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Analysis of the therapeutic strategies used to treat Amyotrophic Lateral Sclerosis caused by TDP-43 aggregation and/or mislocalization

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Abstract

Amyotrophic Lateral Sclerosis (ALS), a fatal neurodegenerative disorder that impairs motor functions, affects 1 in 50,000 people in the world, and has an insignificant hereditary component, with 90% of patients *sans* any family history of the disease. ALS is a debilitating disease due to the limited effectiveness of treatments. *TARDBP*, the gene of interest, encodes The TransActive Response DNA-binding protein 43 (TDP-43 (kDa)). Mutations in this gene result in TDP-43 that may undergo deregulated expression (gain or loss of function), be misfolded, cleaved, aggregated, and/or mislocalized. More specifically, TDP-43 may also be aberrantly ubiquitinated, phosphorylated, cleaved, or nuclear depleted. The resultant proteinopathy is associated in the causal chain of many neurodegenerative disorders, including in > 95% of patients with sporadic ALS. TDP-43 consists of mitochondrial and nuclear localization sequences; RNA recognition motifs; intrinsically disordered regions and is involved in the appropriate splicing and transcription of a host of protein-coding-genes. Preventing and/or correcting dysregulated TDP-43 homeostasis and proteinopathy is near-universally effective *in vitro* and in animal models. This study focuses on analyzing the benefits and limitations of the therapeutic strategies used to treat ALS caused by dysregulated TDP-43 homeostasis and identifying promising methods to target this proteinopathy.

Keywords

Neuroscience, Amyotrophic Lateral Sclerosis, TDP-43, Gene therapy, Neurodegeneration, Nucleocytoplasmic transport, Mislocalization, Lou Gherig's disease, C9orf72, Ran GTPase

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1 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects the function of motor neurons and, in most cases, leads to neuronal cell death. As neurons slowly deteriorate, they are unable to transmit impulses from the central nervous system (CNS) to the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord while the peripheral nervous system consists of the surrounding nerves that branch off from the CNS. When these neurons deteriorate, signals between adjacent cells stop transmitting. Signals are the stimulus that produce muscle movement, hence when the signals are disrupted, the muscles can weaken and twitch (1).

Doctors categorize ALS into two sub disorders: familial ALS and sporadic ALS. Familial ALS (fALS) is caused by hereditary genetic mutations such as mutations in the genes, superoxide dismutase 1 (SOD1) or in the chromosome 9 open reading frame 72 (C9orf72), affecting ~ 10% of patients. Sporadic ALS (sALS), however, is more prevalent among patients, where there are no common underlying cause derived from family history or environmental factors.

Some symptoms of ALS include muscle weakness, spasms, stiffness, and spasticity as well as involuntary movements, respiratory distress, and paralysis. By analyzing their symptoms, physicians are able to diagnose ALS patients, but due to the wide range of causes the disease has, they are unable to target the type of ALS their patients have using a standard treatment process (2). Therefore, many FDA-approved medications for ALS target the broad neurotoxicity linked to the neuronal death in patients in order to slow the development of the patient's symptoms.

The most commonly-used medication for ALS is riluzole, whose function is to block the release of excess glutamate. Glutamate is an amino acid that can attach to two amino acid transporters in the neuron membrane, GLT1 and GLAST (3). Build-up of glutamate in the synaptic cleft, or the space between two neighboring neurons, causes overactivation of glutamate receptors; commonly seen in many patients. Therefore, this medication attempts to reduce the amount of the neurotransmitter present by activating a G-protein transduction process that inhibits the release of glutamic acid, an amino acid used to form glutamate (4). However, rather than specifically targeting glutamate receptors, riluzole also blocks the acetylcholine receptors in neuromuscular junctions, which prevent muscle signal reception (5). This medication is, therefore, has a side effect of exacerbating muscular degeneration in some ALS patients.

Studies have also linked riluzole to higher levels of liver enzyme concentrations, sometimes 2-5 times over the normal limit (6), as well as to rare incidences of acute liver damage, and both have been extensively reported over the last two to three decades. Even though riluzole is moderately effective in increasing the life expectancy of patients, it induces at least one adverse effect in up to 50.3% of patients, including hepatotoxicity and gastrointestinal disturbances (7). Overall, riluzole does not seem to have an exceptional risk-to-benefit ratio and hence is not the most effective treatment pathway for patients.

Edaravone, both intravenous or oral, is another common drug prescribed for sALS. Rather than targeting neurotransmitters, edaravone is a free radical scavenger an antioxidant that may provide cytoprotection to shield neurons from excess glutamate by detoxifying reactive oxygen species (ROS). ROS are known to cause damage to the molecules such as DNA, proteins, and lipids that make up the cells, so it is important that they not be subject to constitutive ReDox attack; which in turn, may accelerate neuronal damage and consequent exacerbation of ALS symptoms. A clinical trial of intravenous edaravone slowed disease progression by 30% after 24 weeks of treatment. However, the medication seemed to offer short-term benefits only to a specific category of patients. Furthermore, the effectiveness of long-term edaravone treatment did not improve the Quality of Life (QoL) using the short-term Revised ALS Functional Rating Scale (ALSFRS-R) scores. ALSFRS-R scores indicate the QoL patients have based on where their symptoms fall on a scale determined by researchers (8).

Even though the edaravone trial did prove the effectiveness of this new treatment design, intravenous edaravone is not the only method used by doctors. Another trial specifically tested the effects of oral edaravone on patients who had not yet undergone severe neuronal degeneration. It had positive outcomes for patients who had the least severe variations of ALS symptoms and had been diagnosed for at most 2 years; the rest of the patients who were not in this subgroup did not present with as successful outcomes (9). Even though edaravone improves the health of many patients with certain types of ALS within certain temporal therapeutic windows, the biggest obstacle that prevents the widespread use of edaravone is its increased cost compared to that of riluzole. A study conducted by the Canadian Agency for Drugs and Technologies in Health showed that in total using an incremental cost-effectiveness ratio, patients will pay approximately $1,957,200 for each year of increased life expectancy gained (10). Edaravone increases the life expectancy of specific categories of ALS patients more effectively than riluzole, but the cost of the treatment relative to the rate of improvement presents an obstacle to increased prescription and usage.

The most recently approved medication for ALS is sodium phenylbutyrate-taur ursodiol, which received FDA approval in 2022. ALS associated epigenetic changes may reduce the upregulation of neuroprotective stress pathways, including HSPs to chaperone misfolded proteins for degradation (11). Sodium phenylbutyrate is a pan-histone deacetylase (HDAC) inhibitor that may ameliorate endoplasmic reticulum stress by upregulating chaperone proteins (12). The primary evidence for the effectiveness of sodium phenylbutyrate-taur ursodiol originated from the CENTAUR phase II trial, which tested the new treatment on patients whose symptoms began less than 18 months before the start of the trial (13). This trial showed that the administration of the medication for 6 months decreased the clinical symptoms of ALS patients and was used to receive FDA approval (14). Based on a subsequent phase III PHOENIX trial, in which the drug failed to meet its primary endpoint (change in baseline of the ALSFRS-R total score) , the drug was

withdrawn from the US market in April 2024. The failure of multiple drugs in the clinic that modulate ALS non-specific pathways (oxidative stress, autophagy, excitotoxicity, inflammation) demonstrates that *specific* ALS mechanism modulating drugs need progression to clinical trials (see Table 1). Among them are approaches involving the inhibition of abnormally transcribed RNA using microRNA or Antisense Oligonucleotides (ASOs), degradation of the abnormally transcribed RNA, removal or inhibition of mutant proteins, and genome editing. These therapeutic pathways are designed to target specific causes of ALS. As an example, QRL-204, QurAlis's drug designed to restore UNC13A function – due to TDP-43 depletion inducing cryptic exon transcription of UNC13A - in nerve cells is being developed by Eli Lilly (Table 1).

Researchers have focused on developing gene therapies for SOD1 mutations because it is one of the most common mutations that cause ALS, affecting 20% of patients with fALS. SOD1 mutations occur in the superoxide dismutase type 1 gene, which is responsible for protecting cells against the damage caused by free oxygen radicals (15). Researchers are proposing the potential of silencing SOD1 mutations or delivering compounds that can activate receptors to protect neurons from SOD1 toxicity. Studies have also focused on modulating the level or expression of proteins that decrease the progression of neurodegeneration symptoms. One of the proteins studied is Exportin 1 (XPO1), which mediates nuclear export signal (NES)-dependent protein transport. Inhibiting XPO1 using RNA and the inhibition of nuclear transport led to a reduction in toxicity caused by mutant C9orf72 (which causes ~40% of fALS cases and 6-8% of sALS cases) (16,17). KPT-350, a XPO1 inhibitor, demonstrated potential in neuroprotection and anti-inflammation in pre-clinical trials, but most of this research was focused on Duchenne muscular dystrophy. More research is required in pre-clinical ALS models before a more definitive conclusion can be reached on the implications of the inhibition of XPO1. ASOs have been used to reduce the sense G_4C_2 RNA foci without reducing the levels of C9orf72, leading to the ASO BIIB078 being approved for clinical trials for ALS patients with the G_4C_2 expansion (16). These treatments, that target specific ALS mechanisms were effective in animal models, but there is no evidence yet about efficacy in the clinic.

