

REVIEW ARTICLE

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Amyotrophic Lateral Sclerosis

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AMYOTROPHIC LATERAL SCLEROSIS (ALS) IS A PROGRESSIVE, PARALYTIC disorder characterized by degeneration of motor neurons in the brain and spinal cord. It begins insidiously with focal weakness but spreads relentlessly to involve most muscles, including the diaphragm. Typically, death due to respiratory paralysis occurs in 3 to 5 years.

Motor neurons are grouped into upper populations in the motor cortex and lower populations in the brain stem and spinal cord; lower motor neurons innervate muscle (Fig. 1). When corticospinal (upper) motor neurons fail, muscle stiffness and spasticity result. When lower motor neurons become affected, they initially show excessive electrical irritability, leading to spontaneous muscle twitching (fasciculations); as they degenerate, they lose synaptic connectivity with their target muscles, which then atrophy.

ALS typically begins in the limbs, but about one third of cases are bulbar, heralded by difficulty chewing, speaking, or swallowing. Until late in the disease, ALS spares neurons that innervate the eye and sphincter muscles. The diagnosis is based primarily on clinical examination in conjunction with electromyography, to confirm the extent of denervation, and laboratory testing, to rule out reversible disorders that may resemble ALS.^{1,2}

A representative case involves a 55-year-old patient who was evaluated for foot drop, which had begun subtly 4 months earlier with the onset of muscle cramping in the right calf as a result of volitional movement (known as volitional cramping) and had progressed to severe weakness of ankle dorsiflexion and knee extension. In addition to these features, the physical examination revealed atrophy of the right calf and hyperreflexia of the right biceps and of deep tendon reflexes at both knees and both ankles. The neurologic examination was otherwise normal. Electromyography showed evidence of acute muscle denervation (fibrillations) in all four limbs and muscle reinnervation in the right calf (high-amplitude compound muscle action potentials). Imaging of the head and neck revealed no structural lesions impinging on motor tracts, and the results of laboratory studies were normal, findings that ruled out several disorders in the differential diagnosis, such as peripheral neuropathy, Lyme disease, vitamin B₁₂ deficiency, thyroid disease, and metal toxicity.³ A full evaluation disclosed no evidence of a reversible motor neuron disorder, such as multifocal motor neuropathy with conduction block, which is typically associated with autoantibodies (e.g., anti-GM₁ ganglioside antibodies) and can be effectively treated with intravenous immune globulin.⁴

The clinical presentation of ALS is heterogeneous with respect to the populations of involved motor neurons and survival (Fig. 2).² When there is prominent involvement of frontopontine motor neurons that serve bulbar functions, a striking finding is emotional lability, indicating pseudobulbar palsy, which is characterized by facial spasticity and a tendency to laugh or cry excessively in response to minor emotional stimuli.

In primary lateral sclerosis, there is selective involvement of corticospinal and corticopontine motor neurons, with few findings of lower motor neuron dysfunction.⁵ Primary lateral sclerosis is ruled out in the representative case described above because of the atrophy and electromyographic findings, which are indicative of lower motor neuron disease. Primary lateral sclerosis progresses slowly, with severe spastic muscle stiffness and little muscle atrophy. This disorder overlaps clinically with a broad category of corticospinal disorders designated as hereditary spastic paraplegias, which are typically symmetrical in onset, slowly progressive, and sometimes associated with sensory loss and other multisystem findings. In primary lateral sclerosis but not hereditary spastic paraplegias, bulbar involvement may be prominent. In progressive muscular atrophy, lower motor neuron involvement is predominant, with little spasticity. The hyperreflexia in the representative case is inconsistent with progressive muscular atrophy.

During the past two decades, it has been recognized that 15 to 20% of persons with ALS have progressive cognitive abnormalities marked by behavioral changes, leading ultimately to dementia.⁶ Since these behavioral alterations correlate with autopsy evidence of degeneration of the frontal and temporal lobes, the condition is designated frontotemporal dementia. It was formerly called Pick's disease.

EPIDEMIOLOGIC FEATURES

In Europe and the United States, there are 1 or 2 new cases of ALS per year per 100,000 people; the total number of cases is approximately 3 to 5 per 100,000.^{7,8} These statistics are globally fairly uniform, although there are rare foci in which ALS is more common. The incidence and prevalence of ALS increase with age. In the United States and Europe, the cumulative lifetime risk of ALS is about 1 in 400; in the United States alone, 800,000 persons who are now alive are expected to die from ALS.⁹ About 10% of ALS cases are familial, usually inherited as dominant traits.¹⁰ The remaining 90% of cases of ALS are sporadic (occurring without a family history). In cases of sporadic ALS, the ratio of affected males to affected females may approach 2:1; in familial ALS, the ratio is closer to 1:1. ALS is the most frequent neurodegenerative disorder of

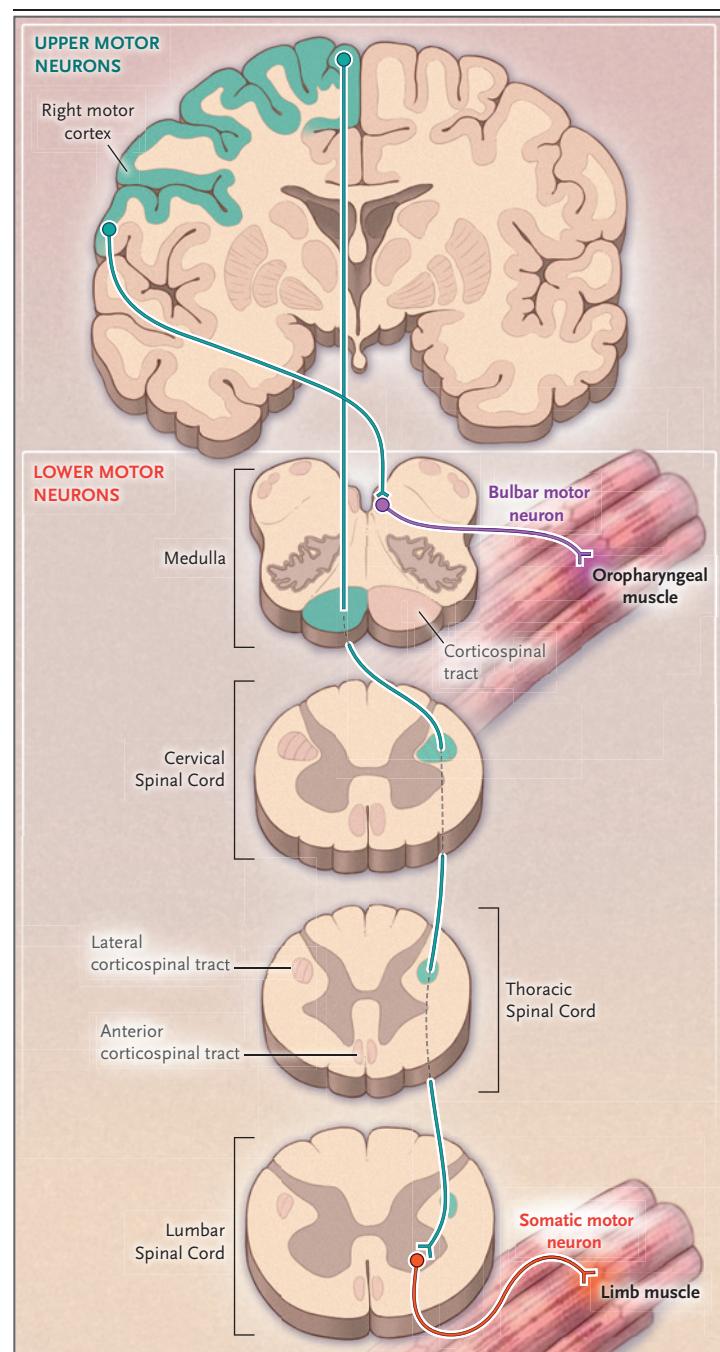


Figure 1. The Motor System.

The motor system is composed of corticospinal (upper) motor neurons in the motor cortex and bulbar and spinal (lower) motor neurons, which innervate skeletal muscle.

midlife, with an onset in the middle-to-late 50s. An onset in the late teenage or early adult years is usually indicative of familial ALS. The time

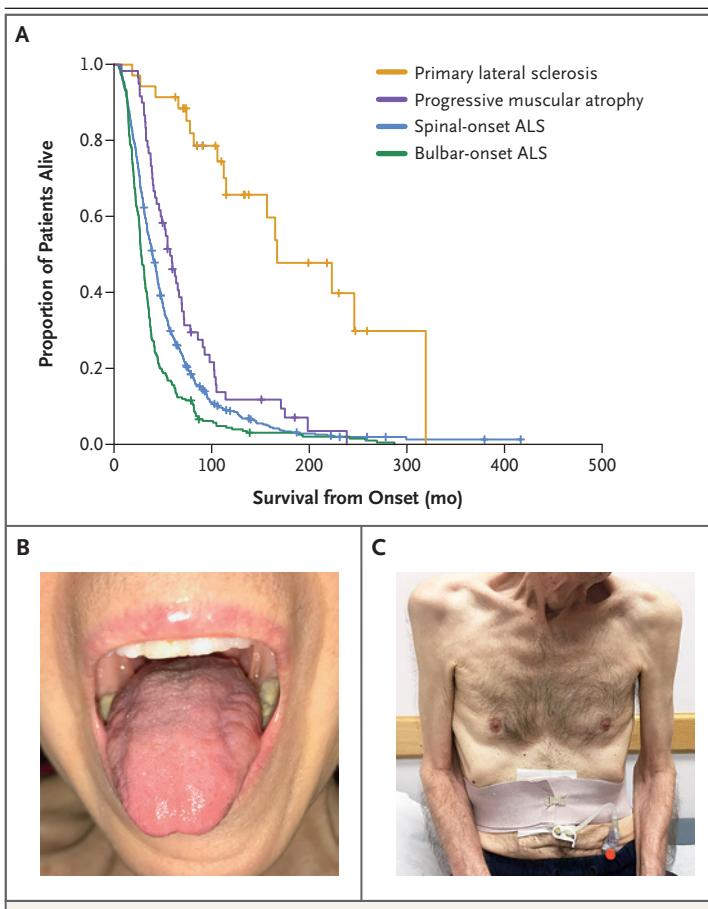


Figure 2. Phenotype and Survival in Amyotrophic Lateral Sclerosis (ALS).

Panel A shows survival curves for two types of ALS (spinal-onset and bulbar-onset) and two other motor neuron diseases (primary lateral sclerosis and progressive muscular atrophy). Panel B shows lateral atrophy and furrowing of the tongue in a patient with ALS, findings that reflect denervation due to degeneration of bulbar motor neurons. Panel C shows thinned arms and shoulders, findings that are typical of the flail-arm syndrome, which occurs in patients with ALS and is associated with protracted survival.

from the first symptom of ALS to diagnosis is approximately 12 months, a problematic delay if successful therapy requires early intervention. Because an abundance of ALS genes have now been identified, it will probably be informative to reanalyze this epidemiologic profile of ALS with stratification according to genetically defined subtypes.

PATHOLOGICAL CHARACTERISTICS

The core pathological finding in ALS is motor neuron death in the motor cortex and spinal cord; in ALS with frontotemporal dementia, neuronal degeneration is more widespread, oc-

curing throughout the frontal and temporal lobes. Degeneration of the corticospinal axons causes thinning and scarring (sclerosis) of the lateral aspects of the spinal cord. In addition, as the brain stem and spinal motor neurons die, there is thinning of the ventral roots and denervational atrophy (amyotrophy) of the muscles of the tongue, oropharynx, and limbs. Until late in the disease, ALS does not affect neurons that innervate eye muscles or the bladder. Degeneration of motor neurons is accompanied by neuroinflammatory processes, with proliferation of astroglia, microglia, and oligodendroglial cells.^{11,12} A common feature in cases of both familial and sporadic ALS is aggregation of cytoplasmic proteins, prominently but not exclusively in motor neurons. Some of these proteins are common in most types of ALS. This is exemplified by the nuclear TAR DNA-binding protein 43 (TDP-43), which in many cases of ALS is cleaved, hyperphosphorylated, and mislocalized to the cytoplasm.¹³ Aggregates of ubiquilin 2 are also common,¹⁴ as are intracytoplasmic deposits of wild-type superoxide dismutase 1 (SOD1) in sporadic ALS.¹⁵ Many protein deposits show evidence of ubiquitination; threads of ubiquitinated TDP-43 are prominent in motor neurons, both terminally and before atrophy of the cell body. Given the diverse causes of ALS, it is not surprising that some types of aggregates are detected only in specific ALS subtypes (e.g., dipeptide aggregates and intranuclear RNA deposits in C9ORF72 ALS).

GENETIC FEATURES

Evolving technologies for gene mapping and DNA analysis have facilitated the identification of multiple ALS genes (Fig. 3). SOD1 was the first ALS gene to be identified, in 1993.¹⁶ More than 120 genetic variants have been associated with a risk of ALS¹⁷ (<http://alsod.iop.kcl.ac.uk>). Several criteria assist in identifying those that are most meaningful. The strongest confirmation is validation in multiple independent families and cohorts. Also supportive are an increased burden of the variant in cases relative to controls and the predicted consequences of the variant (e.g., missense mutation vs. truncation). It has proved almost impossible to predict a variant's relevance to ALS from the biologic features of the gene itself. As shown in Figure 3, at least 25

genes have now been reproducibly implicated in familial ALS, sporadic ALS, or both.^{18–20}

A by-product of the genetic studies that is highly relevant to therapeutic development has been the generation of mouse models of ALS. Strikingly, transgenic expression of mutant SOD1 protein²¹ and, more recently, profilin 1 (PFN1)²² generates a neurodegenerative, paralytic process in mice that mimics many aspects of human ALS. An important lesson from transgenic models of TDP-43 and FUS (fused in sarcoma) is that levels of the normal protein are tightly controlled. In contrast with SOD1, forced expression of high levels of normal TDP-43 by itself triggers motor neuron degeneration.²³ Mouse models of *C9orf72* (the 72nd open reading frame identified on chromosome 9, the most commonly mutated gene in ALS) have now also been generated for *C9orf72* ALS and are discussed below.

Correlations between genetic variants and different clinical profiles in ALS, such as age at onset, disease duration, and site of onset, have been defined (Table 1). An important example is the gene that encodes the enzyme ephrin A4 (*EPHA4*)³³ — lower levels of expression of *EPHA4* correlate with longer survival. Some genetic variants influence both susceptibility and phenotype. For example, progression is accelerated in patients with the common A4V mutation³⁰ of *SOD1* and in patients with the P525L mutation of *FUS/TLS*; the latter may lead to fulminant, childhood-onset motor neuron disease.²⁸

CONCEPTS IN PATHOGENESIS

A comprehensive explanation for ALS must include both its familial and sporadic forms, as well as categories of phenotypic divergence that arise even with the same proximal trigger, such as a gene mutation. A general presumption has been that the disease reflects an adverse interplay between genetic and environmental factors. An alternative view postulates that all cases of ALS are a consequence primarily of complex genetic factors. Several perspectives suggest that the pathogenesis of ALS entails a multistep process.³⁴

LESSONS FROM FAMILIAL ALS

There is striking heterogeneity in the genetic causes of familial ALS, but familial ALS and sporadic ALS have similarities in their pathological features, as well as in their clinical fea-

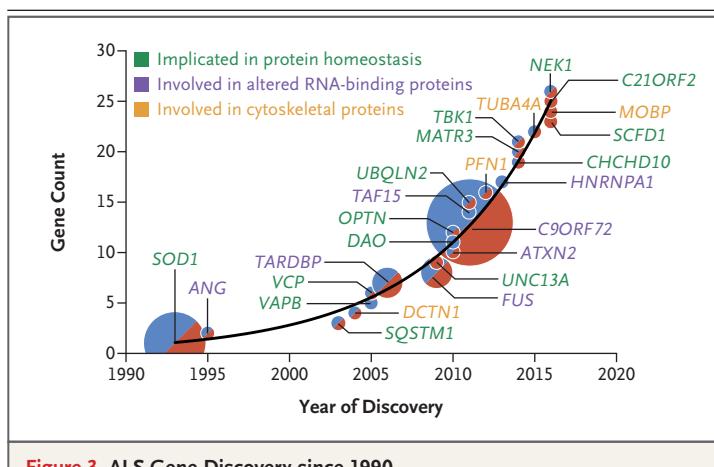


Figure 3. ALS Gene Discovery since 1990.

The cumulative numbers of known ALS genes have increased rapidly. The size of each circle reflects the proportion of all familial ALS cases associated with that gene (e.g., 20% for *SOD1* and 45% for *C9orf72*). Blue circles indicate genes associated only with familial ALS, red circles indicate genes associated only with sporadic ALS, and circles that are half blue and half red indicate genes associated with both familial and sporadic ALS. Each of these genes has been found to be mutated in more than one ALS-affected family or in multiple, unrelated cases of sporadic ALS.

tures, suggesting a convergence of the cellular and molecular events that lead to motor neuron degeneration. These points of convergence define targets for therapy.

A working view of the present panel of ALS genes is that they cluster in three categories,¹⁹ involving protein homeostasis, RNA homeostasis and trafficking, and cytoskeletal dynamics (Fig. 4). These mechanisms are not exclusive. For example, protein aggregates may sequester proteins that are important in RNA binding, thereby perturbing RNA trafficking and homeostasis. Moreover, these mechanisms are detected in the context of both familial ALS and sporadic ALS; some nonmutant proteins also have a propensity to misfold and aggregate in ALS, much like their mutant counterparts (e.g., *SOD1* and TDP-43).

Downstream of each category are diverse forms of cellular abnormalities, including the deposition of intranuclear and cytosolic protein and RNA aggregates, disturbances of protein degradative mechanisms, mitochondrial dysfunction, endoplasmic reticulum stress, defective nucleocytoplasmic trafficking, altered neuronal excitability, and altered axonal transport. In most cases, these events activate and recruit nonneuronal cells (astrocytes, microglia, and oligodendroglia), which exert both salutary and

Table 1. Genetic Variants That Influence the Phenotype in Amyotrophic Lateral Sclerosis.

Gene	Minor Allele Frequency or Expression Level	Phenotype			Study
		Site of Onset	Effect of Minor Allele on Age at Onset*	Effect of Minor Allele on Survival†	
Genomewide association study					
rs3011225-1p34	0.22		2 yr later		Ahmeti et al. ²⁴
UNC13A	0.40			Shorter by 5–10 mo	Diekstra et al. ²⁵
CAMTA1	0.26			Shorter by about 5 mo	Fogh et al. ²⁶
IDE	0.03			Shorter by about 7 mo	Fogh et al. ²⁶
Known ALS genes					
C9ORF72	Up to 0.08	Primarily bulbar			Cooper-Knock et al. ²⁷
FUS-P525L	Rare variation		Many years earlier	Shorter by several months	Conte et al. ²⁸
PFN1	Rare variation	Limb			Wu et al. ²⁹
SOD1-A4V	Rare variation	Limb		Shorter by several months	Cudkowicz et al. ³⁰
SOD1/SOD1	Rare variation		Many years earlier	Shorter by several months	Winter et al. ³¹
Modifier genes					
APOE	Expression increased			Longer by several months	Lacomblez et al. ³²
EPHA4	Expression decreased			Longer by several months	Van Hoecke et al. ³³

* The effect of the minor allele on age is shown relative to a cohort with the major allele.

