also resulted in widespread AAV transduction. In an AAV9 vector dosage study performed in NHPs, it was found that IT injection required a dose 10 times lower to achieve efficacy when compared to that required for an IV injection.[156] An additional study in NHPs found that IT injection of AAV9 resulted in widespread transgene expression in motor neurons within the spinal cord and several brain regions, whereas ICV injection resulted in widespread brain transduction.[157] Although no adverse effects were reported, both studies found evidence of AAV leakage from the CSF compartment into the

peripheral organs and muscle tissue. Although this was found to be less significant compared to IV injection of AAV9, the off-target effects must still be explored to ensure that no toxicity within these peripheral organs is attained. This effect additionally highlights the importance of improving AAV specificity even while more direct injection methods are utilized.

Overall, the recent developments of direct injection methods for AAV gene delivery into the CNS have helped improved the overall safety and efficacy. Although AAV capsid development is also occurring at a rapid rate, it is our opinion that the combination of the novel improved capsids with safe and specific injection methods holds the promise of achieving efficient and specific AAV delivery to patients for CNS indications. Exciting technological leaps forward are additionally being achieved with AAV vectors that are detargeted from peripheral organs (such as AAV.CAP-10), making them safer for IV application and efficient CNS transduction. As the pace of AAV vectorology continues to grow, hopefully future gene-therapy applications will no longer perceive the method of gene delivery to be an obstacle to overcome when creating new and exciting genetic therapies.

5 | CONCLUSION

Despite the extensive research, the precise molecular mechanisms underlying ALS remain incompletely resolved. Dysfunction within protein degradation pathways and RNA metabolism pathways are emerging as pivotal in ALS pathogenesis. Using insights gleaned from studies examining these pathways, gene therapies can be designed to target salient processes and re-establish both proteostasis and ribostasis, possibly inducing cellular remission in the hope to slow down, stop or ideally reverse neurological deficit.

The benefit of using gene therapy over traditional drugs include their duration of action, particularly when targeting post-mitotic cells such as motor neurons. Currently, the use of AAV for gene delivery is showing considerable promise, with an increasing number of trials using this approach. Over the years, there has been great advancement in both ALS research and AAV development which have occurred largely independently of each other. However, integration between these fields of research will facilitate preclinical development of engineered rAAV vectors with optimized and specific targeting capacity for the therapeutic outcome desired. Therefore, ensuring a collaborative effect between discovery science, target validation and optimized AAV delivery will allow for the best chance of therapeutic success in a clinical setting.

ACKNOWLEDGMENTS

L. L. and R. C. was supported by project grants from the Australian National Health and Medical Research Council (NHMRC) (APP1095215, APP1108311, APP1156431, and APP1161583). L. L. was additionally supported by research grants from the Department of Science and Higher Education of Ministry of National Defence, Republic of Poland, ("Kościuszko" k/10/8047/DNiSW/T—WIHE/3) and from the National Science Centre, Republic of Poland (OPUS 13) (UMO-2017/25/B/NZ1/02790). R. C. has received additional funding from FightMND (04_DDG_2020, 25_Impact_2020) and the National Foundation for Medical Research & Innovation Project Grant. R. P. holds an MRC Senior Clinical Fellowship (MR/S006591/1) and a Lister Research Prize Fellowship.

CONFLICTS OF INTEREST STATEMENT

Leszek Lisowski is an inventor on patent applications filed by Children's Medical Research Institute related to AAV capsid sequences and in vivo function of novel AAV variants. Leszek Lisowski is a co-founder and has a sponsored research agreement with LogicBio Therapeutics. Leszek Lisowski is a co-founder of Exigen Biotherapeutics. Leszek Lisowski has IP licensed to biopharmaceutical companies. Leszek Lisowski has consulted on technologies addressed in this review. Leszek Lisowski has stock and/or equity in companies with technologies discussed in this review.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Merjane J, Chung R, Patani R, Lisowski L. Molecular mechanisms of amyotrophic lateral sclerosis as broad therapeutic targets for gene therapy applications utilizing adeno-associated viral vectors. *Med Res Rev*. 2023;1-26. doi:10.1002/med.21937