2 Discussion

2.1 TDP-43

Even though a cause for ALS found in a majority of patients eludes discovery, one of the hallmarks of the disease is aberrant aggregation of the transactive response DNA binding protein of 43 kDa (TDP-43) in the cytoplasm of both sALS and fALS patients. There is not a consensus of whether this aggregation is a direct cause of neurodegeneration seen in ALS. However, it has been shown to cause progression of the disorder's symptoms. TDP-43's function is to regulate mRNA stability; it is necessary for the normal translation and splicing of mRNA and the repression of cryptic exon inclusions (18, 19). Its presence in the nucleus of the neuron is essential for cellular metabolism. When it aggregates in the cytoplasm, it prevents the normal regulation of mRNA splicing and

translation. Cytoplasmic aggregation also causes aberrant nucleocytoplasmic transport (NCT) of itself, as well as of other proteins (19, 20). This prevents and dysregulates normal, functional protein translation and production, which; in turn, can inhibit important cellular functions.

To understand what causes TDP-43 aggregation, it is imperative to first analyze the structure of this protein. It contains two RNA-recognition motifs, called RRM1 and RRM2, that are rich in glycine, and where most mutations that cause TDP-43 aggregation are found (21). However, aggregation of TDP-43 is not simply caused by mutations in the glycine-rich regions of the protein; its aggregation has also been linked to the dysregulation of the protein's production. The production of TDP-43 is highly regulated through an autoregulation process using cryptic exon repression of a 3' untranslated region (UTR) on the *TARDBP* mRNA. When TDP-43 is overexpressed by the cell, the 3' UTR is activated, which results in the proximal poly-A site being excised from the mRNA; this temporarily stops the production of TDP-43 (18). Any anomaly in this autoregulation process can potentially lead to overexpressed and/or mislocalized TDP-43.

The structure of TDP-43, especially when misfolded, leads to aggregation of the protein in the cytoplasm and partial loss of function. TDP-43 aggregation results in cellular stress as evidenced by the formation of stress granules and its co-localization with cytoplasmic protein aggregates. Stress granules typically form to repress the translation of certain RNAs, and proteins that are involved in neurodegeneration interact with these granules (22). Proteomic analysis (which determines when and where proteins are expressed) of the neurodegenerative cells provided evidence that 134 enriched and 17 depleted proteins were present in mice with a nuclear localization sequence for TDP-43. This further provided evidence that stress granule formation was associated with the aggregation of TDP-43 (23). The presence of shortened N-terminal TDP-43 isoforms, termed sTDP-43, are predominantly found in the cytosol of the cell and amplified within at-risk spinal motor neurons. sTDP-43 is created as a normal byproduct of the wild-type protein undergoing autoregulation. Under normal circumstances, it can be cleared by the cell's nonsense mediated RNA decay (NMD). However, some sTDP-43 proteins can evade this mechanism and cause cellular toxicity. Overexpression of TDP-43 coupled with NMD repression was shown to lead to the underproduction of full-length TDP-43 proteins and more sTPD-43 (24). Therefore, this form of the protein represents a viable form of aggregation seen within ALS-affected neurons and must be taken into consideration when designing targeted therapies.

Cytoplasmic aggregation of TDP-43 is also connected to the neuroinflammation seen in the cytokine profile (the measurement of levels of cytokine within the sample), driven by the cytoplasmic DNA sensor cyclic guanosine monophosphate-AMP synthase (cGAS). cGAS is jump started by the ingress of TDP-43 in the mitochondria and the resultant release of DNA within the organelle, leading to the phenomenon known as mitochondrial aggregation of protein. The presence of this form of aggregation was seen in mice with end-

stage ALS degeneration, bordering early lethality. By inhibiting cGAS and its signaling partner, the STING pathway, the upregulation of the nuclear factor NF-kB and type 1 interferon – both molecules associated to the cytokine profile mentioned before and induced by TDP-43 – was prevented (25). This pathway hence represents a significant target for gene therapy due to the elevated presence of signaling metabolite from cGAS in patients' spinal cord samples.

Studies have also demonstrated that there are elevated levels of reverse transcriptase within the cerebrospinal fluid and serum of ALS patients without any exogenous infection. Patients with exogenous HIV-1 were seen to undergo ALS-like symptoms, establishing a connection between HIV and ALS through the human endogenous retrovirus K (HERV-K). HERV-K also has 5 binding sites for TDP-43, suggesting that the protein may be involved with the transcriptional regulation of the retrovirus. The presence of HERV-K resulted in a decrease in TDP-43 mRNA and protein levels; therefore, the retrovirus can regulate both levels through a positive feedback loop, showing that it has some influence on TDP-43 aggregation (26). However, HERV-K is not the only endogenous retrovirus that has been linked to ALS. The human endogenous retrovirus HLM-2 is stored within the gene that codes for the asparaginase-like-1 protein (ASRGL1), whose function is to alter protein folding. The expression of this protein was reduced in the brain samples of patients with ALS, and in the absence of ASRGL1, TDP-43 cytoplasmic aggregation was elevated. However, the overexpression of ASRGL1 restored neuron stability while the overexpression of HLM-2 led to ASRGL1 silencing (27). Therefore, to target this specific relationship, gene silencing and overexpression is not the full answer, and further research is needed.

TDP-43 aggregation is also seen to be driven by its low-complexity domain (LCD), which is one of its four domains. This domain has a high inclination to undergo Liquid-Liquid Phase Separation (LLPS). LLPS is a form of phase transition in which one homogenous solution spontaneously separates into two distinct liquids. The LCD regulates protein-protein interactions, and this impacts gene regulation within the neuron; this domain can also form amyloid fibrils, and recent research has determined that a diagnostic set of aggregation domains of TDP-43, which is described as amorphous aggregates, had characteristics consistent of the ability to form amyloid fibrils (28, 29). Overall, TDP-43 aggregation is affected by a variety of factors, meaning that a complex therapy must be established or major downstream intersection points - or preferably, a point of convergence - of various pathways must be found to affect neurodegeneration deceleration.

This protein aggregation is a significant symptom in both fALS and sALS, and it can progress the neurodegeneration in patients. Genetic mutations causing mislocalization of TDP-43 also increases DNA damage because the localization of certain double-stranded break-repair proteins is disrupted. This mislocalization of TDP-43, whether caused by genetic mutation or other factors, may also lead to the altered splicing, in which exons from the target gene are joined in different combinations

and form different but related mRNA versions (18). Researchers remain divided on two main points of discussion for this protein: whether it is a cause or consequence of ALS or whether consequences of this protein aggregation are caused by the lack of the wild-type protein's function (loss-of-function) or the gain of a new function not seen in the wild-type protein (gain-of-function). It must be emphasized that this explanation is further complicated by the fact that TDP-43 loss of function can occur in the nucleus, but not in the cytoplasm (or *vice' versa*); as can its gain of function; which can manifest in one or the other compartment of the cell.