† The effect of the minor allele on survival is shown relative to a cohort with the major allele.

negative influences on motor neuron viability. The diverse downstream abnormalities may differentially affect subcellular compartments (dendrites, soma, axons, and neuromuscular junctions). One implication of this model is that successful therapy for ALS will require simultaneous interventions in multiple downstream pathways.

GENES THAT INFLUENCE PROTEIN HOMEOSTASIS

The most extensively investigated pathological finding in ALS has been the accumulation of aggregated proteins and corresponding defects in the cellular pathways for protein degradation. Mutant SOD1 frequently forms intracellular aggregates. Genes that encode adapter proteins involved in protein maintenance and degradation are also implicated in ALS. These include valosin-containing protein (VCP)³⁵ and the proteins optineurin (OPTN),³⁶ TANK-binding kinase 1

(TBK1),^{37–39} and sequestosome 1 (SQSTM1/p62)⁴⁰ (Fig. 4A). The TBK1–OPTN axis is interwoven in other neurodegenerative disorders; for example, the Parkinson’s disease gene PINK1 encodes a protein that acts upstream of TBK1 in the mobilization of mitophagy.

GENES THAT INFLUENCE RNA HOMEOSTASIS AND TRAFFICKING

The most rapidly expanding category of ALS genes encodes proteins that interact with RNA. The first protein to be discovered was TDP-43,¹³ whose mislocalization from the nucleus to the cytosol, cleavage, phosphorylation, and ubiquitination were initially illuminated in sporadic ALS and frontotemporal dementia. However, it became apparent that mutations in TARDBP, the gene encoding TDP-43, can cause familial ALS.⁴¹ Mislocalization and post-translational modification of TDP-43 are observed in many neurode-

generative diseases. FUS-TLS encodes another RNA-binding protein, homologous to TDP-43, which in mutant form also causes ALS.^{42,43} Why mutated genes encoding RNA-binding proteins cause ALS is not clear. These proteins have multiple functions in gene splicing, surveillance of transcripts after splicing, generation of microRNA, and axonal biologic processes. Most of these proteins have low-complexity domains that permit promiscuous binding not only to RNA but also to other proteins. The ALS-related mutations heighten this binding propensity, leading to self-assembly of the proteins and the formation of aggregates.⁴⁴ This auto-aggregation is facilitated in stress granules, which are non-membrane-bound structures formed under cell stress that contain RNA complexes stalled in translation.⁴⁵⁻⁴⁷ The self-assembly of mutant RNA-binding proteins may induce toxic, self-propagating conformations that disseminate disease within and between cells in a manner analogous to that of prion proteins.

The most commonly mutated gene in ALS is *C9ORF72*.⁴⁸⁻⁵⁰ The *C9ORF72* protein has a role in nuclear and endosomal membrane trafficking and autophagy. A noncoding stretch of six nucleotides is repeated up to approximately 30 times in normal persons. Expansions of this segment to hundreds or thousands of repeats cause familial ALS and frontotemporal dementia; in addition, these expansions sometimes cause sporadic ALS. Several mechanisms may contribute to the neurotoxicity of the hexanucleotide expansion (Fig. 4B). Transcripts of the offending segments are deposited in the nucleus, forming RNA foci that sequester nuclear proteins. Some of the expanded RNA escapes to the cytoplasm, where it generates five potentially toxic repeat dipeptides through a noncanonical translation process. Recent studies have also shown a defect in transport across the nuclear membrane in cells with the *C9ORF72* expansions.^{51,52} A reduction in the total levels of the normal *C9ORF72* protein may also contribute to neurotoxicity.⁵³⁻⁵⁵ Transgenic mouse models of *C9orf72* recapitulate the molecular features of *C9ORF72* ALS in humans⁵⁶⁻⁵⁹ but, with one exception,⁵⁹ do not show a strong motor phenotype.

GENES THAT INFLUENCE CYTOSKELETAL DYNAMICS

Three ALS genes encode proteins that are important in maintenance of normal cytoskeletal

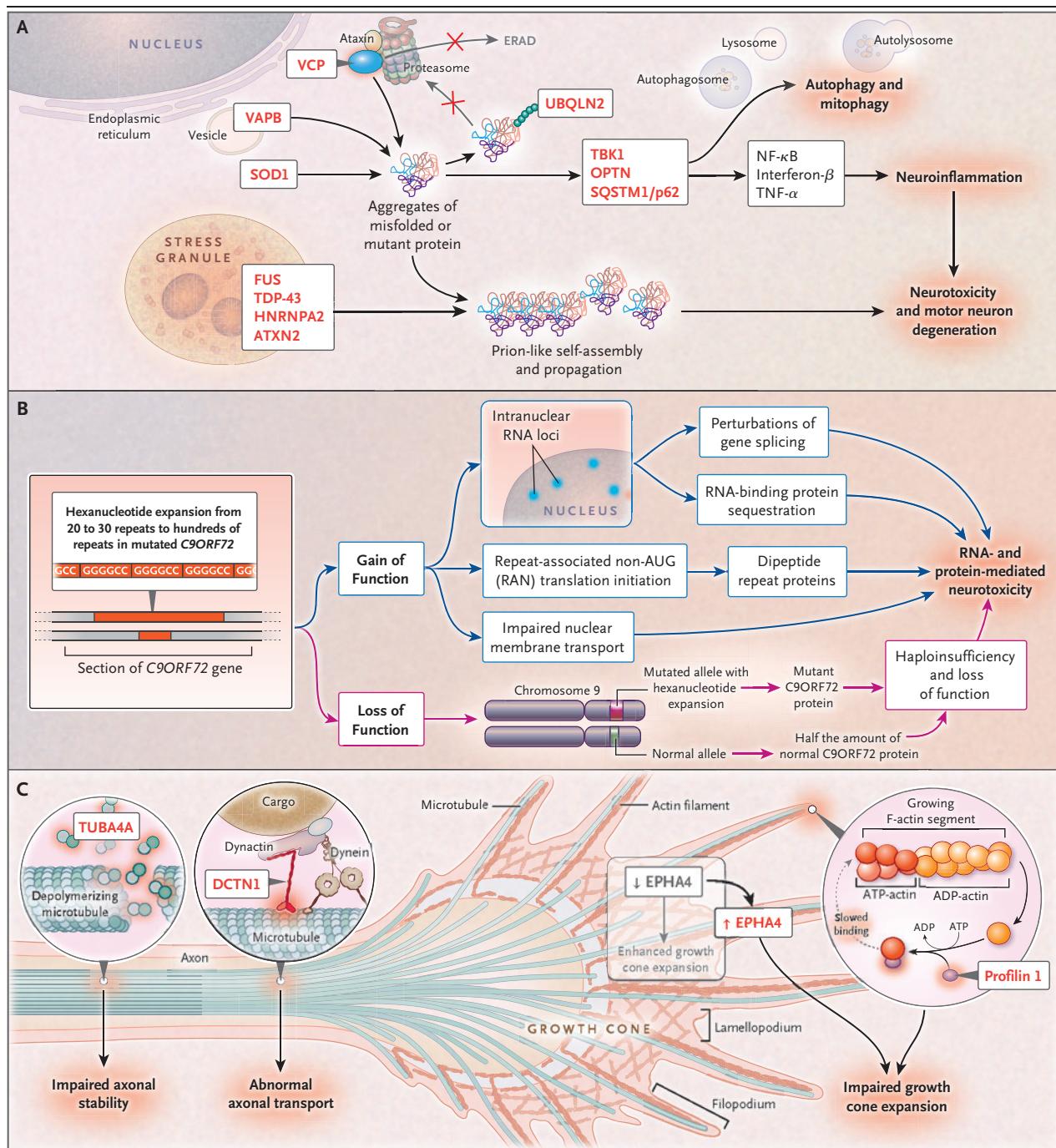
dynamics: dynactin 1 (*DCTN1*),⁶⁰ PFN1,²⁹ and tubulin 4A (*TUBA4A*) (Fig. 4C).⁶¹ *TUBA4A* dimers are components of microtubules, whose integrity is essential for axonal structure; *DCTN1* is implicated in retrograde axonal transport, whereas PFN1 participates in the conversion of globular to filamentous actin and nerve extension. Also implicated is the modifier gene *EPHA4*; lower levels of *EPHA4* expression correlate with longer survival in ALS, perhaps because they permit more exuberant axonal extension.

INSIGHTS INTO SPORADIC ALS

Despite the absence of a family history in sporadic ALS, studies involving twins show that the heritability is about 60%.⁶² Furthermore, mutations usually found in familial ALS can be found in sporadic ALS. This can be partly explained by the difficulty in ascertaining whether patients with late-onset disease have a family history of ALS. The situation is confounded by the observation that some familial ALS gene variants increase the risk of phenotypes other than ALS, such as frontotemporal dementia.^{38,39,48} Unless these other phenotypes are recognized as relevant, the family history may be incorrectly recorded as negative. In addition, several familial ALS gene variants are of intermediate penetrance (e.g., the *C9ORF72* hexanucleotide repeat expansion, *ATXN2* repeat expansions,⁶³ and *TBK1* mutations).³⁷⁻³⁹ Thus, ALS might not be manifested in a gene carrier, in which case, the disease is characterized by familial clustering rather than mendelian inheritance and may appear to be sporadic.⁶⁴ Combinations of such gene variants further increase the risk of ALS and may be another cause of apparently sporadic ALS.⁶⁵

Recent genomewide association studies have shown that rare genetic variation is disproportionately frequent in sporadic ALS.⁶⁶ The genetic architecture of sporadic ALS is markedly different from that of complex diseases such as schizophrenia in which there are additive effects of hundreds of common variants, each with a minute effect on risk. However, common variants still have a part to play in sporadic ALS. For example, variants in the genes *UNC13A*, *MOBP*, and *SCFD1* all increase the risk by a small but significant degree.⁶⁶

Heritability studies also show that a substantial fraction of cases of sporadic ALS cannot be attributed to genetic or biologic factors; these



cases are ascribed to environmental or undefined factors. Attempts to identify occupations or common exposures that might increase the risk of ALS have been inconclusive. Environmental studies are challenging because the number of possible exposures is large, and a critical, disease-related exposure may have happened many

years before the onset of the disease. A particular difficulty is that studies of ALS are susceptible to bias because of the poor prognosis. Patients who live long enough to attend a specialist research clinic are different from those identified in population studies, and this difference can cause bias in the results. For instance, smok-

Figure 4 (facing page). Three Major Categories of Pathophysiological Processes in ALS.

The pathways relating the implicated proteins (red) and key cellular structures and molecules (gray) are shown. Downstream dysfunctional events are black within gray boxes. Panel A shows altered protein homeostasis in ALS. Many ALS genes encode adapter proteins that are critical in protein degradation, acting at the level of the endoplasmic reticulum (endoplasmic reticulum-associated protein degradation [ERAD]) and through proteosomal and autophagic pathways. RNA-binding proteins may self-assemble to form prion-like aggregates. Panel B shows mechanisms of *C9ORF72*-related disease. The toxicity of expanded hexanucleotide repeats in the *C9ORF72* gene is proposed to involve depositions of intranuclear RNA, with resulting perturbations of gene splicing and sequestration of RNA-binding proteins; noncanonical translation of polydipeptides from the expanded DNA, yielding toxic repeat dipeptides; disturbances of nucleocytoplasmic transport; and reduced levels of *C9ORF72* (haploinsufficiency). Panel C shows altered neuronal cytoskeletal dynamics in ALS. Genes encoding dynactin (*DCTN1*) and tubulin 4A (*TUBA4A*) are essential in the maintenance of the structure of the motor nerve axon; mutations in these genes disturb both axonal integrity and axonal transport. Profilin 1 (*PFN1*) is essential for the assembly of filamentous axons and the formation of distal axonal growth cones. *PFN1* mutations and increased expression of ephrin A4 (*EPHA4*) slow the extension of the distal axon. ADP denotes adenosine diphosphate, NF- κ B nuclear factor kappa light-chain enhancer of activated B cells, and TNF tumor necrosis factor.

involving exposure to electromagnetic fields also appear to be at increased risk, but people living near power lines are not. Other risk factors with varying levels of support include pesticide exposure and neurotoxins such as those produced by cyanobacteria. Viruses have been studied as a possible explanation for sporadic ALS. Initial studies suggesting the role of an activated, endogenous retrovirus⁷⁴ were followed by the identification of a possible candidate, human endogenous retrovirus K.⁷⁵

There is increasing evidence that trauma precedes some individual cases of ALS.⁷⁶ A meta-analysis has suggested that trauma overall, trauma occurring more than 5 years previously, bone fracture, and head injury are all associated with an increased risk.⁷⁷ In recent years, it has been observed that persons engaged in sports that entail repetitive concussions or subconcussive head trauma are at increased risk for ALS and a concurrent behavioral disorder marked by impulsivity and memory loss. Autopsy studies in persons with this disorder, called chronic traumatic encephalopathy, have revealed frontotemporal atrophy associated with distinctive deposits of tau protein, as well as TDP-43, the characteristic inclusion protein in ALS.⁷⁸

THERAPEUTICS AND BEYOND

No therapy offers a substantial clinical benefit for patients with ALS. The drugs riluzole⁷⁹ and edaravone, which have been approved by the Food and Drug Administration for the treatment of ALS, provide a limited improvement in survival. Riluzole acts by suppressing excessive motor neuron firing, and edaravone by suppressing oxidative stress. Numerous other compounds that have been investigated have not been shown to be effective.^{80,81} Currently, the mainstay of care for patients with ALS is timely intervention to manage symptoms, including use of nasogastric feeding, prevention of aspiration (control of salivary secretions and use of cough-assist devices), and provision of ventilatory support (usually with bilevel positive airway pressure). Some interventions raise serious ethical issues, such as whether to perform tracheostomy for full ventilation and, if so, when and how to withdraw respiratory support once it has been instituted.

Despite the pipeline of potential treatments for ALS, reflecting the expanded list of targets

ing has been shown to shorten survival in a population study,⁶⁷ so a case-control study selecting participants from clinics would find smokers underrepresented in the ALS group and would thus suggest that smoking either has no effect or might be protective. Similarly, ALS specialists report anecdotally that their patients tend to be athletic, slim, and very fit,⁶⁸ but if these factors slow disease progression rather than increase risk, such patients will be overrepresented at specialist centers.

Notwithstanding the barriers to identifying environmental risk factors, some factors have been associated with ALS in multiple studies.^{69,70} The exposure with the strongest support is military service.^{71,72} In addition, smoking has been implicated as a dose-dependent risk factor for ALS.⁷³ Exposure to heavy metals may be important; blood lead levels and cerebrospinal fluid manganese levels are higher in patients with ALS than in controls.⁷⁰ People with occupations

identified through genetic studies and increasing numbers of ALS investigators, many of whom are in the pharmaceutical sector,^{80,82} no drugs are being investigated in late-phase clinical trials. Several innovative approaches to treating ALS (and other neurodegenerative diseases) are in development. Two examples include the use of adeno-associated viruses (AAV) to achieve widespread delivery of diverse cargoes (missing genes, therapeutic genes, or gene-silencing elements) to the central nervous system and the use of stem cells that provide neurotrophic factors to the central nervous system.⁸³ Studies in cells, mice, and humans support the view that several types of reagents (e.g., antisense oligonucleotides and AAV-delivered microRNA) inactivate production of toxic gene products and thus may be therapeutic in ALS mediated by genes such as *SOD1*⁸⁴⁻⁸⁷ and *C9ORF72*. Indeed, clinical trials investigating the use of antisense oligonucleotides to silence *SOD1* have begun.

One can anticipate continued progress in understanding the biology of ALS. There is no doubt that high-throughput genetics, combined with improved clinical phenotyping, will further refine the genetic landscape of ALS. As thousands of full genome sequences become avail-

able, it will be feasible to explore the possibility that complex interactions among multiple gene variants explain not only familial ALS but also sporadic ALS. The exploration of environmental factors in sporadic ALS will expand, with a focus on the internal environment represented by the microbiome. The ultimate proof of our understanding of the biology of ALS will hinge on our ability to modify the clinical course of the disease.

Dr. Brown reports holding equity in AviTx, Amylyx Pharmaceuticals, and ImStar Therapeutics, receiving fees for serving on an advisory board from Voyager Therapeutics, negotiating a collaborative agreement with WAVE Biosciences, holding patents and receiving royalties for patents on "Method for the diagnosis of familial amyotrophic lateral sclerosis" (US 5,843,641) and "Mice having a mutant SOD1 encoding transgene" (US 6,723,893), holding a patent for "Compounds and method for the diagnosis, treatment and prevention of cell death" (US 5,849,290), and holding a pending patent for "Use of synthetic microRNA for AAV-mediated silencing of SOD1 in ALS"; and Dr. Al-Chalabi reports receiving consulting fees from GlaxoSmithKline, providing unpaid consulting for Mitsubishi Tanabe Pharma, Tree-way, Chronos Therapeutics, and Avanir Pharmaceuticals, receiving consulting fees and serving as principal investigator in an international commercial clinical trial of tirasemtiv in ALS for Cytokinetics, and serving as chief investigator of an international commercial clinical trial of levosimendan in ALS for Orion Pharma. No other potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Novel genes associated with amyotrophic lateral sclerosis: diagnostic and clinical implications

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Summary

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For more on the ExAC database
see <http://exac.broadinstitute.org>

For more on genetic
nomenclature see
<http://varnomen.hgvs.org>

Background The disease course of amyotrophic lateral sclerosis (ALS) is rapid and, because its pathophysiology is unclear, few effective treatments are available. Genetic research aims to understand the underlying mechanisms of ALS and identify potential therapeutic targets. The first gene associated with ALS was *SOD1*, identified in 1993 and, by early 2014, more than 20 genes had been identified as causative of, or highly associated with, ALS. These genetic discoveries have identified key disease pathways that are therapeutically testable and could potentially lead to the development of better treatments for people with ALS.

Recent developments Since 2014, seven additional genes have been associated with ALS (*MATR3*, *CHCHD10*, *TBK1*, *TUBA4A*, *NEK1*, *C21orf2*, and *CCNF*), all of which were identified by genome-wide association studies, whole genome studies, or exome sequencing technologies. Each of the seven novel genes code for proteins associated with one or more molecular pathways known to be involved in ALS. These pathways include dysfunction in global protein homoeostasis resulting from abnormal protein aggregation or a defect in the protein clearance pathway, mitochondrial dysfunction, altered RNA metabolism, impaired cytoskeletal integrity, altered axonal transport dynamics, and DNA damage accumulation due to defective DNA repair. Because these novel genes share common disease pathways with other genes implicated in ALS, therapeutics targeting these pathways could be useful for a broad group of patients stratified by genotype. However, the effects of these novel genes have not yet been investigated in animal models, which will be a key step to translating these findings into clinical practice.