2.2 Cause or consequence

The aggregation of TDP-43 within neurons affected by ALS is an important factor when understanding the progression of the neurodegeneration seen in the disorder. The two most common configurations of TDP-43 aggregation are amyloid-like fibrils (insoluble proteins that tend to aggregate) and soluble oligomers. One study determined that one of the RNA Recognition Motifs (RRMs) contain properties similar to amyloids, which increased the potential for aggregation; fibrils formed with an increase in temperature. However, it is also important to understand that the C-terminal end also undergoes many post-translational changes, which is one of the causes of TDP-43 aggregation (30). Some research supports the hypothesis that the formation of the aggregation of TDP-43 does lead to neurodegeneration in ALS patients, meaning that it can be characterized as a cause for the symptoms of the disorder (18). However, it has also been proposed that the aggregation represents a consequence of the disorder. Semantic differentiation aside, classification into a predominant category (cause or consequence) dictates the trajectory of research and drug discovery. TDP-43 aggregation involves many post-translational changes such as cleavage, ubiquitination, and phosphorylation.The abnormal phosphorylation of TDP-43 increases resistance to degradation by the ubiquitin-proteasome system, which leads to the increased intracellular aggregation of TDP-43 (31). The ubiquitin-proteasome system is involved in the degradation of soluble TDP-43, but once the protein has aggregated, disposal is only possible through autophagy (32). The cleaving of Arg208 (a site on the TDP-43 protein) caused by ALS led to an incapability to fold the protein by RRM2, which made the protein more prone to aggregation (33). It may be concluded that TDP-43 aggregation can cause ALS because aberrant ubiquitination or phosphorylation can lead to the presence of the disorder. However, ALS can cause the cleavage of Arg208, thereby causing TDP-43 aggregation, which implicates aggregation as a consequence of ALS. The process of abnormal phosphorylation and ubiquitination is caused by mutations in regions of TDP-43 observed in ALS diseased neurons. Since the analysis of ALS in this paper does not focus on cleavage but on phosphorylation and ubiquitination, it will be assumed that TDP-43 aggregation is a cause of ALS progression.

2.3 Loss of function

The loss-of-function hypothesis states that the mutant TDP-43 loses some of its primary functions, and it is best tested using common genetic mutations found in *TARDBP*. Previous studies modeled various loss-of-function

TARDBP mutations in non-mammalian and mammalian species (zebrafish, *Caenorhabditis elegans*) by knocking out the gene. They reported that all the KO organisms developed neurodegenerative properties; the same study was simulated in a Drosophila model by knocking down the A315T allele of *TARDBP*, with similar results (34). The loss-of-function hypothesis was also supported by the modeling of the partial knockdown of TDP-43 in a transgenic mouse model because 601 mRNAs were changed out of the 965 altered splicing reactions that were tracked; a large number as would be expected from derepression caused by decreased levels of TDP-43 (35). Another study also provided evidence that a loss in TDP-43 function led to the splicing changes seen in the cryptic exon, UNC13A, which is considered a risk gene for both ALS and fronto-temporal lobe dementia (FTD). This change caused deficits in synaptic transmission, impacting neural function (36). A loss-of-function of TDP-43 therefore, is capable of causing neurodegeneration and ALS symptoms in these models.

In another study, the tdp-1 ortholog in *C. elegans* was silenced because it was similar in function and expression to *TARDBP*. It was observed that its loss led to defects in fertility, growth, and locomotion; transcriptional profiling was also utilized to prove the alterations in the expression of genes involved in RNA processing and protein folding (37). This study was repeated in *D. melanogaster* by knocking out the dTDP-43 ortholog, leading to similar results of impaired locomotor activity and axonal loss, which are all indicators of neurodegeneration (38). The symptoms and TDP-43 proteinopathy expressed in both non-mammalian and mammalian models resembled those of ALS patients. Fragmentation and aggregation of the protein and partial loss-of-function could also be achieved by overexpression of wild-type TDP-43 (39). Therefore, it can be concluded that the mutation of the TDP-43 gene can lead to the loss of the protein's function.

The loss-of-function hypothesis was also evidenced by identifying a set of proteins based on their function, association with ALS, and antibody availability. The impact on these set of identified proteins was studied to determine the effects of TDP-43 aggregation or knockdown. The results showed that when simulating aggregation and TDP-43 knockdown, the chosen set of proteins' reaction to both conditions resembled one another, thereby equating the two conditions in terms of cellular effects (40). Altogether, the general mislocalization of TDP-43 can also be used as proof for the loss-of-function hypothesis. These aggregates are typically shifted from its normal location in the neuronal nucleus, thereby depleting the nucleus of TDP-43. T his manifests as a loss-of-function because because the primary functions of mRNA splicing and repression of cryptic inclusions cannot occur due to nuclear depletion (one-way migration into the cytoplasm).

2.4 Gain of function

The gain-of-function hypothesis is derived from the idea that the TDP-43 gene is overexpressed, which leads to the protein exhibiting additional properties. Some evidence for this hypothesis interprets the toxicity of TDP-43 as novel properties acquired due to the overexpression of TDP-43. These new

properties can include the increased affinity for aggregation, mislocalization, and resistance to proteases or any modified binding interactions with other proteins. These properties can all lead to neurodegeneration.

Many studies have relied on the idea that the overexpression of TDP-43 is what causes the neurodegeneration in cells because *in vivo* experiments have shown that the aggregation caused by overexpression is toxic to neurons. This led to the hypothesis that TDP-43 may be gaining toxic properties not related to its primary function. In *C. elegans*, the expression of the NLS-mutant version of TDP-43 was not toxic though it strictly accumulated in the cytoplasm. This lead to the conclusion that the protein's toxicity due to overexpression is dependent on its RNA-binding domains. Therefore, the gain-of-function hypothesis may be dependent on its normal function related to RNA processing rather than on the protein itself exhibiting - only accumulation dependent - novel toxic properties (38).

One toxic gain-of-function mechanism that is observed due to TDP-43 aggregation is the blockage of intracellular transport in neurons. These aggregates can be found throughout the neuron but they have been observed in both the axons and dendrites. The inhibition of axonal transport is a common symptom in ALS, so this would suggest a link between aggregation and ALS. TDP-43 toxicity has also been linked to its RNA binding abilities because this function regulates its solubility, hence when there is a lack of RNA, oligomers (as precursors of aggregates) of the protein begin to form (18). Research is ongoing to determine whether the gain-of-function mechanism of TDP-43 leads to novel toxic properties, or to toxicity that is related from the protein being unable to perform its normal functions.

2.5 Simultaneous loss-of-function and a gain-of-function

Since both the loss-of-function and the gain-of-function hypotheses rely on different interpretations of similar pieces of evidence, the most logical conclusion is that TDP-43 aggregation can lead to simultaneous gain-of-function and loss-of-function mutations in the TDP-43 protein. This can be explained by how loss-of-function mechanisms found in the protein can lead to aggregates of TDP-43 preventing primary functions, such as transcription and mRNA splicing, and this block can accumulate gain-of-function mechanisms such as the aggregates blocking any intracellular transport in the axons. It may be that this is an example of a continuous feed-forward cycle of loss-of-function and gain-of-function mechanisms. It is hence most effective to consider the possibility of TDP-43 having various properties expressed: gain of toxicity, and the loss of its original primary functions.

2.6 Potential therapeutic strategies

As established, TDP-43 aggregation is a shared proteinopathy found across both sALS and fALS patients, making it an important target for treatment. Targeting this protein's mechanisms through different treatment pathways can potentially be more successful in increasing life expectancy of, and in decreasing the rate of neurodegeneration in patients. Researchers have focused on developing three distinct methods to target TDP-43 aggregation: gene therapy, ASOs, and small molecules and antibodies.

2.6.1 *Gene therapy*

Gene therapy is a form of treatment that aims to manipulate or alter the expression of a gene in order to reverse the effects of diseases and, possibly, cure them (41). It is a relatively new form of treatment since it was created in 1990 to treat a patient with severe combined immunodeficiency (SCID), and has achieved much success in treating other diseases.

Gene therapy can be broadly classified into one where the target cells are removed from the patient's target tissue and edited using the therapeutic gene before being inserted again into the patient; termed *ex-vivo*. Conversely, in *in vivo* gene therapy, the therapeutic gene is inserted into the patient's body, to be carried to its target tissue through the bloodstream using formulation engineering (42). The therapeutic gene referred to is carried to the targeted region of the body using either viral, bacterial, or lipid vectors. The viral vector most commonly used by researchers is the adeno-associated virus vector (AAV).

One study specifically focused on improving a symptom of ALS, namely, the loss of the integrity of corticospinal motor neurons (CSMN), by targeting an enzyme called ubiquitin C-terminal hydrolase-L1 (UCHL1), which maintains the levels of free ubiquitin in neurons. Free ubiquitin allows for normal nervous system development and rapid responses to cell signaling, and the decrease in UCHL1 leads to some ALS symptoms. Mice lacking UCHL1 showed early and selective degeneration in their CSMN due to misfolded SOD1 toxicity and TDP-43 aggregation. By using adenovirus-mediated retrograde transduction (binding of the AAV to receptors on the surface of the axon to begin receptor-mediated gene transfer), the researchers were able to reduce the extent of the loss of integrity due to misfolded SOD1 toxicity and mutated human TDP-43; this was measured by analyzing the neuronal integrity and the stability of the cytoskeleton of the cell in the two different mouse models (43). Another study targeted the human frameshift mutation protein 1 (UPF1) by increasing its production after discovering that it exhibited protective effects in a rat paralysis model. They recreated ALS symptoms by inducing the expression of mutated TDP-43 in the models. UPF1 was then administered using an adeno-associated virus vector. UPF1 treatment demonstrated that the rats regained forelimb motor function, thereby validating the use of UPF1 as a therapeutic strategy to target the symptoms of ALS induced by the expression of mutant TDP-43 (44). These two strategies targeted mutations found in enzymes that protected cells from TDP-43 aggregation.