Where next? The identification of these seven novel genes has been important in unravelling the molecular mechanisms underlying ALS. However, our understanding of what causes ALS is not complete, and further genetic research will provide additional detail about its causes. Increased genetic knowledge will also identify potential therapeutic targets and could lead to the development of individualised medicine for patients with ALS. These developments will have a direct effect on clinical practice when genome sequencing becomes a routine and integral part of disease diagnosis and management.

Introduction

Typically, the disease course of amyotrophic lateral sclerosis (ALS) is rapid, and most patients die within 3–5 years of symptom onset as a result of respiratory failure.¹ Although the disease is considered a rare type of motor neuron neurodegeneration, the number of patients with ALS is rapidly increasing because of population ageing. Most patients are aged between 50 and 75 years at diagnosis and, by 2040, an estimated 400 000 patients will be diagnosed with ALS worldwide.² Approximately 10% of patients with ALS have a family history of disease, whereas the remainder of cases are classified as sporadic.¹ The pathophysiology of ALS—familial or sporadic—is unclear, thus few effective treatments are available. Riluzole and edaravone are the current treatments approved for the disease. Riluzole prolongs survival by 2–3 months at best, with little effect on quality of life,³ whereas edaravone mildly improves patient mobility, but the effect on survival is unknown.⁴ The paucity of effective treatments warrants more genetic and molecular research on the underlying mechanisms of ALS to analyse the disease process at the cellular level. By 2014, 22 genes were implicated in ALS, and mutations in these genes account for about two-thirds of all familial cases and approximately 10% of cases of sporadic ALS.⁵ Since 2014,

seven novel genes associated with ALS—*MATR3*, *CHCHD10*, *TBK1*, *TUBA4A*, *NEK1*, *C21orf2*, and *CCNF*—have been identified. The rapid identification of multiple novel genes associated with ALS reflects improvements in sequencing technologies and, more importantly, provides an opportunity to better understand the disease (figure 1). Such advances are key to the development of disease-modifying treatments.

In this Rapid Review, we summarise the novel genetic discoveries associated with ALS in chronological order. We focus on the technologies and experimental design used to identify these genes, and have cross-checked genetic variants against the Exome Aggregation Consortium (ExAC) public database, which catalogues more than 7 million variants in the protein coding region of the genome identified in more than 60 000 mostly healthy individuals (ie, those without severe paediatric diseases). Genetic screening is becoming more accessible and common in clinical practice, thus understanding how a variant might cause disease within the context of the larger population could help in making reasonable inference about pathogenicity, especially when family history of disease is unknown. We also discuss the importance of these genes for the development of new therapies.

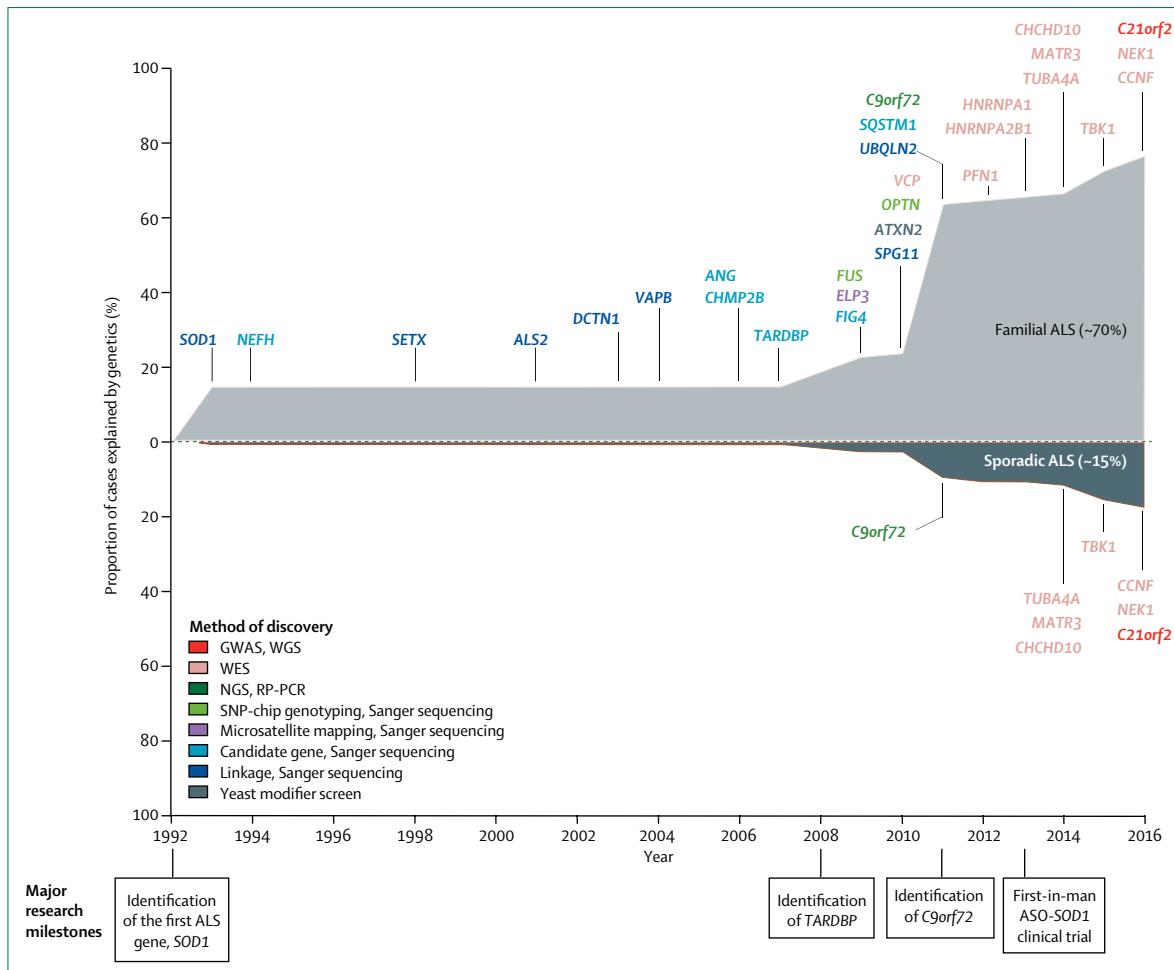


Figure 1: Genetic landscape of ALS between 1993 and 2016

Familial ALS cases constitute about 10% of all cases of ALS. Of this 10%, about 70% can be explained by genetics. Two substantial increases in genetic contribution to ALS were found in 2008 and 2011, corresponding to the identification of *TARDBP* (contributes to about 4% of familial and 1% of sporadic cases) and *C9orf72* (contributing to about 40% of familial cases and 8% of sporadic cases). ALS=amyotrophic lateral sclerosis. GWAS=genome-wide association study. WGS=whole-genome sequencing. WES=whole-exome sequencing. NGS=next-generation sequencing. RP-PCR=repeat-primed polymerase chain reaction. SNP=single nucleotide polymorphism. ASO=antisense oligonucleotide.

Novel ALS genes

Frequency data for these seven novel genes identified since 2014 are scarce because few studies have done large-scale screening of independent patient cohorts. The frequency data available for these genes are likely to be inflated, and we hypothesise that the frequency of mutations in these genes in the population will be lower when additional data is obtained. We estimated that for ALS—assuming full penetrance, no founder mutation effect, and disease prevalence of six cases per 100 000 individuals⁶—a variant observed more than five times per 121 000 alleles in the ExAC database (corresponding to an allele frequency of 0.0033%) is unlikely to cause ALS because it is too common. However, absence of mutations in a gene in the ExAC database does not necessarily infer pathogenicity because rare genetic variants that are unique to one individual or a single family are remarkably common in

the human population (about 3–4 million single nucleotide polymorphisms per individual).⁷

MATR3

In 2014, four mutations (p.S85C, p.F115C, p.P154S, and p.T622A) in *MATR3* were identified by exome sequencing in four families of European descent with either ALS alone or with a combination of ALS and dementia.⁸ Since 2014, 11 additional variants have been described, predominantly occurring in patients with sporadic ALS.^{9–12} In the ExAC database, three variants (p.E664A, p.N787S, and c.48+1G>T) had a reported allele frequency of 0.03–0.05%, p.F115C was reported once, but none of the other variants were listed. Overall, the contribution of *MATR3* to the development of ALS or ALS and frontotemporal dementia is relatively rare, with no significant correlation observed between phenotype and genotype.

In patients with ALS with *MATR3* mutations, upper and lower motor neurons are affected and survival duration ranges from 2–12 years.¹⁰ The concomitant clinical presentation of ALS and myopathic features in individuals with the p.S85C mutation is important because these patients are initially diagnosed with vocal cord and pharyngeal dysfunction with asymmetric distal myopathy, but the presentation of pyramidal tract signs and progressive respiratory failure at end-stage disease usually warrants re-diagnosis.⁸ By contrast to TDP43 and FUS, whereby mutations cause relocation of the mutant protein from the nucleus to cytoplasm, studies^{8,13} have shown that the subcellular localisation of mutant *MATR3* is generally unaffected. Furthermore, *MATR3*-positive inclusions were occasionally observed in histopathological sections from patients with *MATR3* mutations, and in one individual with *C9orf72* expansion.⁸

MATR3 is a 125 kDa nuclear protein with RNA and DNA binding domains that appears to primarily regulate gene expression.¹⁴ Transgenic mice overexpressing human *MATR3* protein develop hindlimb paralysis and muscle atrophy, indicating that neuromuscular function is sensitive to *MATR3* levels.¹⁵ The protein forms a complex with two other ALS-associated RNA-binding proteins, TDP43⁸ and FUS,^{16,17} in a RNA-dependent manner and the p.S85C mutation enhances this interaction.⁸ Thus, overlap might occur in the upstream regulatory proteins or downstream effector targets among ALS-RNA binding proteins. Elucidation of this potentially shared set of proteins might identify molecules suitable for therapeutic intervention.

CHCHD10

CHCHD10 was first linked to ALS in a study¹⁸ of a large French family who had a complex phenotype of ALS, ataxia, mitochondrial myopathy, parkinsonism, and sensorineural hearing loss. Exome sequencing identified a p.S59L mutation within *CHCHD10*.¹⁸ Subsequently, 20 additional missense variants, clustered in exon 2—which encodes an internal hydrophobic helical segment important for mitochondrial membrane binding¹⁹—have been reported in a broad range of neurodegenerative disorders, including ALS and frontotemporal dementia,^{18,20–28} frontotemporal lobar degeneration,²⁹ parkinsonism,^{26,27} Alzheimer's disease,³⁰ autosomal dominant mitochondrial myopathy,³¹ adult-onset spinal muscular atrophy,³² and Charcot-Marie-Tooth type 2.³³ The pathogenicity of p.S59L,¹⁸ p.R15L,^{20–22} and p.G66V²⁰ has been validated in family studies, whereby the mutations were shown to segregate with ALS. Additionally, the mutations were absent in the ExAC database. However, mutations in *CHCHD10* appear to be a relatively rare cause of ALS, but might be more frequent among patients diagnosed with frontotemporal dementia.^{27,34}

CHCHD10 is a 14 kDa nuclear-encoded, mitochondrial protein localised to the mitochondrial intermembrane space. The protein is important for the maintenance of

mitochondrial dynamics and cellular bioenergetics.³⁵ Patient fibroblasts expressing mutant *CHCHD10* protein (p.S59L) have a fragmented mitochondrial network and disrupted mitochondrial cristae.¹⁸ These effects are similar to abnormalities in mitochondrial dynamics induced by mutations in TDP43.³⁶ *CHCHD10* also interacts with TDP43, which promotes retention of TDP43 in the nucleus,³⁷ but this localisation is disrupted in the presence of *CHCHD10* mutations, causing an accumulation of TDP43 in the cytoplasm and synaptic damage.³⁷ Further study is necessary to investigate the mechanistic association between these proteins and their involvement in mitochondrial dysfunction and TDP43 proteinopathy. This insight could identify therapeutic targets susceptible to manipulation by small molecules, to rescue the observed cellular defects involved in ALS.

TUBA4A

TUBA4A was implicated as a novel gene for familial ALS on the basis of exome sequencing data obtained from a large cohort of European and American patients with ALS and controls.³⁸ This finding was replicated in an independent Belgian cohort,²⁶ but not in Asian patients with ALS.³⁹ All variants were absent or had very low frequency in the ExAC database and had adequate segregation data, with the exception of p.K430N. The overall frequency of *TUBA4A* mutations suggests it is a rare cause of ALS. Little information is available about the clinical presentation, prognosis, or neuropathological evaluation of patients with *TUBA4A* variants, and although patients often present with typical features of ALS, some also present with features of frontotemporal dementia.^{26,38}

The main cytoskeletal scaffold in cells is comprised of microtubules, composed of polymerised α-tubulin and β-tubulin subunits. In primary motor neurons, expression of missense mutation *TUBA4A* interferes with tubulin dimerisation, resulting in a weakened microtubule network.³⁸ Mutations have been found to cluster in the protein domain responsible for the interaction with other tubulin subunits and the axonal transport proteins dynein and kinesin.⁴⁰ This finding highlights the crucial role of cytoskeletal and axonal transport defects in the pathogenesis of ALS. Therapeutic approaches enhancing cytoskeletal integrity might be crucial for halting progression or reversing the disease course.

TBK1

A whole exome sequencing study⁴¹ revealed that *TBK1* was implicated in ALS. Enrichment of nonsynonymous variants in patients with ALS compared with healthy controls was found across the entire coding region.⁴¹ This finding was validated by another whole exome sequencing study,⁴² which reported segregation of the pathogenic variants within affected families. Mutations in *TBK1* are found in about 1% of patients with familial

ALS and in approximately 1% of patients with sporadic ALS.^{42–53} The clinical phenotypes associated with *TBK1* mutations are heterogeneous, with variable age of onset, differing progression, and irregular length of survival time.^{39,43–46} Extrapyramidal symptoms, ataxia, and psychiatric symptoms have also been reported in some patients with *TBK1* mutations.⁴⁶ Neuropathological examination of CNS tissue from patients with a *TBK1* mutation showed *SQSTM1/p62* and TDP43-positive inclusions,^{42,45,46} which are indicative of abnormal TDP43 protein aggregation and defective protein clearance pathways.⁴⁷ Since these inclusions are also observed in other patients with ALS without *TBK1* mutations,⁴⁸ this suggests that a common disease mechanism might exist, and a broad treatment approach to restore defective proteostasis might also benefit patients with *TBK1* mutations.⁴⁹

TBK1 is a homodimeric multidomain protein with a kinase domain, a ubiquitin-like domain, and two coiled-coil domains.⁵⁰ The protein acts as an interaction platform for multiple proteins and regulates the activities of downstream protein targets involved in key cellular processes that have been implicated in ALS, including neuroinflammation, ubiquitin-proteasome systems, and autophagy pathways involving other genes also associated with ALS—ie, *OPTN*, *SQSTM1/p62*, *VCP*, and *UBQLN2*.⁵⁰ Most pathogenic variants identified in *TBK1* are concentrated within the kinase and the coiled-coiled domains,⁴² suggesting that these mutations might operate by altering these downstream regulatory pathways. We identified some variants (p.K291E, p.I305T, p.L306I, p.H322Y, p.T322I, p.R444Q, and p.A535T) in the ExAC database that had a frequency higher than our estimated threshold of 0·0033%, suggesting that they are unlikely to be pathogenic. Pathogenicity of the other variants will require further investigation in families and in cells or animal models.

NEK1 and C21orf2

Heterozygous loss-of-function mutations in *NEK1* have been implicated in sporadic ALS.⁴¹ *NEK1* interacts with two proteins known to be associated with ALS, *ALS2* and *VAPB*,⁴⁵ which are involved in endosomal and endoplasmic reticulum lipid trafficking; this interaction provides some functional evidence of *NEK1* involvement in ALS pathogenesis. Two independent case-control studies^{51,52} provided corroborative evidence that *NEK1* is associated with ALS, and indicated that it might account for up to 2% of all ALS cases. Clinical descriptions of patients with *NEK1* mutations are scarce, but patients appear to present with typical ALS without dementia.^{51,52}

Concomitant with the identification of *NEK1*, a large case-control study⁵³ using the genome-wide association approach found that *C21orf2* was associated with increased ALS risk. *NEK1* and *C21orf2* interact with each other and are involved in microtubule assembly, DNA damage response and repair, and mitochondrial function.^{54,55}

Additional genetic replication studies in independent cohorts and functional and clinical studies for both *NEK1* and *C21orf2* are required to fully understand the contribution of these variants in the pathogenesis of ALS.

CCNF

CCNF was identified as a causative gene for ALS on the basis of exome sequence analysis⁵⁶ of a large family of European descent who had ALS, frontotemporal dementia, or both diseases, with an autosomal dominant pattern of inheritance. The authors reported additional, potentially pathogenic variants in *CCNF* in familial cases (all absent or less than the 0·0033% threshold in the ExAC database), with an overall mutation frequency that ranged between 0·6 and 3·3% in white populations.⁵⁷ Clinically, these patients presented with either typical ALS, ALS with frontotemporal dementia, or frontotemporal dementia alone.⁵⁶

CCNF is the substrate-recognising component of the Skp1-cullin-F-box E3 ubiquitin-ligase complex, which is responsible for tagging proteins with ubiquitin and marking them for degradation via the ubiquitin-proteasome system.⁵⁷ Neuronal cells overexpressing mutant *CCNF* show an increase in ubiquitin-tagged proteins, which include TDP43. This increase suggests that these variants affect the proteasomal degradation pathway by either aberrantly tagging all proteins with ubiquitin or failing to transfer ubiquitin-tagged proteins to the proteasome complex for removal.⁵⁶ This finding indicates that mutations in *CCNF* might lead to abnormal proteostasis, which might be exacerbated by TDP43 proteinopathy. Therefore, therapies that enhance protein clearance or reduce ubiquitination might be viable approaches to treatment.

Role of genetics in therapy development

With the exception of riluzole, which was shown to prolong survival for 2–3 months,³ and edavarone, which was shown to decrease the rate of patient immobility,⁴ currently no treatments are available for ALS that can effectively stop or reverse the disease progression. Diagnosis of ALS is only possible through assessment of clinical symptoms after a substantial number of motor neurons have died. Thus, for a drug to be effective, early or presymptomatic diagnosis would be necessary to prevent further motor neuron degeneration and to preserve the function of remaining motor neurons. However, this presents a challenge because no reliable molecular biomarkers have been identified for presymptomatic diagnosis or for patient stratification in clinical trials. The genetic landscape of ALS is slowly evolving in response to novel genetic discoveries, helping to identify pathogenic cellular pathways (figure 2, table), and to provide both potential biomarkers and targets for drug discovery.