TDP-43-induced neurodegeneration could be rescued by lowering the expression of the gene coding for the protein NPTX2. Lentivirus transfected NPTX2 exhibited neurotoxicity due to overexpression of the gene. Correcting NPTX2 misregulation partially rescued neurons from TDP-43-induced neurodegeneration, thereby suggesting that NPTX2 was a driver of TDP-43 toxicity through a downstream pathway (39).

The inhibition or overexpression of signaling pathways can be achieved using gene therapy. The casein kinase 1 epsilon gene (CK1ε) protein is tightly correlated to hyper-phosphorylated TDP-43 aggregation; the

inhibition of the CK1 kinase activity with siRNA led to a decrease in phosphorylated TDP-43 aggregation from both its insoluble and soluble isoforms, thus demonstrating the potential effectiveness of this therapeutic strategy (45). Another study inhibited the cAMP/PKA signaling pathway by targeting two negative downregulators – phosphodiesterase *dunce* and the subunit *PKA-R2*. Results indicated a decrease in TDP-43 aggregation and mislocalization within larval motor neuron cell bodies (46). Overall, the targeting of signaling pathways related to the nuclear pore complex and the phosphorylation of intracellular proteins and enzymes, using gene therapy, appears to have a significant effect in the pathophysiology of ALS.

The vector used to deliver the gene; and the promoter used to drive its expression, are as important as the gene itself. The vector determines the amount of intact gene cargo that is ultimately delivered to the targeted cell compartment. When targeting neuron cells specifically, the vector must include a promoter that is specific to the cell-type. A study using an AAV9 vector with a synapsin promoter determined that the vector was targeting other tissues along with the CNS because traces of the promoter were found in the liver 4 weeks after the first round of results. This is because with the combination of this particular vector, the promoter synapsin behaves in a neuron-selective; not in a neuron-specific fashion. In another study, intravenous delivery – rather than intracerebroventricular delivery - was more advantageous for efficient expression of TDP-43 when delivered by a AAV-PHP.B vector because this method of administration – coupled with the enhanced CNS tropism of the vector itself - increased the strength and specificity of the included promoter (47). Though AAV-PHP.B is a more effective method of transport of genetic material, it is significantly more expensive than AAV9, which decreases the possibility of making it a widespread solution. Therefore, future research in this field should focus on either decreasing the production cost of the vector or finding a less expensive but equally effective variant of AAV9.

Gene therapies were also used by researchers to study the effectiveness of targeting TDP-43 and its relationship with other proteins such as SARM1, which is implicated in the degeneration of axons. One study demonstrated the effects of knocking out the SARM1 ortholog in Drosophila, wherein axon degeneration was prevented, thereby implicating SARM1's participation in neurodegeneration (48). Another study used TDP-43 to manipulate the levels of SARM1 in neurons through the presence of Stathmin-2 (STMN2). TDP-43 mediates the mRNA splicing of STMN2, which is a protein that is significantly reduced in ALS and whose decreased levels can be associated with the aggregation of TDP-43. Loss of STMN2 was replicated in murine models. Results showed that there was a connection between motor neuropathy and protein loss. STMN2 is normally coregulated with another protein called NMNAT2, which can stimulate axon protection if overexpressed and can also inhibit the function of SARM1. However, due to TDP-43 aggregation, STMN2 is dysregulated, which negatively impacts the expression of NMNAT2 , thus leading to the expression of SARM1 in the patient's neurons. This specific study

proposed the theoretical strategy to increase the expression of STMN2 in order to overexpress NMNAT2. It was postulated that such a strategy would inhibit the function of SARM1 and reduce motor neuropathy in patients (49). The results from this study showed that an indirect gene therapy pathway that does not directly modulate TDP-43 - but a related protein - could be effective as a standard therapy in animal models. The one limitation to this approach is that it fails to address the aggregation of TDP-43 already present in the neuron.

Researchers also utilized the deletion or suppression of the expression of certain proteins related to TDP-43 in order to control the toxicity of the protein. One study found that the most successful suppressor of TDP-43 toxicity was the deletion of DBR1, which coded for an RNA lariat debranching enzyme (50). Intronic lariats accumulate in the cytoplasm upon overexpression of DBR1, which prevent TDP-43 from normally modulating essential cellular RNA and RNA-binding proteins. The various gene therapies that researchers have developed in order to provide potential treatment for TDP-43 toxicity have generally met proof-of-principle in *in vitro* or animal studies. However, they still need to be translated to humans.

2.6.1.1 *Benefits*

Gene therapy is a very specific treatment option because it is able to target any specific gene implicated in the causal chain of ALS and, therefore, is only applicable to a certain class of patients who meet specific criteria. Since certain genes that affect TDP-43 are targeted, the patient must present with the imbalance and/or dysregulation between the target genes and TDP-43. Patients must also pass many diagnostic tests to confirm that the patient fits all the criteria to receive the gene therapy. This can increase the success rate of the therapy since it can more effectively address the symptoms. The current medications used to treat ALS address the general symptoms of all ALS patients, hence there is no guarantee that they will be as successful in all patients. However, with gene therapy targeting smaller subsets of patients, based on diagnostic genetic markers, researchers can target a particular gene to suit that particular patient.

Each of the suggested gene therapies have also been supported by multiple trials of the treatment in animal models that replicate the pathology of the target category of ALS patients. This shows that the effectiveness is replicable in animal models. The most significant benefit of gene therapy is that it may not have to be administered numerous times; once the vector has been delivered to the target neurons, then the effects can persist for extended periods of time because this treatment manipulates the genetic material of the neuron.

2.6.1.2 *Limitations*

Although genetic therapies do have multiple significant benefits, they also present with many limitations. These therapies target a specific subset of ALS patients, they cannot be applied to the general population of ALS patients. Though this is beneficial since it can increase success rates, it also makes treatment more inefficient, time-consuming, and expensive because more effort is spent in deducing which gene(s) needs to be modulated

in a particular patient. The gene therapies target the symptoms of ALS related to that particular gene(s). There is no guarantee that such genetic pressure will not cause neuronal degeneration due to an adaptation response, wherein other genes and gene products intervene to maintain the ALS status quo.

There are two distinct barriers to developing gene therapy: sequencing and developing the treatment. To apply gene therapy, the patient's genome must be analyzed to discover the mutation(s) that could be causing TDP-43 toxicity. But since many genes could be connected, multiple variants of the treatment must be developed with new delivered genetic material and adjustments of the vectors. Therefore, a very small subset of patients will benefit from this treatment since each proposed treatment targets a specific gene in the chain of causation of ALS.

2.6.2 *Antisense Oligonucleotides*

ASO are single-stranded DNA designed to be complementary to certain sections of target mRNA in order to bind effectively. They are typically used to regulate gene expression through, for instance, the inhibition of mRNA translation (51). They were developed in 1978 when research showed that if synthetic oligonucleotides were complementary to mRNA, they could inhibit viral replication. One of the most well-known applications of ASOs is nusinersen, a drug used to treat spinal muscular atrophy.

ASOs act by causing RNA cleavage, RNA blockage, mediated cleavage, RNA interference, or splice modulation in order to manipulate the expression of genetic material. Similar to gene therapies, the oligonucleotides are also typically delivered in vectors, either viral or bacterial, in order to protect them from degradation. ASOs are administered either through intravenous infusion, subcutaneous or intravenous injections (52).

The administration of anti-SOD1 ASOs through either direct delivery or packaged in AAV9 vectors showed promising results in animal models. The results of administration of subpial injections of the ASO using viral vectors showed that the progression of ALS could be prevented or completely stopped in the models depending on whether the ASO was administered before or after the onset of the disease. Therefore, the use of anti-SOD1 ASOs appeared to be more effective in reducing the symptoms of the disease when compared to the previous gene therapies mentioned. This research also showcased the success of adenovirus vectors in the effective delivery of ASOs to neurons (53), although the application of this specific treatment is limited due to the immunogenic response to viral vectors. ASOs can also be delivered in non-viral vector formulations.