Pathogenicity in some cases is likely to be driven by the acquisition of a toxic function through genetic

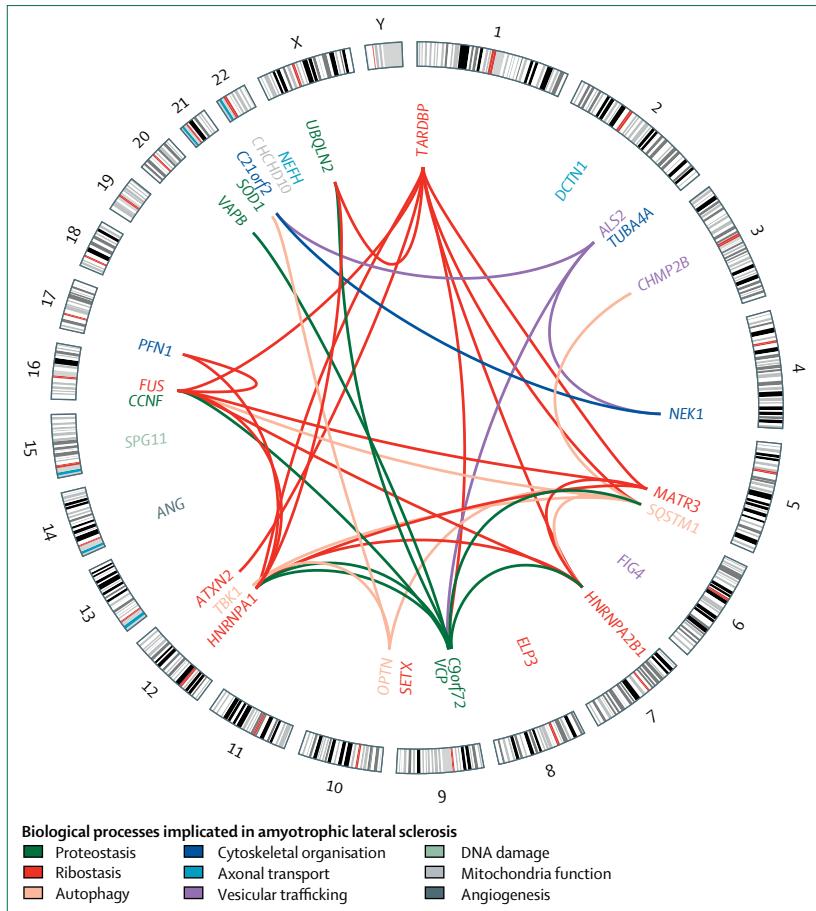


Figure 2: Interactions between genes associated with amyotrophic lateral sclerosis

The outer circle is a karyotype ideogram showing 24 chromosomes (22 autosomal chromosomes, X chromosome, and Y chromosome); the inner circle shows the location of each gene. Links between genes represent interactions at the protein or gene level. Interaction data was obtained from the Biological General Repository for Interaction Datasets. Black lines indicate cytogenetic band patterns. Biological processes implicated in either the gene or interactions are indicated by colour.

mutation, which forms the basis for antisense oligonucleotide treatment.⁵⁸ By reducing the production of toxic species, the pathogenic process driven by these species can be modified or stopped to prevent further cellular damage. In animal models of *SOD1*-associated ALS,⁵⁹ antisense oligonucleotide treatment significantly delayed disease onset, improved neuromuscular function, and prolonged survival. These effects were accompanied by a corresponding decrease in *SOD1* in cerebrospinal fluid, indicating that the concentration of *SOD1* in this compartment might be a pharmacodynamic biomarker for future prognostic and efficacy assessments.⁶⁰ The first clinical trial⁶¹ of antisense oligonucleotide treatment in human beings had favourable safety outcomes, and a trial to assess the safety, tolerability, and pharmacokinetics of a second generation *SOD1* antisense oligonucleotide is currently in progress (ClinicalTrials.gov, NCT02623699). Phase 2 and phase 3 trials are needed to establish whether

the efficacy observed in experimental models can be achieved in human beings.

Similar biomarker development and antisense oligonucleotide studies targeting *C9orf72* are in development.^{62–64} Although it remains unclear which toxic species drive pathogenicity,⁶⁵ a single dose of antisense oligonucleotide that specifically targets the expanded allele was sufficient to alleviate behavioural symptoms in transgenic *C9orf72* mice and reduce the number of RNA foci and dipeptide repeat proteins.^{66,67} Patients with *C9orf72* expansion also showed increased toxic RNA accumulation in tissues and circulating dipeptide repeat proteins in blood and cerebrospinal fluid,⁶⁷ suggesting that *C9orf72* could be a candidate biomarker of disease diagnosis, treatment efficacy evaluation, and prognosis.⁶²

Genetic discoveries have been directly applied in clinical settings to alleviate disease—eg, riboflavin therapy for Brown-Vialetto-Van Laere syndrome,^{68,69} which is an inherited variant of ALS. The syndrome is a rare progressive neurodegenerative disorder that typically manifests as childhood ALS in combination with sensorineural deafness.⁷⁰ Brown-Vialetto-Van Laere syndrome is caused by mutations in two riboflavin transporter genes (*SLC52A2* and *SLC52A3*)⁷⁰ that result in a reduction of plasma flavin and acylcarnitine concentrations.⁶⁸ Patients treated with high-dose oral riboflavin had marked motor improvements and an overall alleviation of clinical symptoms.⁶⁸

TBK1 is a key regulatory molecule upstream of *OPTN*, *SQSTM1/p62*, and *IRF3* in the autophagy and neuro-inflammatory pathways that are implicated in ALS.^{42,50} Manipulation of *TBK1* might potentially compensate for defects caused by other ALS-associated proteins in these pathways—eg, *VCP* and *UBQLN2*. *NEK1* and *C21orf2* are known to interact at the protein level and, in addition to *TUBA4A*, *PFN1*, *NEFH*, and *PRPH*, they represent the building blocks of the cellular scaffold. Administration of small molecules that enhance cytoskeletal integrity could represent a viable therapy for stopping progression or reversing the disease course in patients with these mutations.

Conclusions and future directions

ALS research has been largely driven by advances in our understanding of the genetics underlying the disease. This, in turn, has been fuelled by technological developments in next generation sequencing. Since 2014, seven novel genes—*MATR3*, *CHCHD10*, *TBK1*, *TUBA4A*, *NEK1*, *C21orf2*, and *CCNF*—associated with ALS have been identified using these techniques. However, the precise disease mechanisms attributed to these genes are unclear, and further elucidation from in-vivo and in-vitro functional studies is required. The collective identification of these novel genes is important within the context of other established genes that are associated with ALS to enable investigation of the disease process at the cellular level (figure 2).

Gene	Loci	Genetic effect	Familial amyotrophic lateral sclerosis (%) [*]	Sporadic amyotrophic lateral sclerosis (%) [*]	Implicated amyotrophic lateral sclerosis pathway	Disease features [†]	Other associated allelic disorders	DNA, RNA, and proteins found to interact with target gene [‡]
2016 <i>C21orf2</i>	21q22.3	Autosomal dominant, risk factor	NA	NA	Cytoskeletal organisation	Typical amyotrophic lateral sclerosis; frontotemporal dementia	Down syndrome; spondylometaphyseal dysplasia; retinal degeneration	ALS2, NEK1
2016 <i>CCNF</i>	16p13.3	Autosomal dominant	4	2	Proteostasis	NA	None	None
2016 <i>NEK1</i>	4q33	Autosomal dominant, autosomal recessive	2	2	Cytoskeletal organisation; DNA damage and cell cycle	Typical amyotrophic lateral sclerosis	Short-rib thoracic dysplasia 6 with or without polydactylyism; polycystic kidney disease	ALS2, C21orf2
2015 <i>TBK1</i>	12q14.2	Autosomal dominant, de novo	<1	<1	Autophagy; neuroinflammation	Typical amyotrophic lateral sclerosis; frontotemporal dementia	None	OPTN, SQSTM1
2014 <i>CHCHD10</i>	22q11.23	Autosomal dominant	<1	<1	Mitochondrial function; cellular bioenergetics	Typical amyotrophic lateral sclerosis; frontotemporal dementia; ataxia; myopathy	Spinal muscular atrophy (Jokela type); autosomal dominant isolated mitochondrial myopathy	None
2014 <i>MATR3</i>	5q31.2	Autosomal dominant	<1	<1	Ribostasis	Slow, progressive typical or atypical amyotrophic lateral sclerosis; frontotemporal dementia; myopathy	Vocal cord and pharyngeal dysfunction with distal myopathy	HNRNPA2B1, HNRNPA1, TARDP, FUS
2014 <i>TUBA4A</i>	2q35	Autosomal dominant	<1	<1	Cytoskeletal organisation; axonal transport	Typical amyotrophic lateral sclerosis; frontotemporal dementia	None	None
2013 <i>HNRNPA1</i>	12q13	Autosomal dominant, de novo, risk factor	NA	NA	Ribostasis	Typical amyotrophic lateral sclerosis; myopathy; cognitive impairment	Inclusion body myositis with early-onset Paget disease without frontotemporal dementia 3; multisystem proteinopathy	HNRNPA2B1, TARDP, UBQLN2, MATR3, FUS, PFN1
2013 <i>HNRNPA2B1</i>	7p15	Autosomal dominant, risk factor	NA	NA	Ribostasis	Typical amyotrophic lateral sclerosis; myopathy; cognitive impairment	Inclusion body myositis with early-onset Paget disease with or without frontotemporal dementia 2; multisystem proteinopathy	HNRNPA1, TARDP, FUS, MATR3, SQSTM1
2012 <i>PFN1</i>	17p13	Autosomal dominant	<1	<1	Cytoskeletal organisation; axonal growth and transport	Typical amyotrophic lateral sclerosis	None	FUS, HNRNPA
2011 <i>C9orf72</i>	9p21	Autosomal dominant	40	7	Intracellular trafficking; autophagy; proteostasis; global RNA alterations; nucleocytoplasmic transport	Typical amyotrophic lateral sclerosis; frontotemporal dementia	None	HNRNPA1, HNRNPA2B1, UBQLN2, SQSTM1
2011 <i>SQSTM1</i>	5q35	Autosomal dominant	1	<1	Autophagy; neuroinflammation	Typical amyotrophic lateral sclerosis	Frontotemporal dementia; amyotrophic lateral sclerosis; distal myopathy; childhood-onset neurodegeneration with ataxia, dystonia, and gaze palsy; Paget disease of bone-3	TBK1, TARDP, OPTN, CHMP2B, FUS, HNRNPA2B1, NEFM
2011 <i>UBQLN2</i>	Xp11	X-linked autosomal dominant	<1	<1	Proteostasis	Typical juvenile or adult onset amyotrophic lateral sclerosis	None	HNRNPA1, TARDP
2010 <i>ATXN2</i>	12q24	Autosomal dominant, risk factor			Ribostasis	Typical amyotrophic lateral sclerosis	Spinocerebellar ataxia type 2	TARDP
2010 <i>OPTN</i>	10p13	Autosomal dominant, autosomal recessive	<1	<1	Autophagy; neuroinflammation	Slow, progressive atypical amyotrophic lateral sclerosis	Adult-onset primary open angle glaucoma	TBK1, SQSTM1, SOD1
2010 <i>SPG11\$</i>	15q14	Autosomal recessive	NA	NA	DNA damage	Slow, progressive juvenile amyotrophic lateral sclerosis, mainly affecting upper motor neurons	Hereditary spastic paraparesis; Charcot-Marie-Tooth disease, axonal, type 2X; spastic paraparesis type 11	None

(Table continues on next page)

Gene	Loci	Genetic effect	Familial amyotrophic lateral sclerosis (%) [*]	Sporadic amyotrophic lateral sclerosis (%) [*]	Implicated amyotrophic lateral sclerosis pathway	Disease features [†]	Other associated allelic disorders	DNA, RNA, and proteins found to interact with target gene [‡]
(Continued from previous page)								
2010 VCP	9p13	Autosomal dominant, de novo	1	1	Proteostasis	Typical amyotrophic lateral sclerosis; frontotemporal dementia	Charcot-Marie-Tooth disease type 2Y; inclusion body myopathy with early-onset Paget disease and frontotemporal dementia 1	FUS, TARDBP, VAPB, HNRNPA1
2006 ANG	14q11	Risk factor	NA	NA	Angiogenesis	Typical amyotrophic lateral sclerosis; frontotemporal dementia	None	None
2009 ELP3	8p21	Undefined	NA	NA	Ribostasis; cytoskeletal integrity	Typical amyotrophic lateral sclerosis	None	None
2009 FUS	16p11	Autosomal dominant, autosomal recessive, de novo	4	1	Ribostasis	Juvenile and adult onset typical or atypical amyotrophic lateral sclerosis; frontotemporal dementia; dementia	Hereditary essential tremor-4	MATR3, TARDBP, TAF15, SMN, VCP, SQSTM1, PFN1
2008 TARDBP	1p36	Autosomal dominant, autosomal recessive, de novo	4	1	Ribostasis	Typical amyotrophic lateral sclerosis; frontotemporal dementia	None	ATXN2, MATR3, UBQLN2, HNRNPA, 2B1, HNRNPA1, SQSTM1, UBQLN2, VCP, FUS
2006 CHMP2B	3p11	Autosomal dominant	NA	NA	Vesicular trafficking; proteostasis	Typical amyotrophic lateral sclerosis	Frontotemporal dementia	None
2004 VAPB	20q13	Autosomal dominant	NA	NA	Proteostasis	Typical or atypical amyotrophic lateral sclerosis	Finkel type spinal muscular atrophy	VCP
2003 DCTN1	2p13	Autosomal dominant, risk factor	NA	NA	Axonal transport	Slow, progressive juvenile amyotrophic lateral sclerosis	Distal hereditary motor neuropathy type VIIb; Perry syndrome	None
2001 ALS2§	2q33	Autosomal recessive	NA	NA	Vesicular trafficking	Slow, progressive, infantile and juvenile amyotrophic lateral sclerosis (mainly affecting upper motor neurons)	Juvenile primary lateral sclerosis; infantile hereditary spastic paraparesis	C21orf2, VCP, NEK1
1998 SETX	9q34	Autosomal dominant	NA	NA	Ribostasis	Slow, progressive, juvenile amyotrophic lateral sclerosis	Autosomal recessive spinocerebellar ataxia type 1	None
1994 NEFH	22q12	Autosomal dominant, risk factor	NA	NA	Axonal transport	Typical amyotrophic lateral sclerosis	Axonal Charcot-Marie-Tooth disease type 2CC	None
1993 SOD1	21q22	Autosomal dominant, autosomal recessive, de novo	12	1–2	Proteostasis; oxidative stress	Typical amyotrophic lateral sclerosis; cognitive impairment (very rare)	None	OPTN

NA=not available. *Proportion of cases attributed to mutations in the corresponding genes. †Data extracted from the Online Mendelian Inheritance in Man (OMIM) database. ‡Data extracted from the Biological General Repository for Interaction Datasets (BioGrid). §ALS2 and SPG11 genes are associated with a phenotype that differs from typical amyotrophic lateral sclerosis, which mainly affects the upper motor neurons, with disease onset at a young age.

Table: Genes associated with amyotrophic lateral sclerosis identified between 1993 and 2016

For the OMIM database see
<https://omim.org>

For the BioGrid database see
<https://thebiogrid.org>

The considerable advances in genetic identification seen in the past decade are likely to continue as whole genome sequencing becomes more accessible. Such progress will facilitate the analysis of larger cohorts leading to a better understanding of the molecular defects that cause motor neuron degeneration. In particular, these techniques will help to identify rare

polymorphisms in the non-coding intergenic regions of the genome and structural variants, such as repeat expansions, copy number variants, and indels that might contribute to ALS. The availability of well phenotyped cohorts and efforts in large-scale genomic sequencing are essential to improve our understanding of ALS pathophysiology, and thus, to identify therapeutic targets.

Search strategy and selection criteria

We searched PubMed for articles published in English between Dec 1, 2013, and Aug 31, 2017, using the search terms "ALS AND genetics" and "motor neuron disease AND genetics". We selected articles that reported the identification of the novel amyotrophic lateral sclerosis genes *MATR3*, *CHCHD10*, *TBK1*, *TUBA4A*, *NEK1*, *C21orf2*, and *CCNF*. We also searched for articles describing the function and implications of mutations in these selected genes in neurological and non-neurological diseases, and associations with known amyotrophic lateral sclerosis genes identified before 2014. We selected the most relevant articles on the basis of subjective appraisal of their quality and mechanistic insight that could be relevant to amyotrophic lateral sclerosis.

Increased knowledge about the genetic profiles that protect or confer disease risk in patients with ALS will change the way clinical trials are done and how therapy is prescribed to patients. The most important change will be the stratification of patient and control cohorts by genotype, which will increase the success rate of clinical trials. Because ALS is a genetically heterogeneous and complex disease, a personalised medicine approach is emerging, whereby treatment is tailored to the specific mutation that causes disease in an individual patient. Thus, genetic screening for known variants or mutations will be integral to diagnosis, treatment, and prevention of ALS. Many advances have been achieved in the past 5 years, such as the application of gene silencing for *SOD1* and *C9orf72*, the development of viable biomarkers for the diagnosis of patients with ALS who have mutations in those genes, and the evaluation of the efficacy of potential treatments. More breakthroughs are expected in the future when more genes are identified through these large-scale genetic studies.

Contributors

All authors contributed equally to the preparation and writing of the manuscript. All authors approved the final version.

Declaration of interests

AC received personal fees from Biogen Idec, Mitsubishi, and Neuraltus. BJT received grants from Merck, Microsoft Research, the Amyotrophic Lateral Sclerosis Association, the Packard Center for Amyotrophic Lateral Sclerosis Research, the Muscular Dystrophy Association, the Center for Disease Prevention and Control, the Myasthenia Gravis Foundation, the Italian Football Association, and the Italian Amyotrophic Lateral Sclerosis Association; and holds the European patent (16204118.0-1404) on the clinical testing and therapeutic intervention for the hexanucleotide repeat expansion of *C9orf72* and the USA patent is pending. RC declares no competing interests.

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Review article

The panoramic view of amyotrophic lateral sclerosis: A fatal intricate neurological disorder



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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurological disease affecting both upper and lower motor neurons. In the United States alone, there are 16,000–20,000 established cases of ALS. The early disease diagnosis is challenging due to many overlapping pathophysiologies with other neurological diseases. The etiology of ALS is unknown; however, it is divided into two categories: familial ALS (fALS) which occurs due to gene mutations & contributes to 5–10% of ALS, and sporadic ALS (sALS) which is due to environmental factors & contributes to 90–95% of ALS. There is still no curative treatment for ALS: palliative care and symptomatic treatment are therefore essential components in the management of these patients. In this review, we provide a panoramic view of ALS, which includes epidemiology, risk factors, pathophysiologies, biomarkers, diagnosis, therapeutics (natural, synthetic, gene-based, pharmacological, stem cell, extracellular vesicles, and physical therapy), controversies (in the clinical trials of ALS), the scope of nanomedicine in ALS, and future perspectives.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a highly progressive neurodegenerative disease that destroys the nerve cells responsible for controlling voluntary muscle movements. Both upper and lower motor neurons are affected which results in paralysis and ultimately death. These nerve cells termed “motor neurons” run from the brain or spinal cord to muscles which control movements in the mouth, throat, chest, arms, and legs. In ALS patients, these cells degenerate and cause atrophy, resulting in the degeneration of the associated muscles. The disease name is derived from Greek terms where “amyotrophia,” means no muscle nourishment, “lateral” indicates the affected area of the spinal cord, and “sclerosis” means hardening of the tissue. ALS is also known as Lou Gehrig’s disease, named after the famous baseball player. According to the ALS Association, every 90 min, someone is diagnosed or passes away with ALS, which sums up to 16 people per day, 112 per week, and 5824 per year [1]. ALS has an incidence rate of 2 in 100,000 people and a prevalence rate of 4–7 in 100,000 people [2]. ALS is more common in men than in women. In the European population, ALS occurrence is

2.6–3.0 cases per 100,000 people. Countries like Uruguay, New Zealand, and the United States (US) have a higher prevalence of ALS as compared to Serbia, China, and Taiwan. ALS is expected to increase by 69% from 2015 to 2040 [3]. Every year, 6000 new ALS patients are identified in the US. The prevalence rate in the US is 5 in 100,000 people and 16,000 to 20,000 with established ALS. Moreover, ALS is more common in white males. In the US, ALS highly prevails in the Midwest and Northeast regions as compared to South and West which might be due to the greater presence of whites [4]. ALS usually occurs between the age of 40 and 70, however, the presence of ALS has also been reported in younger patients. The disease progresses aggressively with patients surviving for only 3–5 years post diagnosis [5]. The survival of ALS patients depends upon various factors. Lower survival risk is associated with older age of onset, the onset of bulbar ALS, low score of Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS or ALSFRS-R), and respiratory muscle dysfunction. Longer survival factors include younger age at disease onset, limb onset ALS, and longer diagnosis time [6–8].

The common symptoms of ALS include muscle weakening, muscle twitching, muscle cramping, muscle spasticity, muscle stiffness,

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depression, insomnia, fatigue, dysarthria, dyspnea, dysphagia, and pain [9]. Some of the clinical changes (muscle atrophy) are shown in Fig. 1. Progressive muscular atrophy chiefly affects lower motor neurons. The muscles get weak as the disease progresses. Weakening starts in the hands and spreads to other parts of the body. The sign and symptoms of ALS coexist in both upper motor neurons (UMN) as well as in lower motor neurons (LMN) (<https://www.ncbi.nlm.nih.gov/books/NBK556151/>). In ALS, the muscles weaken and waste over time and spread with the progression of the disease. The weakness starts in the distal muscles of the limbs and then in the proximal muscles [10]. Depending upon the phenotype there are 4 categories of ALS, limb onset (LO) ALS is the most dominant type and is generally present in 70% of ALS patients. Weakening and wasting of LMN are the main characteristics of this type. LO ALS progresses symmetrically from distal to

proximal muscles [11]. In this type, the involvement of other organs progresses slowly [12]. The other phenotype is known as bulbar onset (BO) ALS which is present in 25% of ALS patients and affects both upper and lower motor neurons. The patients with this form typically show dysarthria and dysphagia (difficulty in speech and swallowing) and limb impairment with disease progression [13]. Progressive muscular atrophy (PMA) is believed to be another form of ALS which extensively involves LMNs [14]. Primary lateral sclerosis (PLS) is a type of ALS which is exclusive to UMN. Disease progression is slow with no evident weight loss and no symptoms in LMN within 4 years of disease onset, that's why it is known as UMN dominant ALS [15]. The last category is ALS plus syndrome which comprises of patients having additional symptoms (such as dementia, sensory loss, autonomic dysfunction, ocular motility disturbance, extrapyramidal) to LMN and UMN disease [16].

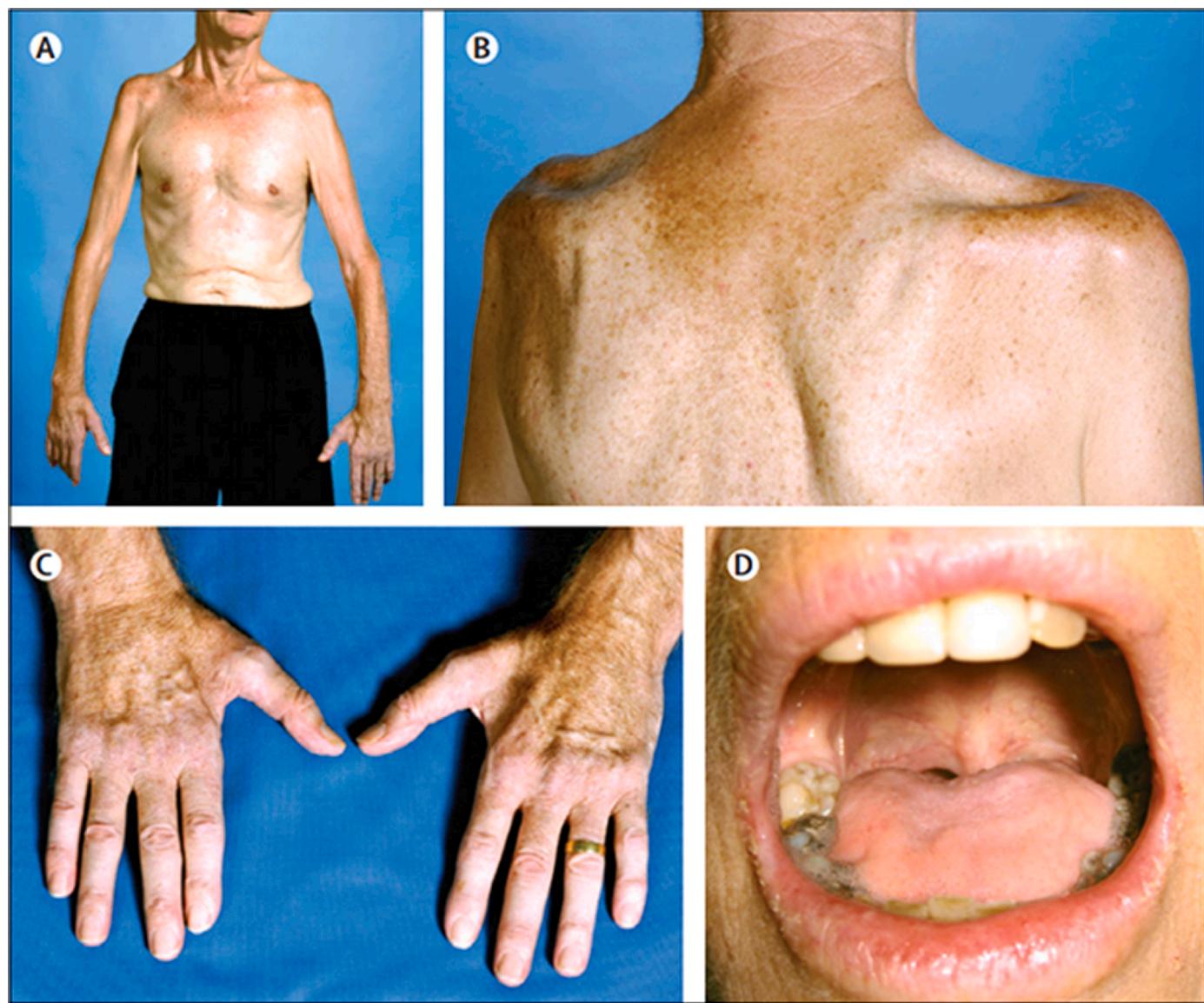


Fig. 1. Clinical features of muscles weakening in ALS patients. (A) Weakening in upper limb muscle, resulting in an inability to lift arms. (B) Weakened supraspinatus, infraspinatus and deltoid muscles leading to prominent glenohumeral joint which is prone to subluxation. (C) Weakened thenar muscles along with the first dorsal interossei, depicting "split-hand" feature of ALS which may have a corticomotoneuronal origin. Thenar muscles and first dorsal interossei may be more prone to glutamate-mediated endotoxicity as they have more extensive corticospinal connections (D) tongue muscles in bulbar-onset ALS. Wasting of tongue muscle leads to dysphagia, thus supplementary feeding is required. In Bulbar onset ALS patients, tongue is most disproportionately affected as compared to other oropharyngeal muscles. This may be due to the fact that tongue has more extensive corticospinal connections than any other muscles in oropharyngeal area which also supports the corticomotoneuronal origin theory.

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Respiratory muscle dysfunction along with BO ALS is the major cause of death as well as reduced survival in ALS patients. 50% of ALS patients also have cognitive injuries. Some ALS patients also develop fronto-temporal dementia (FTD), which gives an idea that ALS-FTD might be related as they also share the common pathophysiology [9]. ALS is a very vast topic. More than 50,000 articles are published related to the term 'ALS' when searched on PubMed. In this review, we tried to crystallize that information and present it concisely.

2. Risk factors for ALS

There are 2 forms of ALS. The predominant form is known as sporadic ALS (sALS). Most ALS cases (90%) are sporadic, the remaining 10% of cases belong to the familial ALS (fALS) type. The causes of sALS are mostly unknown while fALS usually has an autosomal dominant inheritance pattern [17]. The risk factors of ALS may be genetic or non-genetic (Fig. 2). Non-genetic factors include lifestyle risk factors such as smoking (smoking poses a higher risk of ALS in women, especially post-menopausal) [18,19], dietary factors (high antioxidant intake such as Vitamin E and polyunsaturated fatty acids relates to low risk of ALS) [20,21], body mass index (lower BMI and reducing BMI rate are prognostic factors of ALS) [22] physical fitness (football/soccer players and athletes have higher risk of developing ALS) [23], occupation (occupations where workers are exposed to various chemicals, pesticides,

metals, electromagnetic field such as carpenters, tobacco, rubber, construction, military, farm, electrical, leather, power plant workers, laboratory technicians, nurses, painters welders, veterinarians, herdsman etc.) [24], environmental factors (viruses, toxicants) [25], pesticides (organochlorine derivative, herbicide, fumigants, pyrethroids are associated with ALS) [26,27], viruses (HHV6, HHV8, HIV, HTLV1, HERV-K), and medical conditions (head trauma, metabolic diseases, cancer, neuroinflammation) [24].

The main culprit genes involved in ALS pathogenesis are superoxide dismutase 1 (SOD1), TAR DNA Binding Protein (TARDBP/TDP43), fused in sarcoma (FUS), Ubiquilin2 (UBQLN2), and C9ORF72. In addition to these, various other genes (almost 20) are also known to be involved in the generation of ALS. Of all the cases of fALS, 20% are due to SOD1 mutation, 4–5% are due to TDP43 & FUS mutation, and 30% are due to C9ORF72 mutation. Based on the gene mutation, ALS is subdivided into genetic subtypes [28] and their frequencies in fALS are mentioned in percentages [5,29]. ALS1/SOD1 (20%), ALS2/ALSIN, ALS4/Senataxin (SETX), ALS5/spastacsin (SPG), ALS6/FUS (5%), ALS8/vesicle-associated membrane protein-associated protein B (VAPB), ALS9/angiogenin (ANG) (<1%), ALS10/TDP43 (5%), ALS11/FIG4, ALS12/optineurin (OPTN) (4%), ALS14/valosin containing protein (VCP) (1–2%), ALS15/ALSX/UBQLN2 (<1%), ALS16/SIGMAR1, ALS17/charged multivesicular body protein 2B (CHMP2B), ALS18/profilin 1 (PFN1) (<1%), ALS19/receptor tyrosine-protein kinase erbB 4 (ERBB4),

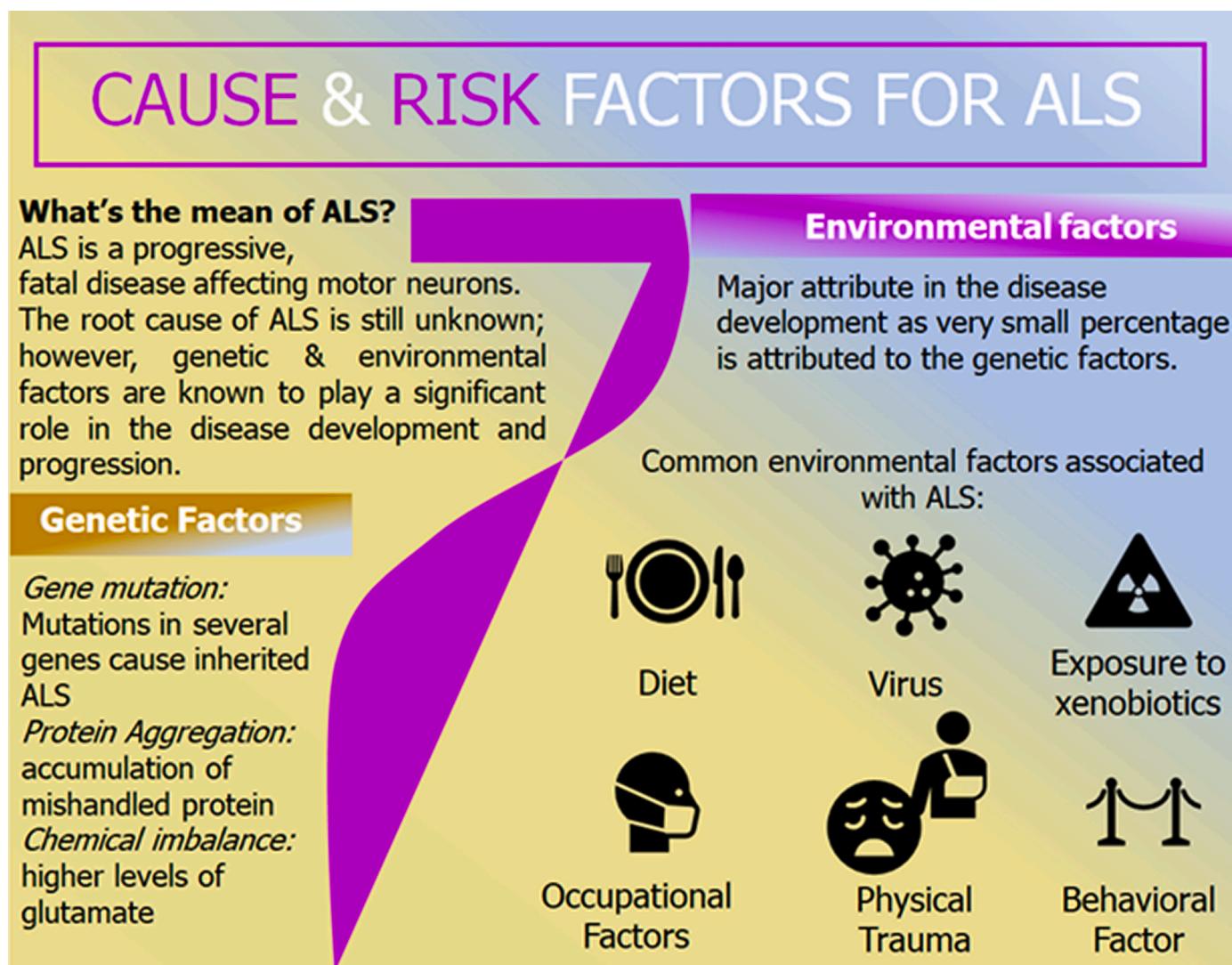


Fig. 2. Pictorial representation of the causes and risk factors associated with ALS. The factors are categorized in two categories: Genetic and Environmental.

ALS20/heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) (<1%), ALS21/matin 3 (MATR3)(1–2%), ALS22/tubulin α 4A (TUBA4A)(1%), ALS23/ANXA11 (1%), ALS24/NEK1 (1–2%), ALS25/KIF5A, ALS-FTD1/C9orf72 (30%), ALS-FTD2/coiled-coil-helix-coiled-coil-helix domain-containing 10 (CHCHD10)(2%), ALS-FTD3/sequestosome 1 (SQSTM1) (<1%), and ALS-FTD4/serine/threonine-protein kinase TBK1 (TBK1)(3%) [28,30]. In most cases of sALS, occurrence have an unknown cause, however, some genes are known to be involved such as SOD1, TDP43, FUS, and C9ORF72 [31]. In some cases, there is an interaction between genetic and non-genetic factors leading to the development of ALS [24]. The common genetic mutations that are reported in sALS are single nucleotide polymorphism (SNP), polyglutamine repeats (PolyQ), the abnormal copy number of genes, deletion, and insertion [30].

Genome-wide association studies suggest that non-coding chromosomal regions also contribute towards the development of ALS [32]. It is well recognized that a large part of human DNA is non-coding and any mutation occurring in non-coding regions can affect biological fitness [33]. Regions of non-coding DNA control the expression of coding genes such as enhancers. Enhancers regulate gene expression via the binding

of transcription factors (TFs) [34]. DNA looping enables the physical interaction between enhancer and promoter [35]. A study conducted by Cooper-Knock et al. in patient-derived cells reported a valid ALS-associated genetic variation within enhancer regions of caveolin1/caveolin 2 (CAV1/CAV2) (CAV1 and CAV2 has a major role in the organization of intercellular signaling) and coding regions of CAV1 leading to reduced expression of CAV1/CAV2 resulting in neurodegeneration [36]. The most common cause of fALS and FTD is C9ORF72 which has the mutation (hexanucleotide repeat expansion) in the non-coding region [37]. Patients with different ethnicities exhibit phenotypic variability. A large-scale genetic association study conducted among Japanese ALS patients revealed the presence of a functional SNP in the enhancer region of ZNF512B at chromosome 20q13 [38]. Mutation resulted in the reduced expression of ZNF512B which in turn downregulated TGF- β signaling. In general, activated TGF- β promotes neuronal survival, therefore its deficiency leads to neuronal degradation [39].