Loss of TDP-43 induced synaptic dysfunction that could be rescued by UNC13A splice-switching ASOs (36). Another study demonstrated that ASOs that blocked STMN2 cryptic splicing could be used in combination therapies to target the levels of TDP-43 without reducing the levels of STMN2 (54). This method was an improvement over previous studies where (toxic TDP-43 cytoplasmic aggregation was targeted using Ataxin-2-ASOs or small molecules) the levels of STMN2 decreased in tandem with the decrease in

toxicity of TDP-43. Hence, both the loss-of-function and gain-of-function effects seen in the ALS pathology can be simultaneously targeted using ASOs.

TDP-43 proteinopathies have been linked to the de-repression and inclusion of cryptic exons in mRNA which can lead to the loss of neuronal proteins such as STMN2. Targeting the STMN2 cryptic exon using ASOs led to an increase in STMN2 expression, and restored axonal regeneration in induced pluripotent stem cells (iPSC). Oligonucleotides can also bind to pre-mRNA in cells to reduce the levels of toxic proteins, and this allows the ASO to edit both the exons and introns of the RNA before it is translated into mRNA (55). This can increase the effectiveness of treatment against TDP-43 since the administered ASO effectively takes over the function of targeting (repressing) cryptic exon inclusion into mRNA. Using multiple small effectors, which are cells that respond to stimulus, multiple ASOs, or small nuclear RNA (snRNA) could be packaged to target multiple cryptic exons simultaneously (56). This means that rather than either indirectly targeting or partially using gene therapy, multiple vectors with genetic material can now be delivered. Again, the approach seems to work in animals but has not yet been translated to humans.

2.6.2.1 *Benefits*

ASOs focus on inactivating or silencing specific genes. Because of this increased specificity, they are very successful in the process of silencing genetic mutations. They can lead to the restoration of protein function and expression, reduce the expression of toxic proteins found in the cell, or modify protein expression; therefore, they can target both gain-of-function and loss-of-function mutations. This is important in order to address the pleiotropic characteristics shown byTDP-43 proteinopathies.

2.6.2.2 *Limitations*

Although ASOs are effective in targeting mutations, they requires continuous dosing to maintain the response. This may be undesirable for many patients since many methods of dosing of ASOs, such as subcutaneous, intravenous, intrathecal, or subpial routes, are invasive. The cellular uptake of the ASOs also cannot be precisely predicted even as the delivery of the ASOs become more precise, because of their inherent instability in biological fluids and the necessity to cross the blood brain barrier when administered peripherally. Even though the implementation of liposomal or other forms of non-viral vector assisted delivery improves stability, it still presents a disadvantage since it involves expensive formulation engineering.

2.6.3 *Small molecules and protein drugs*

Small molecules interact with target proteins in a specific way. They came into widespread use during the golden age of drug discovery with the first ones produced being antidepressants and antipsychotics in the 20th century. They are easy to administer (usually orally) and their interaction with the target protein can be extensively modeled in the lab with a reasonable certainty of similar interaction occurring *in vivo*. There is no easy way to group the different small molecules together based on their function because each small molecule interacts with each protein differently; many, for instance, are capable of

crossing the blood-brain barrier in order to target large proteins in the CNS and alter their activity.

Calcium channel agonists have been used in animal studies to protect against neuromuscular dysfunction. A channel agonist is a substance that binds to the channel receptors of the cell, in this case the neuron, to cause a specific biological response. When the ALS mutation, mut*TARDBP*, was expressed in zebrafish larvae, its motor function improved after treatment using the calcium channel agonists, FPL 64176 or Bay K 8644 (57).

End stage cytoplasmioc stress granules containing aggregated TDP-43 were enriched in the RNA binding protein CLUH, and a group of 18 CLUH targets. These CLUH targets, in turn, were enriched for catabolic enzymes involved in the branched chain amino acid and ketone body pathways. These enzymes are critical for switching from glucose to fat metabolism under starvation conditions. CLUH recruitment into the TDP-43-associated SG fraction would diminish its physiological role and contribute to excessive impairment in mitochondrial function, as well as neurodegeneration seen in ALS. The phenomenon can hence be thought of as a stress-related feedback loop resulting in persistent and progressive SG and CLUH granule recruitment with mitochondrial disruption leading to neuronal starvation and ATP crisis. Uridine supplementation was used to prevent ATP loss within cells and cell death in neurons with low levels of glucose. The treatment successfully extended the survival of animal subjects, proving its effectiveness (23).

TDP-43 can co-aggregate with other proteins such as the guanine exchange factor and the RNA-binding rho guanine nucleotide exchange factor (RGNEF). Specifically, the N-terminal fragment of RGNEF interacts with the RRMs of TDP-43. This finding led to the use of the protein RGNEF as an inhibitor for the TDP-43 overexpression phenotype. This potential treatment method reduced the toxicity seen in ALS animal models (58). Another study showed that the simultaneous expression of mutant TDP-43 and SOD1 led to the tryptophan dependent aggregation of SOD1, Tryptophan residues are found in both SOD1 and the RRM1of TDP-43. Therefore, rather than directly targeting TDP-43 aggregation to reduce neurodegeneration, it was found that 5-fluorouridine could be used to inhibit SOD1 aggregation by targeting its tryptophan residues; specifically SOD1 Trp32 (59).

It was found that AIM4, a derivative of acridine, can interact with amino acid residues in TDP-43's C-terminal domain and prevent protein aggregation (60). The compound rTRD01 improved neuromuscular function in *Drosophila* larva, which have the disease-linked *c9orf72* gene (61). Additionally, a study found that the flavonoid compound, baicalein, could target the misfolded domains of TDP-43 to redirect them towards their functional oligomeric physiological conformations (62). These are all examples of different small molecules' effectiveness in reversing neurodegeneration in animal trials.

Small molecule drugs have also been used to target the retroviral elements such as HERV-K. It has been hypothesized that antiretroviral therapeutics may be successful in reversing

neurodegeneration and improving patients' symptoms. One such antiretroviral, Triumeq®, contains two reverse transcriptase inhibitors – abacavir and lamivudine – that inhibit the formation of the double-stranded DNA inside the cytoplasm of cells with activated HERV-K, thereby preventing the subsequent activation of the cGAS/STING neuroinflammatory pathway. A phase IIa clinical trial was completed on 40 patients, with a resultant decrease in ALSFRS-R progression by 21.8%. Additionally, there were no reported drug-adverse effects experienced by patients, proving that long-term Triumeq® administration was safe and tolerable for the trial patients. The antiretroviral also had some success in reducing the rate of disease progression, and this could potentially lead to an increase in life expectancy (26).

Another study assessed the effect of methylene blue (MB) and Latrepirdine (Dimebon®) on TDP-43 aggregation. MB is a medication used to treat methemoglobinemia, a condition where hemoglobin slowly loses its ability to carry oxygen, while Dimebon® is an antihistamine that has also been tested for its therapeutic potential in Alzheimer's. These two small molecule medications have been successful in phase II Alzheimer clinical trials, and their combined use reduced the TDP-43 aggregation in neuroblastoma cell lines by 80% (63). Therefore, it can be concluded that both MB and Dimebon® may be effective in ALS, but a clinical trial has yet to be conducted.

However, sometimes the small molecules analyzed do not necessarily have to be created but can be proteins already found in the body; these protein drugs function in a manner similar to small molecule drugs because they have high specificity to their target. Patients with both fALS and sALS caused by TDP-43 aggregation have impaired autophagosome formation and the accumulation of glutamate receptors. One study showed that an anticoagulation-deficient form of activated protein C, which is a glycoprotein that controls blood clotting, can reduce the presence of these defects in induced motor neuron models; proteostasis and low glutamate levels are both also accomplished in gain- and loss-of-function fALS models with mutant C9orf72 (64). Similar to gene therapy, this protein drug utilizes a blood test to determine whether a patient fits the criteria. Another study investigated the use of the polyglutamine binding peptide 1 (QBP1), which is an octapeptide that prevents amyloid formation. It proved to be efficient in binding to the Q/N-rich segment of the C-terminal domain of the TDP-43 protein, preventing aggregation (65, 66). Therefore, this treatment method does have some increased benefits due to its specificity and relatively simple mechanism of action.

Another more recent and similar treatment method for ALS is antibodies; proteins created to counteract an antigen in the bloodstream. Monoclonal antibodies were first used to prevent kidney transplant rejection in 1986, eventually progressing to exhibit a broad range of applications such as diagnosis, research, and disease treatment. They are able to recognize and target specific proteins in cells, and they have been used in multiple different cell lines such as cancer cells or cells found in the immune system. Antibodies can also be categorized as a protein drug when administered to alleviate the symptoms of ALS.