3. Pathophysiologies underlying ALS

To develop an efficient therapy, it is important to know the

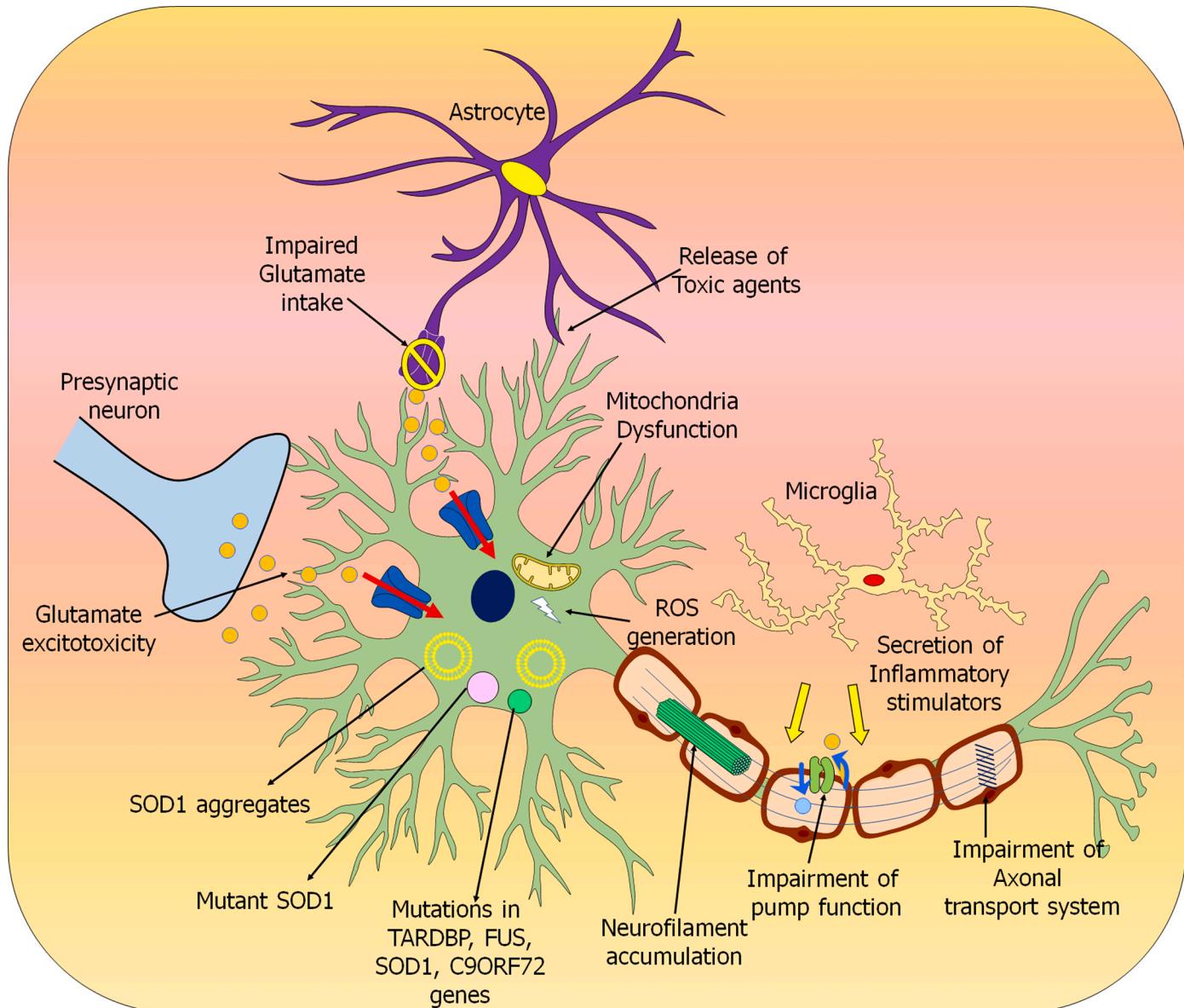


Fig. 3. Common pathophysiologies underlying ALS.

molecular mechanism that results in motor neuron degeneration. An overview of ALS pathophysiologies has been beautifully sketched in the video by nature (<https://www.youtube.com/watch?v=xrljFVMLiOQ>). SOD1 mutations were linked to the pathogenesis of ALS for almost two decades, however, neurodegeneration/neurotoxicity following SOD1 mutation's mode of action is unclear. Based on several hypotheses it is concluded that ALS is a multifactorial disease, and several mechanisms collectively contribute to the neurodegeneration underlying ALS [40]. Some common pathophysiology underlying ALS are summarized in Fig. 3 and are discussed below.

3.1. Mitochondrial dysfunction

In the spinal motor neurons and skeletal muscle of both ALS patients as well as in ALS murine models, mitochondrial changes are observed such as a change in morphology (vacuolation, dilation, distorted cristae & membrane, discontinuous network, swelling) [41–43]. SOD1 protein is present in the intermembrane space and matrix of mitochondria [44]. The abnormal deposition of mutant SOD1 interferes with the normal physiological function of mitochondria in cell metabolism including unusual ATP (Adenosine triphosphate) & ROS production, impaired energy & calcium homeostasis, altered apoptosis, and mitochondrial transport along the axons [45–48]. Differential protein expression is also associated with mutant mitochondria [49–51]. Mutant SOD1 on the surface of mitochondria might restrict the protein import as most of the functional proteins required by mitochondria are synthesized in the cytoplasm and then imported to the mitochondria [52].

3.2. Glutamate excitotoxicity

Glutamate is an excitatory neurotransmitter that is produced in the presynaptic terminal from where it diffuses and activates specific receptors and triggers an action potential. The glutamate concentration is tightly regulated in the synaptic cleft to avoid excitotoxicity. The clearance of glutamate is achieved by glial and neuronal cell transporter protein and excitatory amino acid transporters [53]. Overactivation of glutamate receptors might result in the degeneration or death of the neurons involved [54]. Elevated levels of intracellular calcium prompt enzymatic and mitochondrial damage leading to reactive oxygen species (ROS) generation and activation of various devastating biochemical pathways that establish neuronal degeneration [55,56]. To prevent neuronal damage, the glutamate level should be regulated. In the nervous system, isoform 2 of the astroglial glutamate transporter (EAAT2) maintains the glutamate levels below the excitotoxic level. Reduced EAAT2 levels are observed in the spinal cord and motor cortex of ALS patients and SOD1 mouse models. However, loss of functional EAAT2 is not the principal cause of motor neuron degeneration in ALS [57].

3.3. Oxidative stress

Oxidative stress occurs when the generation of ROS is more than the cell could remove, resulting in the accumulation of ROS which could potentially damage cell structures and macromolecules like DNA/RNA and protein. The enzyme majorly responsible for the prevention of oxidative damage as well as reduction of superoxide leakage from mitochondria is SOD1. Mutated SOD1 has altered activity which causes cytotoxicity. Previously it was known that mutant SOD1 leads to complete protein loss however, newer studies revealed that mutant SOD1 proteins were active and stable, thus, promoting neuronal apoptosis [58–60]. This suggested that ALS involves both losses of function and gain of toxic function of SOD1. Mutant SOD1 can produce toxic superoxides by extracting electrons from other antioxidants and contributing them to molecular oxygen [61,62]. Cerebrospinal fluid, serum, and urine samples of ALS patients exhibit enhanced levels of free radicals and oxidative damage [13].

3.4. Protein aggregation

Protein aggregation is a hallmark of various neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's, and ALS. Aggregates form when misfolded proteins oligomerize and get accumulated, thus gaining overall toxic functions [63]. Mutated SOD1 protein-rich, inclusions are observed in the tissues of ALS patients (both sALS and fALS) as well as in the mouse model of SOD1 (mutant) [41]. The mutated proteins escape the degradation due to their aberrant accumulation. Along with SOD1, TDP43 and FUS mutated proteins also form aggregates. TDP43 is a nuclear protein and plays an essential role in transcription, splicing, mRNA transport, and DNA damage prevention [64]. 80% of ALS cases are positive for TDP43 inclusions. Mutated TDP43 mislocalizes to the cytoplasm which is due to the mutation in the 3' UTR region of the gene [65,66]. Similar to TPD43, FUS is also a nuclear protein but mislocalizes to the cytoplasm which might be due to the mutation affecting their nuclear import [67]. The protein aggregates also include chaperones, mitochondrial proteins, ubiquitin, and neurofilaments, thus resulting in the impairment of their normal functions leading to more and more accumulation of protein and death of motor neurons [57].

3.5. Prion propagation

The implication of protein aggregate formation is loss of function as well as gain of toxic function. Along with these concepts, an emerging facet of aggregate-induced toxicity is the damage of neighboring cells through the prion-like mechanism. Prion disease is characterized by seeding and self-propagation, similarly, ALS initiates at a specific location and then spreads in an orderly manner [68,69]. TDP43, as well as FUS, is known to have prion-like domains thus allowing their toxic aggregates to enter neighboring cells. Several criteria are to be fulfilled, before considering ALS a prion-like disease. Firstly, the misfolded proteins should have properties of seeding and self-aggregation which are quite fulfilled for SOD1, TDP43, and FUS [70–73]. Secondly, the protein inclusions should be able to transmit to neighboring cells which was well observed in the case of TDP43 in cultured cells [74]. Thirdly, the propagation does not necessarily require the cell-to-cell contact. In NSC34 cells, mutant SOD1 is secreted extracellularly and transported to spinal neurons via exosomes where it induces the death of motor neurons [75,76]. Whether FUS and C9ORF72 qualify for the prion-like molecules is still unknown [77].

3.6. Endoplasmic reticulum (ER) stress

ER is the site for protein (membrane and secretory) synthesis and folding with the aid of various chaperones, foldases, and cofactors expressed in the ER. These factors ensure the normal folding and prevent the aberrant aggregation of proteins. The ER also serves as a calcium reserve and site for lipid synthesis. Stress conditions may interfere with the normal functioning of this organelle and induce ER stress [78]. Aggregation of misfolded proteins, increases protein burden prolonging ER stress which drastically affects cell survival in neurodegenerative diseases [79]. ER stress activates the unfolded protein response (UPR) pathway that increases the protein folding capacity and quality control mechanism of ER [80]. ER stress in ALS has been confirmed from the studies conducted on postmortem tissues of ALS patients. The studies reveal the activation and upregulation of UPR signaling pathways, ER chaperones, and cell death signals associated with ER stress [81–83]. ER has a physical and functional connection with the mitochondria. Both are commonly involved in calcium homeostasis and the synthesis of lipids. Mitochondrial dysfunction is one of the pathophysiologies involved in ALS pathogenesis. Studies show that ER stress and mitochondrial dysfunction are connected as there is a crosstalk between both the organelles which ultimately leads to neuronal degeneration [84].

Sigma-1 receptors (Sig-1Rs) are transmembrane proteins present in

ER and act as chaperones. Sig-1Rs are widely expressed in motor neurons. Mutation in Sig-1R (E102Q) has been reported in juvenile ALS [85]. Neura2A cells with Sig-1R-E102Q mutation exhibit reduced mitochondrial ATP production disrupted mitochondrial structure and enhanced neuronal death via ER stress. The development of mitochondrial stress leads to neuroinflammation as mitochondrial-dependent signaling controls the innate and adaptive immune response [86]. Accumulated Sig-1Rs were found in the C terminal and ER structures of motor neurons in ALS patients and the SOD1 mice model. Knockouts of the Sig-1R gene in ALS mouse model suggest its role in the pathological progression of ALS. Similarly, slow ALS progression was observed after administration of Sig-1R agonist pRE-084. This indicates that Sig-1R is not only involved in ALS progression but can also serve as a potent target for ALS therapy [87,88].

3.7. Autophagy dysregulation

Accumulation of protein inclusions and dysfunctional organelles (such as mitochondria) is the signature of many neurodegenerative diseases which might be toxic to the cells. Removal of these aggregates might be a good therapeutic intervention [89]. Cells have two major clearance pathways, autophagy, and the ubiquitin-proteasome system (UPS) that prevent the accumulation of the aberrant proteins and establish homeostasis in eukaryotes. UPS clears the short-living proteins and autophagy removes long-living, aberrant proteins and is also important for organelle turnover [90]. Autophagosomes are double bilayered vesicles containing the substrate to be degraded [91]. The degradation is achieved by its fusion with the lysosome. Mutation in two, autophagy essential genes resulted in the development of neurodegenerative phenotype in mice, which suggests that the role of autophagy in neurons is very important [92,93]. Motor neurons of fALS patients and mutant SOD1 mice exhibit a high number of autophagosomes [94–97]. Autophagy regulatory genes SQSTM1 and UBQLN2 were found to be mutated in both sALS and fALS patients [98–101].

3.8. Accumulation of neurofilaments

Aggregated and mislocalized, phosphorylated, accumulation of neurofilaments (NFs) are the hallmark of the pathogenesis underlying ALS. NFs are mostly found in the cytoskeletal components of large, myelinated axons. NFs have 3 components, light, medium, and heavy subunits (NF-L, NF-M, NF-H). NF-L is responsible for filament assembly and NF-M & NF-H are responsible for link formation with other NFs in the axon [102]. Abnormal phosphorylation occurs in the NFs in ALS conditions, which results in the altered axonal transport of NFs leading to their accumulation in cell bodies and proximal axons [103]. In ALS, altered stoichiometry (upregulation/downregulation) of NFs leads to their accumulation [104]. SOD1 (mutant) mouse model overexpressing NF-L subunit shows the aberrant accumulation of NFs, on the other hand, overexpression of NF-L and NF-H subunits slowed the progression of ALS in the mouse models [105,106]. NFs disorganization is involved in the pathogenesis of ALS, however, the correlation between NF accumulation and neurodegeneration is not very clear.

3.9. Neuroinflammation

Neuroinflammation in ALS is characterized by the presence of activated microglia, astrogliosis, and cells of the immune system at the neuronal injury site [107]. There is a regulated communication between motor neurons and glial cells, any miscommunication may compromise neuronal homeostasis and endurance. Mutation in the SOD1 gene in only motor neurons was insufficient to develop the disease in ALS mouse models which indicated the involvement of non-neuronal cells. Mutated (SOD1) glial cells neighboring normal motor neurons developed a pathological phenotype whereas the reverse of it (normal glial cells neighboring mutated motor neurons) did not develop the diseased

phenotype [108]. At the initial phase of ALS, glial cell, and motor neuron communication exhibits neuroprotection but when the damage of motor neurons becomes worse, motor neurons along with astrocytes start releasing misfolded proteins and toxic molecules, leading to microglial activation. Thus, this release of misfolded proteins and toxic molecules switches the initial relationship between the glial cells and motor neuron from one that was anti-inflammatory and neuroprotective to one that is pro-inflammatory and neurotoxic [109,110]. Astrocytes reside in CNS and make the majority of glial cell components. Astrocytes maintain low glutamate levels in the synaptic cleft mediated by EAAT2 receptors. In ALS conditions, astrocytes downregulate the EAAT2 transporter which results in less removal of glutamate leading to excitotoxicity [111]. Astrocyte activation in ALS leads to the down-regulation of neurotrophic factors and overexpression of neurotoxic factors [112].

3.10. Protein homeostasis

Proteome integrity and homeostasis are essential for the normal functioning of any cellular system. Besides other pathophysiolgies contributing to ALS, perturbation of protein homeostasis by environmental, infectious, or endogenous factors also contributes to the ALS complexity. Proteins like TDP43, SOD1, and FUS are highly prone to aggregation and form insoluble aggregates which lead to dysregulation of protein homeostasis failing synaptic plasticity, and general synaptic function maintenance. The aggregated proteins lose their normal functionality and adversely affect other processes (in response to neurophysiological and environmental triggers), they are involved in [113]. Fig. 4 represents the consequences of protein homeostasis dysregulation.

3.11. RNA metabolism

Mutations influencing the risk of ALS and/or FTD occur in some RNA binding genes, such as TAR DNA binding protein (TARDBP), FUS RNA-binding protein (FUS), TATA-box binding protein associated factor 15 (TAF15), EWS RNA-binding protein 1 (EWSR1), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), HNRNPA2B1, Ataxin 2 (ATXN2), and TIA1 cytotoxic granule associated RNA-binding protein (TIA1) [114]. Mutations in TARDBP and FUS lead to the formation of aberrant stress granule, pathogenic RNA foci, aberrant nucleocytoplasmic shuttling, etc. [115,116]. Mutation in the C9ORF72 gene (GGGGCC hexanucleotide repeat expansion in the first intron region of the chromosome 9 open reading frame 72) is very common in ALS and FTD patients. Normal individuals have 2–23 repeats whereas ALS/FTD patients have hundreds to thousands of copies [117]. The toxicity induced by repeat expansion is directly proportional to age, repeat length, and degree of expression. Induced pluripotent stem cell (iPSC)-derived neurons from ALS/FTD patients and motor neurons of C9orf72-ALS patients exhibit RNA foci enriched with RNA-binding proteins [114]. This suggests that the RNA foci might have trapped the bound RNA-binding proteins, resulting in toxicity (splicing defects, dysregulated RNA metabolism) [118]. Accumulation of non-AUG (RAN) repeat-associated proteins is observed in the brain and spinal cord of C9orf72 mutation carriers. These exhibit various toxic properties such as impaired nucleocytoplasmic transport which may serve as a driving force for neurodegeneration [117].

4. ALS pathogenesis controversy: the origin of disease

According to Charcot, ALS occurs concomitantly in upper and lower motor neurons, however, the origin of ALS is of great controversy to date. Unveiling this controversy will enable the scientists to have a better understanding of the disease which could be applied towards the diagnostic and therapeutic interventions. There are two proposed hypotheses: 'dying-forward' and 'dying-back' (Fig. 5). According to the dying-forward hypothesis, ALS mainly occurs in corticomotor neurons

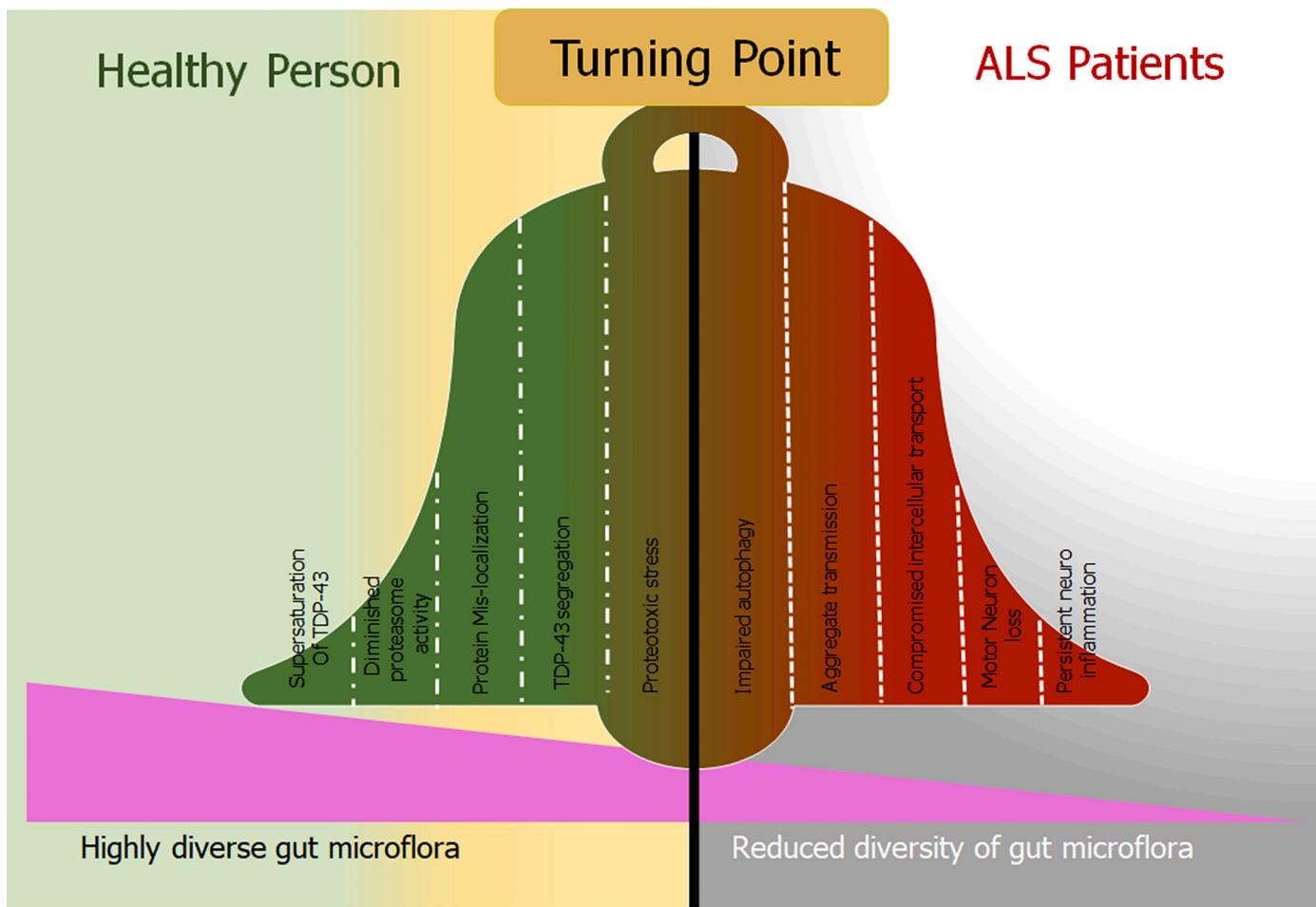


Fig. 4. Dysregulation of protein homeostasis and its consequences.

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that connect to anterior horn cells monosynaptically, thus, mediating degeneration of anterior horn cells through glutamate excitotoxicity. The dying-forward hypothesis was supported by magnetic stimulation studies done in ALS patients which showed that sALS patients exhibited cortical excitability as an early feature and fALS patients exhibited cortical excitability before the clinical onset [119]. Other clinical observations supporting this hypothesis are, motor neurons lacking monosynaptic connection with corticomotor neurons (oculomotor, abducens, and Onuf's nuclei) are spared in ALS, lack of naturally occurring ALS animal models poses scarcity of corticomotoneuronal-anterior horn cell connections, absence of pure LMN forms of ALS [6].

According to the dying-back hypothesis, ALS initiates within muscle cells or at the neuromuscular junction. To be more specific, this hypothesis states that neurotrophic factors are scarce. This hypothesis is supported by observations showing the occurrence of synaptic denervation before anterior horn cell degeneration and evidence of accumulation of aberrant SOD1 protein in Schwann cells which might lead to synaptic denervation. Due to these contrasting hypotheses, it is also proposed that UMN and LMN degeneration occurs independently [56].

5. Biomarkers

ALS biomarkers have grabbed immense attention in the past 20 years. Identification of sensitive and specific biomarkers will provide a better understanding of disease to clinicians and researchers, which in turn can help in, improved designing of clinical trials, development of novel biotherapeutics, and improvement of the overall health of ALS patients. Extensive research is available on the biomarkers but only a

few validated biomarkers are available due to the limitations of different methodologies adopted, non-standardized procedures, smaller sample size, and lack of longitudinal studies. For a biomarker to be validated, there is a need to overcome these limitations. Biomarkers could be classified into different categories [120] for ALS. Systemic prognostic biomarkers include body weight (malnutrition) [121] and respiratory function (respiratory insufficiency) [122]. Cerebrospinal fluid (CSF) biomarkers which include neurofilament proteins (accumulation) [123,124], Tau (increased expression) [125,126], proteomics (increased neurofilament complement C3, secretogranin I, Chitinases and glutamate receptor 4; decreased cystatin C), metabolomics (low acetate; high pyruvate and ascorbate), oxidative stress biomarkers (increase in mutated SOD1, reduced Glutathione), biomarkers of neuroinflammation (IL-10, IL-6, GM-CSF, IL-2, IL-15, CHIT-1 and C3, IL-17, bFGF, VEGF, MIP-1 β , MIP-1 α , MCP-1 β , IFN- γ , ollistatin, IL-1 α , and kallikrein-5), C9ORF72 (misfolded) [120], and MicroRNAs (upregulation of miR-338-3p & miR181a-5p and downregulation of miR21-5p & miR15b-5p) [127,128]. Blood biomarkers include C9ORF72, SOD1, TDP43, DNA methylation (increased), neurofilament proteins (high), and inflammatory markers include high CD3+, CD4+, CD8+, CD4+CD28+, CD3+CD56+ T-cells, CD8+CD45RA+ naïve T cells, TNF- α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, and IL-13; low IFN- γ . Muscle denervation biomarkers include Lower serum creatinine. Metabolic biomarkers include low glucose, glutamine, uric acid, high glutamate, ferritin, low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (apoB), and apoB/apoA-I ratio. Urine biomarkers include high 8-hydroxy deoxyguanosine (8-OHdG), F2-isoprostan, extracellular domain of neurotrophin receptor p75 (p75ECD), low Collagen type 4,

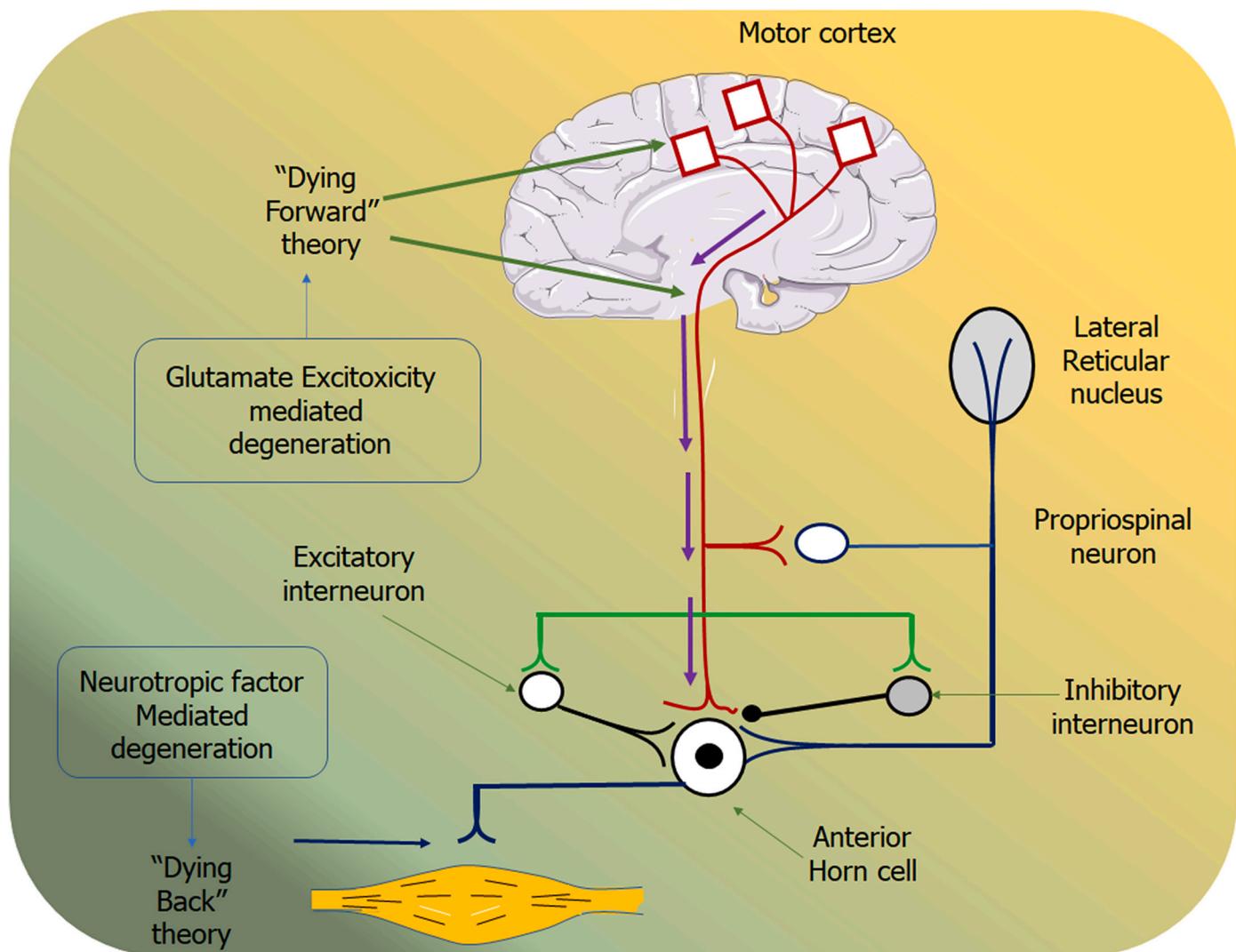


Fig. 5. Demonstration of dying-forward and dying-back hypothesis.

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glucosylgalactosyl hydroxylysine. Imaging biomarkers depict anatomical and pathological changes and processes *in vivo* and includes Magnetic resonance imaging (MRI), Diffusion tensor imaging (DTI), combination of MRI & DTI, Magnetization Transfer Imaging (MTI), Functional MRI (fMRI), Magnetic resonance spectroscopy (MRS), Muscle imaging & spectroscopy and Positron emission tomography (PET). Electrophysiology biomarkers include motor unit number estimation (MUNE), neurophysiological Index, Axonal Excitability, Electrical impedance myography (EIM), and transcranial magnetic stimulation (TMS) [120].

The gut microbiome plays a pivotal role in health and diseases, and studies have been conducted to link gut microbiome abnormality and ALS [129]. Gut microbiome analysis is an emerging biomarker study in ALS. Every human body harbors (nose, eyes, mouth, throat, gastrointestinal tract (GIT), and skin) almost trillions of bacterial cells which are more than the human cells altogether. Generally, these bacteria benefit (produce vitamins, help in digestion, maintain the immune system, and protect from other harmful pathogens) the body unless there are other factors involved such as stress, viral infection, chronic disease, and/or immunocompromised body [130]. The GIT microbiota is the most diverse one. It was already known that the brain regulates gut function, however, now it is known that it goes the other way round too. The Human Microbiome Project establishes the fact that the microbiome, the

gut, and the brain communicate via immunological (cytokines), endocrine (hypothalamic–pituitary–adrenal axis), and neural (vagus) pathways. Recent studies show that there is a link between the gut microbiome and the neurodegeneration process [131]. In ALS, gut microbiota and metabolites via the gut-brain axis, play a pivotal role in its pathogenesis [132,133]. NGS analysis shows that cyanobacteria are present in ample amounts in ALS patients and play a key role in neurodegenerative diseases pathogenesis, especially ALS. The cyanobacteria produce β-Methylamino-L-alanine (BMAA) and BMAA is found to be in higher concentrations in the brain of patients with ALS/PDC (ALS combined with PD and dementia) [134]. The presence of BMAA in the brain and CSF of ALS and AD patients suggests that cyanotoxins may promote the progression of neurodegenerative diseases [135–138]. Apart from this, cyanobacteria also produce neurotoxins, such as saxitoxin (which causes paralysis of voluntary muscles), microcystin (toxic for the brain), and nodularin (damages cytoskeleton). Some intestinal bacteria that have an immunomodulatory function are imbalanced such as Verrucomicrobia phylum, Verrucomicrobiaceae family, and the Akkermansia genus. Higher Akkermansia microbial groups are found in ALS, which contributes to the inflammatory condition in ALS. Lower Verrucomicrobia microbial group in ALS patients contributes to immunocompromise. The higher abundance of Enterobacteriaceae leads to intestinal inflammation. High fecal Enterobacteriaceae and low levels

of a brain-derived neurotrophic factor in the blood were found in ALS patients. ALS patients have a lower number of Eubacteriaceae members which could be linked to reduced short-chain fatty acids production, thus, lower energy of the host [139]. These changes in gut microbiome architecture can be used as a potential biomarker for the early diagnosis of ALS [140]. Also, a deep understanding of the connection of the gut microbiome in the development of various neurodegenerative diseases could provide alternative strategies to treat such diseases by the introduction of probiotics or microbial transplantation [141].

ALS is characterized by the generation of stress granules. Aggregation of non-translating mRNAs and RNA binding proteins leads to the formation of stress granules. Aggregation of misfolded and mislocalized proteins increases the cellular stress which results in the change in the composition of extracellular vesicles (EVs). The stress granule proteins are packaged in EVs (such as exosomes) and released in the extracellular environment [142]. Exosomes are small membranous EVs, cup-like nanovesicles, 30–200 nm in diameter secreted by cells [143]. SOD1, TDP43, C9ORF72, and FUS proteins form aggregates in the cytoplasm and form stress granules. SOD1, TDP43, C9ORF72, and FUS are secreted in exosomes suggesting a prion-like spread of these misfolded proteins [144,145]. EVs are continuously released in biofluids like blood and CSF (cerebrospinal fluid), carrying the biomolecular signature (nucleic acid/proteins) depicting the physiological state of the source cell, thus, making them an attractive choice to be used as biomarkers [144]. Exosomes derived from CNS can cross BBB (Blood Brain Barrier) and enter systemic circulation, thus, being easily detected in blood, CSF, and urine [146].

P75ECD is a biofluid (urine) based biomarker for ALS that is gaining the attention of researchers. Elevated levels of p75ECD are found in the urine of ALS patients as compared to healthy controls and other diseases (Parkinson's and Multiple Sclerosis) [147]. To evaluate the progression of p75ECD a follow-up study was conducted in ALS patients, sampled a median of 2-time points over 2–3 years. The results show an increase in p75ECD over time and suggest disease progression [148]. p75ECD is the only biofluid-based biomarker that reflects the disease progression. Therefore, it has the potential to be used as a prognostic as well as a progressive biomarker for ALS [148]. A study conducted by Jia R et al. reports elevated levels of urinary p75ECD in Chinese ALS patients [149]. The development of p75ECD as a progressive biomarker will enable its

use in clinical trials. The effectiveness of a treatment regimen against ALS can be evaluated based on its ability to increase, decrease, or stabilize the urinary p75ECD levels over time [150]. Advantages of urinary biomarkers over other biomarkers are its easy accessibility [151] as well as cost-effectiveness as compared to imaging biomarkers [149]. P75ECD is more specific and sensitive as compared to neuro-imaging markers such as MRI [152].

6. Diagnosis of ALS

ALS diagnosis is the major challenge in ALS patients. ALS is not diagnosed in the early stages due to the slow progression of the disease or due to the presence of other neurological comorbidities. 7–8% of ALS cases are misdiagnosed because of the ALS mimicking syndromes. Therefore, it is a prerequisite to rule out other ALS mimicking syndromes to correctly diagnose ALS. There are 3 principles on which ALS diagnosis is based, malfunctioning of a particular body part, the manifestation of LMN and UMN signs in one or more segmental anatomic areas, and malfunction progression. If these requirements are not fulfilled, then the diagnosis might be doubtful and needs to be revised [153]. ALS diagnosis starts with the evaluation of medical history, physical examination, electromyography (EMG), and neuroimaging (Figs. 6, 7) [10]. EMG is used to identify the ALS mimicking diseases as well as the loss of motor neurons [153]. With the help of neuroimaging, (brain and spinal cord) structural lesions affecting the motor system can be eliminated. Biomarkers play a key role in the diagnosis and prognosis of ALS. Some markers (such as phosphorylated neurofilament heavy subunit in cerebrospinal fluid) may provide clues of ALS onset even in the initial phase when there are no clear symptoms of UMN disease but only recent onset of muscle weakness. Genetic testing of the most common mutated genes in ALS (C9orf72, SOD1, TDP-43, FUS, TBK-1) may also be used to diagnose ALS [10].

7. Therapeutic options

There is no definite treatment available for ALS. The available therapies aim at managing the symptoms and providing palliative care [154]. The symptoms that are managed medically include painful muscle spasms, hypersalivation, pseudobulbar affect, cognitive

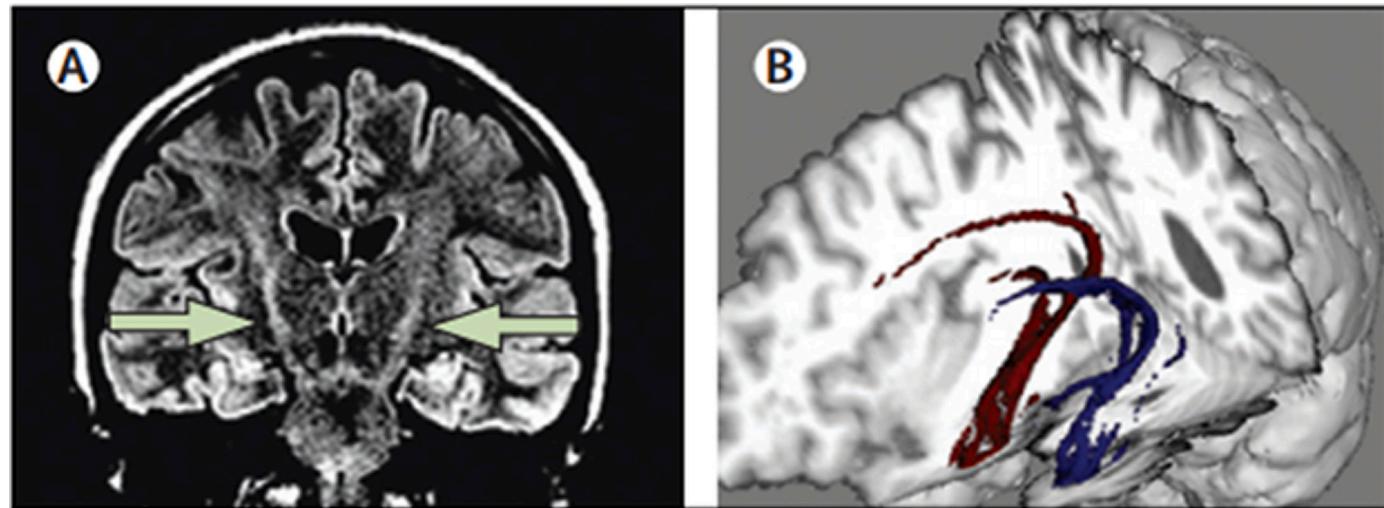


Fig. 6. Standard v/s investigational MRI images (A) coronal view (arrows) shows hyperintense corticospinal tracts in ALS patients observed by T2-weighted fluid attenuated inversion recovery (FLAIR) sequence. (B) Diffusion tensor tractography image of superior oblique cut-out brain section (viewed from left) shows lesser white matter projection fibers on left (blue) side as compared to the right (red) side in an ALS patient with unusual phenotype such as prominent aphasia and refurbishment of the temporal lobe white matter projection fibers.
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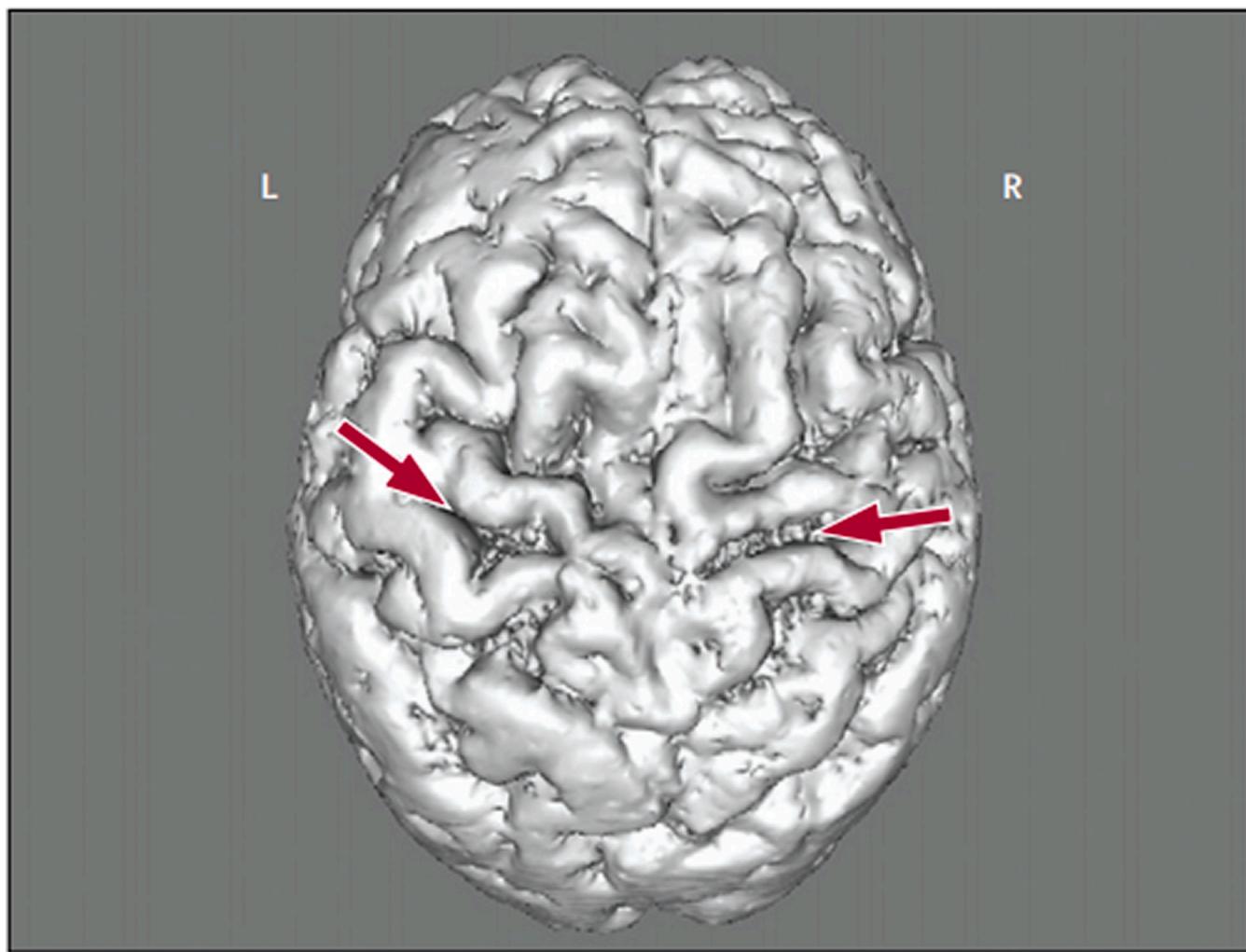