One study investigated the effectiveness of the single-chain antibodies (scFv) targeting the RRM1 of the TDP-43 protein in order to reduce its cytoplasmic aggregation. When delivered using an adenovirus vector, the antibodies were able to reduce toxic aggregation, neuroinflammation, motor defects, and cognitive impairment in murine models (67). These results suggest that this specific antibody may be useful in treating patients with ALS and that antibodies can be an effective method to target neurodegenerative disorders like ALS.

A new method of treatment using heterobifunctional molecules has also been proposed by researchers in recent years. The term used is a proteolysis targeting chimera (PROTAC). A PROTAC has two active domains with a linker, and it is able to remove any unwanted proteins – in this study, the C-terminal TDP-43 aggregates. Multiple types of PROTACs characterized by different sizes of linkers were tested for the degradation efficiency of C-TDP-43 aggregates. It was determined that one of the PROTACs bound to the C-TDP-43 aggregates and a ligase molecule to begin ubiquitination; it also decreased how compact the oligomers of the aggregates were and the number of aggregates present (68). This method was successful in this study, but it is limited to the population of ALS patients who have mutations in the C-terminal of the TDP-43 molecule.

2.6.3.1 *Benefits*

The current research on both small molecules and antibodies shows that when TDP-43 aggregation is targeted, they can benefit both fALS and sALS, indicative of broad effectiveness. It also has as much success as other more established methods of treatment such as gene therapy, so there should be a greater push for its use for two main reasons. Primarily, it is easier to administer because small molecules can be administered orally while patients are given monoclonal antibodies through intravenous injections. This is simpler than the administration of ASOs or gene therapies because most do not require the use of extensive formulation engineering involving vectors, promoters and the necessity to be imported into the nucleus. Additionally, small molecules are already an accepted treatment model, hence it is easier to convince patients to consent to this new form of treatment compared to experimental gene therapy. Small molecules and antibodies delivery models have already been established, but the model of gene therapy changes depending on the target tissue and the type of disease that is being treated. Overall, the advantages that small molecules and antibodies present as treatment methods are greater compared to gene therapies in both the medical and societal context.

2.6.3.2 *Limitations*

Though antibodies and small molecules both have significant advantages, these treatment methods lack the specificity presented by other models. Typically, medication containing small molecules or antibodies induce off-target effects because these species have some affinity for other proteins as well as the target protein. Though they have been tested extensively in animals, limited or no testing has been performed on humans. Another limitation is the need for repeated administration. Many patients may not prefer

the need for repetitive treatment because it increases costs and discomfort.

2.7 Potential research focus

The treatment methods proposed previously by different research groups have exibited varying degrees of success in fALS and/or sALS cases caused by TDP-43 aggregation. However, as previously described, they each present with some important limitations that must be improved upon in order to be the most effective treatment model for treating TDP-43 aggregation and ALS. The following section will provide a discussion of nucelocytoplasmic transport and related pathways, which may be more amenable to intervention success.

2.7.1 *Nucleocytoplasmic transport*

The neuron needs to maintain constant transportation of cellular products and waste while performing other important functions such as mRNA splicing, protein production, and receiving and sending signals to other neurons. However, when TDP-43 aggregates in the cytoplasm, it inhibits important functions such as NCT; performed predominantly by transport through the nuclear pore complex (NPC) (20). The NPC is critical in maintaining homeostasis, and one specific pathway, the ESCRT-III, is significantly involved in ensuring that the functionality of the NPC is maintained. The ESCRT-III pathway also influences the reduction in specific nucleoporins that are related to neurodegenerative pathologies (69), showing that targeting the dysfunction of the NCT may increase the effectiveness of therapeutic strategies.

NCT is the phenomenon of importing and exporting proteins and other cellular cargo between the nucleus and the cytoplasm. In order to carry out these functions, proteins must have peptide signals called nuclear localization signals (NLS) and nuclear export sequences (NES) (70). The importance of NCT in the context of ALS can be explained both by its relationship with TDP-43 and other factors of the disease. One of the dominant mutations, Fused in Sarcoma (FUS), is a contributing factor for ALS, and its presence leads to a decrease in the NCT. FUS interacts with nucleoporins in the cytoplasm of mutant neurons at higher rates while most interactions focus on the nucleus of wild-type cells. It has been hypothesized, therefore, that mutations linked to ALS are related to the mislocalization of FUS rather than to a change in the intrinsic properties of the protein itself (71).

In iPSC-derived motor neurons with the *TARDBP* mutation, there is higher redistribution of mRNA and splicing alterations, which can both be linked to the dysfunction of the TDP-43 protein; in turn due to its observed cytoplasmic mislocalization. A VCP ATPase inhibitor, ML240, was used to partially restore protein localization and mRNA distribution (72). Mutant TDP-43 can cause further aggregation and mislocalization of nucleoporins, which are the proteins that make up the NPC, and transport factors. These mislocalizations can increase the cytoplasmic aggregation and further mislocalization of TDP-43, creating a cycle of toxic protein production. Fly and yeast models of C9orf72 G_4C_2-repeat expansion pathology and sporadic ALS patient cells both present with NCT dysfunction, which may be a common indicator

of the disease (73). In the sporadic ALS cells, the presence of the components of the NCT and NPC was observed (74). Injury to the NPC brought about by the reduction in the transmembrane nucleoporin POM121 in iPSC derived neurons portrays its symptoms and effects within the neuron in a similar manner that TDP-43 loss of function does. This observation connects the integrity of the NPC to the protein loss of function (75). Research has also shown that the enzyme, glycerophosphodiester phosphodiesterase 2 (GDE2), is involved in the nuclear localization of TDP-43, and the deactivation of this enzyme caused sustained activation of the Wnt signaling pathway and TDP-43 abnormalities. The Wnt pathway's activation has been seen with abnormalities in iPSC from ALS patients, indicating that it may play an important role in the disease. Therefore, GDE2 has been characterized as a regulator of the Wnt pathway, and the activation of this pathway poses an important target for preventing TDP-43 aggregation (76). Taken together, ALS and the mislocalization of TDP-43 is associated with important functional attributes of NCT and NPC.

However, not every study has succeeded in demonstrating the influence of the NCT on ALS and neurodegeneration, but these differences in observations may be caused by multiple factors including specific cell characteristics, sorting signals, and difference in the assays conducted. The conclusion that NCT dysfunction is associated with ALS is difficult to establish because of technical limitations of studies that highlighted this theory (77). Another study showed that TDP-43's aggregation actually caused the inhibition of NCT. A long-time exposure to amyloid-like fibrils leads to the formation of liquid droplets of the protein within the cytoplasm, inducing the mislocalization of components of the NCT (78). Therefore, there are two contrasting conclusions existing about the role that NCT plays in TDP-43 aggregation and ALS neurodegeneration. But, since this protein aggregation is found within the cytoplasm while TDP-43 must shuttle between the nucleus and cytoplasm, the misregulation of NCT should be a main target for potential therapeutics, and this can be done by considering it either an effect or a cause of protein aggregation. In the following section, NCT dysfunction will be treated as a cause for protein aggregation.

2.7.2 *Therapeutic strategies targeting NucleoCytoplasmic Transport dysfunction*

It was initially hypothesized that nuclear export must be downregulated in order to compensate for the nuclear import impairment. A decrease in neurodegeneration was observed when KPT-276, a selective inhibitor of nuclear export (SINE), was administered to ALS simulated neurons. Hence, an association was postulated between regulations on nuclear export and neuronal degeneration (79). Another study demonstrated that a SINE that targeted XPO1 partially rescued motor deficits in rats with TDP-43 induced paralysis (80). However, downregulation of nuclear export increased toxicity in *Drosophila* models with the G_4C_2-repeat expansion. These studies all draw on a relationship between ALS and the nuclear export protein, XPO1. However, TDP-43 can be exported independently of the receptor CRM1/XPO1 by passive diffusion (81). Therefore, although not completely understood,

it does appear that there is a relationship between NCT, nuclear export proteins, TDP-43, and neurodegeneration that needs more investigation (79).

Ran GTPase, appears to be the master regulator of the nuclear transport of cellular material in order for nuclear localization of TDP-43 and regulates nuclear transport using the NPC. Its accessory proteins are necessary for the normal localization of TDP-43 (82). The Ran protein is a Ras-related GTPase, and it switches between RanGTP and RanGDP based on whether it is bound to GTP or GDP. The Ran GTPase system is involved in loading the contents of nuclear transport receptors on and off, and this process allows for transport to be performed against the concentration gradient. This function makes Ran GTPase increasingly important in communicating between the nucleus and cytoplasm and in reducing the toxic properties of TDP-43 to target the symptoms of ALS.

It was observed that the presence of G_4C_2-repeat RNA reduces one nucleoporin, leading to a decrease in seven other nucleoporins; this phenomenon affects the localization of Ran and makes cells sensitive to cellular toxicity. Additionally, an injury of the NPC in fALS and sALS was linked to the accumulation of CHMP7, which mediates quality control for the NPC. Inhibiting the export of CHMP7 led to TDP-43 dysfunction, along with other consequences (79). This specific strategy highlights how the mutated version of NCT may promote aggregation, and targeting NCT may improve ALS symptoms. Much research has also been done to target RAN dipeptide repeats (DPR) – specifically the types GA, GR, and PR – through immunotherapy. One study determined that α-GA antibodies reduced the formation of GA aggregation in C9orf72 autopsy tissue. Mice with the overexpression of GA were vaccinated with ovalbumin-$(GA)_{10}$ peptides in another study, which led to the prevention of motor deficits. Using α-GA treatment also led to a decrease in GR and PR proteins and since there was no change in sense or antisense RNA levels, it could be concluded that RAN proteins drive both ALS and FTD (83). Another study demonstrated that the loss of C9orf72 disrupted both the Ran GTPase gradient and NCT, and it was enhanced specifically *in vivo* by the formation of importin granules (84). Both studies proposed the use of proteins to regulate the mutations in NCT because the proteins constituting the NCT played a critical role in the development of neurodegeneration. Though most of this research has been done on a specific mutation related to fALS, there is scope to connect the abnormal functions of RAN proteins to sALS as well. However, the potential future steps for ALS treatment should focus on the relationship between ALS caused by C9orf72 and the Ran GTPAse gradient.

2.8 Potential future steps

These potential future steps have two-parts. First, the existing aggregates of TDP-43 must be degraded in order to clear the cytoplasm and restore NPC and NCT functionality. Since TDP-43 aggregates will still be produced as the existing aggregates are removed, this must be carried out simultaneously with the second step. The most effective way to break down these aggregates would be to utilize synthetic peptides or small molecules, as shown in Figure 1, that can be administered orally (85).

Artificial peptides can be designed to target mutated domains of TDP-43, such as the low complexity domain, that are known to cause aggregation (86). Therefore, peptides or small molecule administration is the most efficient method to clear mutated TDP-43 from the cytoplasm. These drugs could be delivered orally using several avenues of formulation engineering so that they are absorbed into the bloodstream from the gut, cross the blood brain barrier and can affect lysosomal escape into the cytoplasm.

The initial part of clearing aggregated TDP-43 is key to re-establishing protein homeostasis, but the mislocalization of TDP-43 is still a challenge that must be resolved with a second phase since the protein will continue to cluster in the cytoplasm due to mutations or other causes of aggregation (Figure 1).

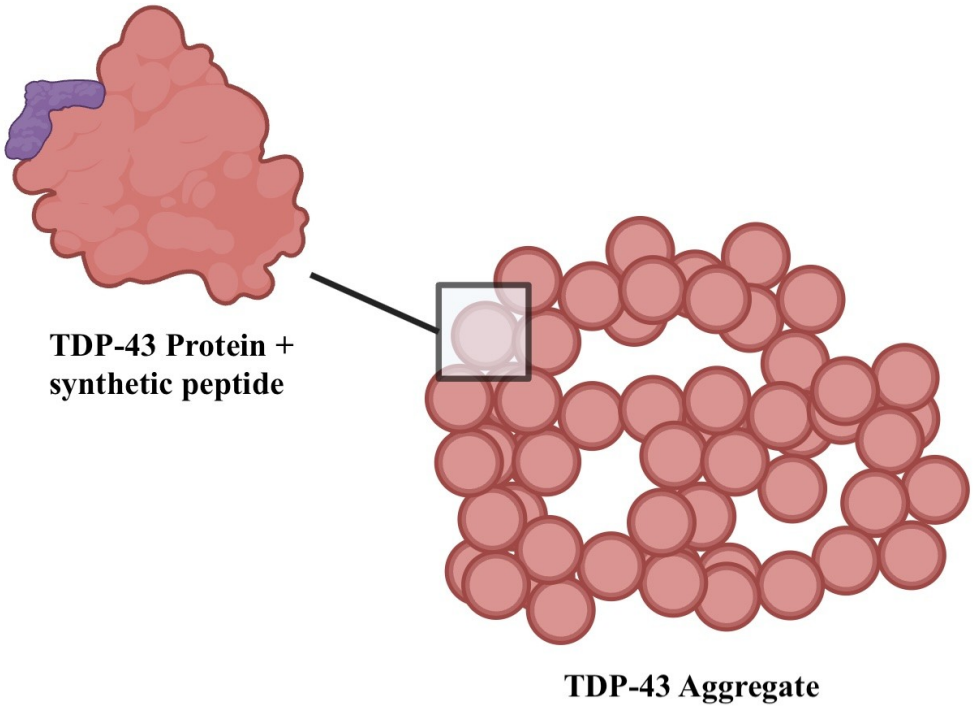

Figure 1: The first part of the proposal includes the breakdown of the TDP-43 aggregation using synthetic peptides or small molecules. These peptides or molecules will target mutated domains of the protein in order to prevent aggregation.

The second part of this proposal targets the relationship between the Ran GTPase system and C9orf72. Levels of Ran GTPase were depleted in a case study of FTD patients (82), and FTD shares TDP-43 pathology with sALS (87). In both disorders, the protein shares a pathogenic role and exacerbates the symptoms by inhibiting necessary functions in neurons.

Therefore, it can be hypothesized that the depletion of Ran GTPase can contribute to the mislocalization of TDP-43 seen in ALS. Research has shown that the inhibition of $(G_4C_2)30$ RNA, which led to increased levels of Ran GTPase, rescued the impairment of nuclear import. This strategy was successful in patients with C9orf72 mutations (17), but a more recent study did highlight the fact that the loss of C9orf72 as a whole did impact the gradient formed by Ran GTPase and cytoplasmic transport (84). However, ALS is a multi-faceted neurodegenerative disease since the defects in motor neuron capabilities are caused by a multitude of issues such as mutations or protein aggregations. Therefore, a simple increase in Ran GTPase through direct delivery may not allow for a decrease in TDP-43 aggregation since other factors may continue to cause this protein aggregation. Instead, more research should be conducted to determine how to manipulate the gradient of Ran GTPase to decrease TDP-43 aggregation and alleviate the motor symptoms of ALS.

This treatment proposal targets the challenge of TDP-43 aggregation, which involves both the protein shape and its mislocalization. Although the mislocalization of TDP-43 may be corrected with this therapeutic pathway, structural mutations in the TDP-43 protein may present another limitation. Overall, this potential treatment model may hold potential to target and correct the dysfunction and/or dysregulation of gene/protein players in the causal chain of ALS.

Table 1 shows various TDF-43 mediated pathways in the development and progression of ALS and opportunities for therapeutic intervention in those pathways.

Table 1: The pathways implicated by TDP-43 in ALS, the potential therapies and their mechanisms of action.

Potential Therapy	Mechanism of Action
Ubiquitin C-terminal hydrolase-L1 directed gene delivery	Using adenovirus-mediated retrograde transduction, the extent of integrity loss due to misfolded SOD1 toxicity and mutated TDP-43 decreased (43).
Human frameshift mutation protein 1	Delivery of AAV9 human mycUPF1 showed that they regained forelimb motor function (44).
Casein kinase 1 epsilon gene	Inhibition of the CK1 kinase activity with siRNA decreased the phosphorylated TDP-43 aggregation with both insoluble and soluble aspects (45).
cAMP/PKA signaling pathway inhibition	Inhibiting the cAMP/PKA signaling pathway by targeting phosphodiesterase *dunce* and the subunit *PKA-R2*, and TDP-43 aggregation and mislocalization within larval motor neuron cell bodies decreased (46).
CHMP7 ASO	Reduction of ESCRT-III nuclear surveillance protein, CHMP7 can both prevent and reverse TDP-43 dysfunction in ALS iPSNs. ASO-mediated reduction of CHMP7 following the emergence of NPC injury, but prior to the detectable development of TDP-43 dysfunction, was sufficient to completely prevent the emergence of molecular hallmarks of TDP-43 loss of function (69).
Calcium channel agonists	Characteristics of motor function prior to treatment and after treatment using the

	calcium channel agonists, FPL 64176 or Bay K 8644 were stabilized after treatment (57).
Uridine prodrugs such as Triacetyluridine (also see 5-fluorouridine below for another mechanism of action)	Metabolic mitochondrial enzymes trapped in the insoluble fraction (of TDP-43 stress granules) from the brain via a CLUH (mRNA transport protein) dependent mechanism results in motor neuron death by starvation in ALS. Uridine prodrug Triacetyluridine may therefore have a neuroprotective effect in ALS-induced neuronal starvation (23).
UNC13A splice switching ASOs	TDP-43 depletion induces a severe reduction in synaptic transmission, leading to an asynchronous pattern of network activity, largely driven by a single cryptic exon in UNC13A. ASO targeting the UNC13A cryptic exon robustly rescues UNC13A protein levels and restores normal synaptic function (36).
Tranilast (Rizaben®) as an NMD-activating drug	In human SH-SY5Y neuroblastoma cells and in mouse brains, expression of glycine-arginine with 36 repeats (GR36) was sufficient to cause NMD inhibition. sTDP43-encoding transcripts that escape NMD can lead to toxicity within neurons (24).
5-fluorouridine (also see Uridine prodrugs above)	Tryptophan-68 becomes antibody-accessible in aggregated TDP-43 in sporadic ALS motor neurons and cell culture. 5-fluorouridine inhibits aggregated TDP-43–induced G85R-GFP SOD1 aggregation *via* its interaction with SOD1 tryptophan-32 (59).
ML240, a VCP ATPase inhibitor, partially restored mRNA and protein localization.	ALS phenotypes with *TARDBP* and VCP-mutated iPSMNs exhibited extensive nucleocytoplasmic mRNA redistribution, RBP mislocalization, and splicing alterations (72).
STING inhibitor, H-151	TDP-43 cytoplasmic mislocalization results in mitochondrial DNA release that also activates the cGAS/STING pathway, resulting in the upregulation of NF-kB and IFN pathways; in turn causing faster neurodegeneration (25).
Reverse transcriptase inhibitors (Triumeq®), Also see ASRGL1 mechanism of action below.	The presence of 5 binding sites for TDP-43 on the consensus sequence of HERV-K LTR suggests that TDP- 43 may be involved in HERV-K transcriptional regulation. Increased ERV expression within glia resulted in increased cellular release of neuronal toxic factors and accelerated the symptoms of ALS (26).
HML-2 and ASRGL1 inhibition and overexpression	ASRGL1 is expressed in the brain and harbors an intronic copy in an antisense direction of the HERV-K, subtype HML-2, which has been associated with the pathogenesis of ALS. Overexpression of HML-2 leads to ASRGL1 silencing. TDP-43 is a substrate of ASRGL1. Loss of ASRGL1 triggers misfolding, fragmentation, phosphorylation and mislocalization of TDP-43 (27).
NPTX2 inhibitors.	The strongest misregulated target encoded the synaptic protein NPTX2, the levels of which are controlled by TDP-43 binding on its 3′ untranslated region. LOF of TDP-43 due to aggregation, increases NPTX2, leading to ALS symptom acceleration (39).
RGNEF inhibitors	The protein RGNEF inhibited the phenotypes in TDP-43 overexpression; this potential treatment method reduced the toxicity levels seen in ALS (58).
Baicalein	Conformational switching of TDP-43 prone-to-aggregate domains, results in LLPS, insoluble and non-functional aggregates and cytoplasmic mislocalization. Targeting the misfolded TDP-43 to redirect them towards their functional

	oligomeric physiological conformations is an effective therapeutic intervention in ALS (62).
WNT pathway inhibitors	GDE2 maintains TDP-43 nuclear localization by regulating the dynamics of canonical Wnt signaling. Ablation of GDE2 causes aberrantly sustained WNT activation in adult neurons, which is sufficient to cause NCT deficits, nuclear pore abnormalities, and TDP-43 nuclear exclusion (76).

3 Conclusion

A large body of accumulated knowledge and evidence has demonstrated a correlation between cytoplasmic TDP-43 mislocalization and/or aggregation with neurodegenerative symptoms seen in ALS. The failure of multiple drugs in the clinic that modulate broad ALS non-specific pathways (oxidative stress, autophagy, excitotoxicity, inflammation) demonstrates that perturbing *specific* players in the above pathways, which are mechanistically nearer to ALS loci, is more likely to be effective. Potential targets with the most promise are TDP-43 with associated proteins/ RNA that are present in TDP-43 stress granules and/or agglomerates and proteins transcriptionally regulated by TDP-43 that feed-back or feed-forward into TDP-43 levels, nonsense mediated decay, the NuceloCytoplasmic Transport pathway and associated proteins and splice switching and cryptic exon modulating mechanisms. Targeting TDP-43 mislocalization and/or aggregation using multiple therapeutic strategies such as gene therapy, ASOs, small molecules, and protein drugs, could reduce or reverse the symptoms of ALS and extend the lifespan and QoL of patients. A multitude of strategies developed so far were presented an discussed in this paper, and overall, each was effective in animal models with specific criteria. An important limitation to many of these therapeutic strategies was that they targeted known genetic mutations. Therefore, after analyzing the common thread in TDP-43 aggregation, a potential research focus was detailed in this paper that suggested the use of a simultaneous two-part process: part one would involve synthetic peptides or small molecules delivered orally with appropriate formulation engineering to penetrate the blood brain barrier, target the ALS neuronal cells, escape the endosome into the cytoplasm and degrade cytoplasmic TDP-43 aggregates. Part two involves a proposal to investigate the means to alter the Ran GTPase gradient with an objective to restore NucleoCytoplasmic Transport homeostatis. Since ALS is a debilitating disease that still has no effective treatment, any therapeutic pathway that extends the lifespan of patients would be a huge advancement in the field.

4 Abbreviations

ALS: Amyotrophic Lateral Sclerosis, PNS: Peripheral Nervous System, CNS: Central Nervous System, fALS: familial Amyotrophic Lateral Sclerosis , sALS: sporadic Amyotrophic Lateral Sclerosis, SOD1: Superoxide Dismutase 1, C9orf72: Chromosome 9 open reading frame 72, ROS: Reactive Oxygen Species, ALSFRS-R: Revised ALS Functional Rating Scale, XPO1: Exportin-1, NES: Nuclear Export Sequence, ASO – AntiSense Oligonucleotides, TDP-43:

Transactive response DNA binding Protein of 43 kDa, NCT: NucleoCytoplasmic Transport, UTR: UnTranslated Region, NMD: Nonsense Mediated RNA Decay, cGAS: cytoplasmic DNA sensor cyclic Guanosine monophosphate-AMP Synthase, HERV-K: Human Endogenous Retrovirus K, ASRGL1: ASpaRaGinase-Like-1 protein, LCD: Low-Complexity Domain, LLPS: Liquid-Liquid Phase Separation, RRM: RNA Recognition Motif, NLS: Nuclear Localization Sequence, AAV: Adeno-Associated Vector, SCID: Severe Combined ImmunoDeficiency, CSMN: CorticoSpinal Motor Neurons, UCHL1: Ubiquitin C-terminal Hydrolase-L1, STMN2 – STathMiN-2 protein, snRNA: small nuclear RNA, MB: Methylene Blue, PROTAC: PROteolysis TArgeting Chimera, DPR: DiPeptide Repeats, FTD: FrontoTemporal Dementia, SINE: Selective Inhibitor of Nuclear Transport, GLT1: GLutamate Transporter 1, GLAST: GLutamate ASpartate Transporter, QoL: Quality of Life, HSP: Heat Shock Proteins, STING: STimulator of INterferon Genes, RRM: RNA Recognition Motif, NPTX2: Neuronal PenTraXin II, cAMP: cyclic Adenosine MonoPhosphate, PKA: Protein Kinase A, PhP.B: Peptide sequence TLAVPFK, SARM1: Sterile Alpha- and aRmadillo-Motif containing protein, NMNAT2: NicotinaMide Nucleotide AdynylylTransferase 2, DBR1: DeBranching RNA lariats 1, RGNEF: Rho Guanine Nucleotide Exchange Factor, CLUH: CLUstered mitochondria Homolog, AIM4: [4,5-bis{(N-carboxy methyl imidazolium)methyl}acridine] dibromide, QBP1: polyGlutamine Binding Peptide 1, PROTAC: PROteolysis TArgeting Chimera, ESCRT III: Endosomal Sorting Complexes Required for Transport, VCP: Valosin Containing Protein, POM121: nuclear envelope POre Membrane protein, RAN: RAs related Nuclear protein, RDR: Ran Dipeptide Repeats, CHMP7: CHarged Multivesicular body Protein 7, FUS: FUsed in Sarcoplasma

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