Fig. 7. Top view of a patients' (primary lateral sclerosis) brain as observed by 3D MRI. Arrows indicate the broadened precentral sulci and atrophy in the adjacent gyri (motor strips). Macroscopic atrophy is a rare feature of typical ALS but projects the late stage of a corticomotoneuronal process that is hypothesized to be a characteristic of ALS.

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impairment, and depression. Other interventions include ventilation, percutaneous endoscopic gastrostomy, and counseling of patients as well as family members for palliative care [155]. Fig. 8 illustrates the pathophysiology of neuronal injury and the possible therapies that might be used against it. Some of the therapies are discussed briefly below.

7.1. Natural compounds

Oxidative stress plays a major role in the development and progression of neurodegeneration in ALS. Mutations in SOD1 (antioxidant enzyme-superoxide dismutase) are results in oxidative stress in ALS. The mutation of this gene results in structural instability which leads to misfolding and mislocalization of the protein. The protein forms cytotoxic aggregates (alone or with other proteins). This enzyme is lost with disease progression, leading to ROS generation, which affects vital cellular processes and causes mitochondrial dysfunction. Due to the influence of oxidative stress in the development and progression of ALS, natural compounds with antioxidant properties draw attention towards their exploration in the treatment of neurodegenerative disorders. Epidemiological study data shows that the incidence rate of neurological disorders significantly reduces among the ethnic group intaking an

antioxidant-rich diet. Some of the natural compounds are listed in Tables 1 & 2 below [156].

7.2. Genetic therapy

7.2.1. Genome editing based approach

The genome editing-based therapeutic approach is an innovative approach to treat ALS as it could be used very precisely to correct the genetic mutations that are causative of the disease. The homology-directed repair (HDR) approach was used to correct the variety of SOD1, FUS, and TARDBP mutations in ALS patient-derived induced pluripotent stem cells (iPSCs). The improved cellular phenotype in motor neurons derived from gene-corrected iPSCs was observed, however, the efficiency was low (less than 1%) [167]. CRISPR/Cas9 nuclease-based approach was also used to knock out the mutant SOD1 gene in a mouse model of ALS, but this approach could not differentiate between the wild type and the mutated type SOD1 gene and targeted the gene universally. Such therapy could lead to dangerous SOD1 downregulation. Loss of SOD1 activity has been reported to cause motor activity loss in infants. The CRISPR-based approach was also used to delete the expanded repeats of the C9ORF72 gene. This approach also caused the deletion of repeats in wild-type genes, but there was no negative

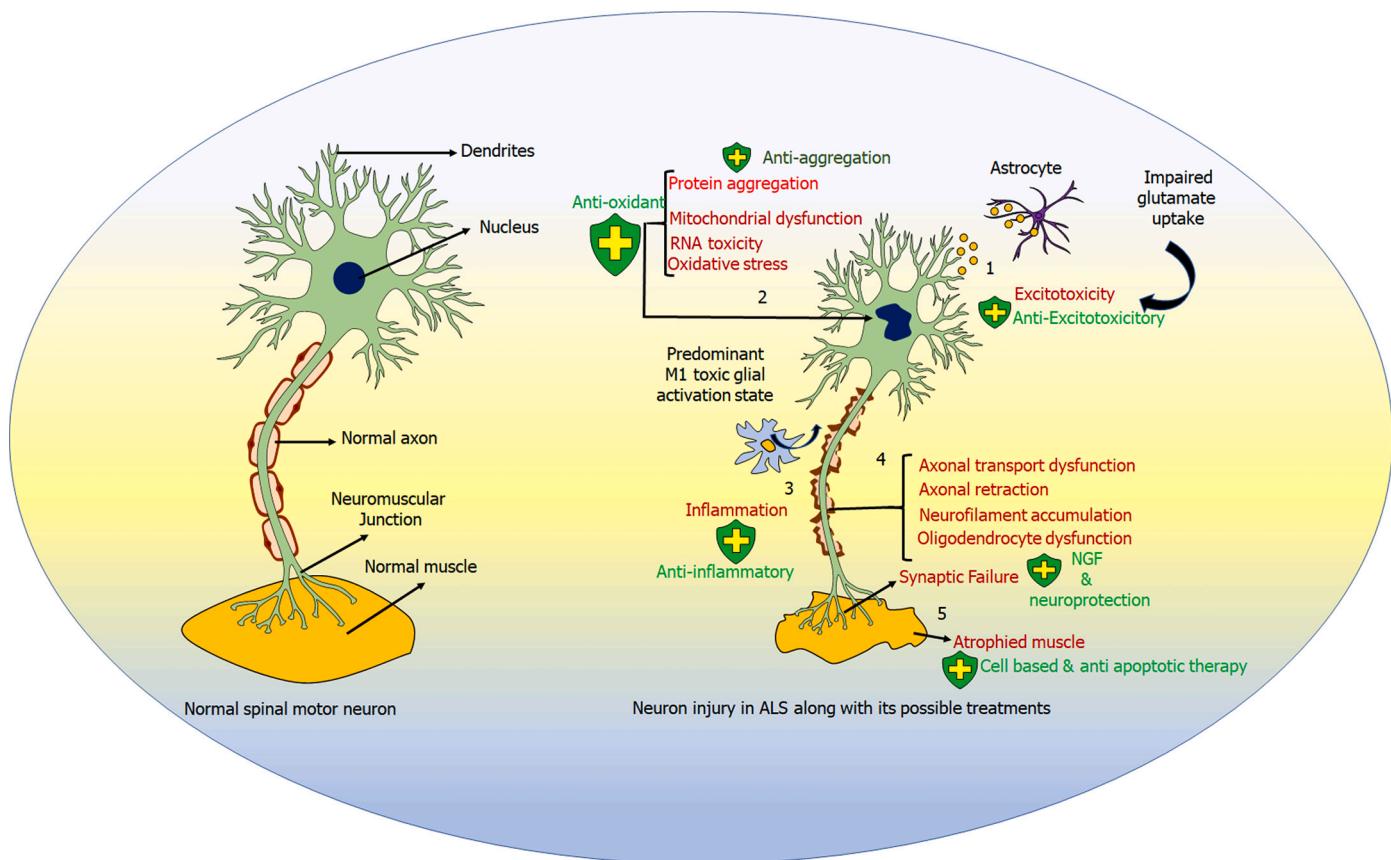


Fig. 8. Schematic representation of healthy spinal cord motor neuron and ALS affected spinal cord motor neuron along with the main approaches to ALS treatment. The therapies are mainly based on pathophysiological models of the disease. The groups include anti-apoptotic, anti-inflammatory, anti-excitotoxicity, antioxidant, anti-aggregation, neurotrophic growth factor, and neuroprotective approaches.

outcome to this. Also, a correction in C9orf72 promoter methylation was observed along with reduced excitotoxicity in motor neurons. In an HDR-based approach, a donor template containing normal expands of C9orf72 was introduced in ALS iPSCs. This approach also targets wild-type alleles, but the advantage is that it avoids the generation of deletions of variable degrees. The motor neurons derived from these corrected iPSCs exhibited reduced RNA foci and aggregation. The motor neurons also showed improvement in cell survival and response to cellular stress. The major challenges of the genome editing-based approaches are limited editing efficiency, and poor delivery of CRISPR/Cas-9 based enzymes. The major advantage of this approach is that it could be a one-time treatment [168].

7.2.2. Gene therapy

RNA silencing (miRNA and siRNA) based strategies are of great interest to limit the course of the pathology of ALS. For siRNA-mediated gene therapy, a lentivirus carrying siRNA (specific against SOD1-mRNA) is used. When the mouse model carrying the mutated SOD1 gene was injected (muscle or spinal cord) with this virus, it showed controversial results for the slowing of disease progression and survival. In conclusion, this approach could be beneficial for fALS, but not for sALS [169].

In the SOD1 rat model of ALS, the introduction of antisense oligonucleotide against SOD1-mRNA led to decreased SOD1 mRNA/protein concentration in the spinal cord and prolonged survival [170]. Phase I clinical trial with intrathecal administration of antisense oligonucleotide on a small group of patients showed that it was safe and well-tolerated [171]. One disadvantage with antisense technology is that it cannot cross the BBB, therefore, to overcome this, it should be delivered directly to the central nervous system. Another disadvantage is that this

treatment requires multiple doses. Gene therapy can be very successful, but it needs to be optimized [57].

7.3. Pharmacological therapy

7.3.1. FDA approved drugs

There are only two FDA-approved drugs (Table 3) for ALS treatment, Rilutek (riluzole) and Edaravone [172]. Riluzole ($C_8H_5F_3N_2OS$) was the first FDA-approved oral formulation used to treat ALS in 1995. It is a glutamate antagonist i.e., it inhibits the release of glutamate by inactivating sodium channels, thus, resulting in the inhibition of the downstream processes triggered by the binding of glutamate to the excitatory amino acid receptors. The approval of riluzole was based on two studies where survival increased by 2–3 months [173]. Later, studies suggested that the survival increases by 6–7 months. A Cochrane review of 4 randomized trials suggested the increase in survival by 1 year in 9% of the trial group. In the current scenario, the patient database shows even more promising benefits of this drug, which ranges from 6 to 21 months [155,174]. Riluzole is more effective in patients with bulbar onset. Patients treated with riluzole exhibited less muscle strength deterioration. The side effects of riluzole include asthenia, spasticity, mild increase in the aminotransferase level and drug-related withdrawal rate was significantly higher among the study group [175].

Radicava (Edaravone) is a free radical scavenger and has a protective effect on neurons. Edaravone is a member of the substituted 2-pyrazoline-5-one class ($C_{10}H_{10}N_2O$) which is provided as a colorless liquid sterile injection solution supplied for intravenous (IV) infusion. It is proposed that its neuroprotective role might be due to its ability to inhibit the opening of the mitochondrial permeability transition pore (mPTP) in the brain along with its radical scavenging property [176]. In

Table 1

List of some natural compounds along with their roles towards neuroprotection.

S. no	Category	Compounds	Role	Source
1	Alkaloids	Nicotine, Morphin, Caffeine, 8-methoxy-N-methylflindersine, Zanthodioline, Alternamide A, Alternamide B, Alternamine A, Hydroxytyrosol, Formamide, Alternamine B, Uridine, Mitrphylline, Isomitrphylline, Iraquiine, and Kareemine	Free radical scavenging activity.	Animals (prosobranch), bacteria, plants (<i>Zanthoxylum rhoes</i> , <i>Alternanthera littoralis P. Beauv</i> , <i>Uncaria tomentosa</i> , <i>Alphonsea cylindrical King</i>), and fungi
2	Flavonoids	Flavin, anthocyanin, kaempferida, kaempferol, fisetin, compound 22, 7,8 dihydroxy flavone, myricetin, quercetin, naringin, and 3,5,4'-trihydroxy-6,7,3'-trimethoxy flavone	Decreased spinal cord injury, SOD1 inhibition, inhibition of motor neuron aggregation, inhibition caspase3, and decrease reactive oxygen species.	Strawberry, Brazilian green propolis, <i>Panax ginseng</i> , <i>Godmania aesculifolia</i> and <i>Tridax procumbens</i> , <i>Achillea fragrantissima</i>
3	Coumarins	Scopoletin, trigocoumarin, esculetin, coumarin, fraxetin, methyl-3-coumarin, caraganolide A, psoralen, isoporalen (36), esculin, hinokitol, felopterin and auraptene	Antioxidant, reduction of (1,1-diphenyl-2-picryl-hydrazil) DPPH, inhibition of induced peroxidation, reduction of glutathione-induced death, and induction of neuroprotection	<i>Canarium patentinervium</i> , <i>Trigonella foenum-graecum</i> , <i>Ganoderma lucidum</i> , <i>Amburana cearenses</i> , <i>Angelicae dahuricae radix</i> , <i>Angelica gigas</i> , <i>Caragana turfanensis</i> , <i>Ducrosia ismaelis asch</i>
4	Tannins		Antioxidant activity	<i>Sida cordifolia</i> , <i>Evolvulus alsinoides</i> , <i>Cynodon dactylon</i> , <i>Terminalia chebula</i> (combreteaceae), <i>Spatholobus suberectus</i> (SSE), <i>Uncaria rhynchophylla</i> (URE), <i>Alpinia officinarum</i> (AOE), <i>Drynaria fortune</i> (DFE), <i>Crataegus pinnatifida</i> (CPE), <i>Solanum macrocarpon</i> , <i>Glaucium acutidentatum</i> , <i>Tripterygium wilfordii</i> , <i>Asparagus adscendens</i> , <i>Tussilago farfara</i>
5	Terpenoids	Celastrol, ECN (7β-(3-ethylcis-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-Z-notonipetranone)	Antiamnesic, and antioxidative activities	
6	Lignans	Sesamine	Peroxidation of dismutase-SOD1, antioxidative activities	<i>Sesamum indicum</i> L., <i>fagara</i> , <i>Broussonetia papyrifera</i>
7	Quinones	Anthraquinones (emodin), dianthraquinones (aloe-emodin), benzoquinone	Antioxidant effects	<i>Rheum officinale</i> baill, <i>Myrsine</i>
8	Saponins	Madecassocide, ginsenosideo, and astragalosideo IV	Cell survival, improving neurite growth and recovering the activities of axons and synapses.	<i>Centella asiatica</i> (L.) Urban, <i>Panax ginseng</i> , <i>Astragalus membranaceus</i>
9	Methylxanthines	Caffeine, theophylline, and theobromine	Protection against excitotoxicity,	<i>Camellia sinensis</i> L., <i>Coffea</i> sp., <i>Theobroma cacao</i> L.
10	Glucosinolates	Glucomoringin (GMG)	Immunomodulatory, anti-inflammatory, antioxidant, and antiapoptotic	<i>Moringa oleifera</i> , <i>Brassicaceae</i> , <i>moringaceae</i>
11	Fatty acids	Omega-3, omega-6, triheptanoin, and docosahexaenoic acid (DHA)	Delays the loss of motor neurons and the appearance of motor symptoms, anti-inflammatory	Food

preclinical studies, SOD1 rodent models of ALS showed improved motor function, slower disease progression, and reduced motor neuron degeneration [177,178]. Edaravone was approved by the FDA in May 2017 and is currently being used for the treatment of ALS in Japan and South Korea. An oral formulation of edaravone in Phase I clinical trial showed positive results, meaning it's well tolerated when taken orally and is safe. Two clinical trials, Phase I & Phase III are in progress for the pharmacokinetic evaluation of a single dose of edaravone (oral) given to ALS patients with gastrostomy and evaluation of long term safety & tolerability of edaravone (oral) in ALS patients over 24 to 48 weeks [179].

7.3.2. Investigational drugs

With a better understanding of the disease pathology and pathophysiology, many potent therapeutics are under development that particularly target oxidative stress, neuroinflammation, mitochondrial dysfunction, apoptosis, glutamate excitotoxicity, localization of proteins, DNA damage, RNA metabolism, etc. Various clinical trials are underway for new treatment molecules. Table 4 tabulates some investigational drugs that are currently ready for the phase 3 trial [172,175,180–182].

7.4. Stem cell therapy

Stem cell therapy is emerging in the field of ALS. Stem cells can self-renew, inhabit damaged sites, differentiate in response to external stimuli, and stimulate tissue repair and regeneration. Commonly used stem cells to treat neurological disorders are embryonic stem cells (ESC),

neural stem cells (NSC), mesenchymal stem cells (MSC), and induced pluripotent stem cells (iPSC) [183]. The stem cells may be exploited for their ability to release trophic factors and to reduce neurotoxic particles, thus, offering neuroprotection. Out of various stem cells, MSCs are of most interest [184]. Their main source includes skeletal muscle, placenta, umbilical cord, blood, and adipose tissue. MSC derived from adipose tissue (ASC) is gaining interest as it could be extracted in large amounts from liposuction facilitating autologous transplantation. The main advantages of MSCs are that they can be safely isolated, simply expanded in vitro, differentiated to neural-like, glial-like and astrocytic-like cells (both in vivo and in vitro) [185,186], and be less susceptible to tumorigenicity, thus, do not require immunosuppressive treatment. Following transplantation and migration, MSCs are drawn towards the damaged site and increase the release of neurotrophic factors [187]. Some of the completed clinical trials are listed in Table 5. Stem cell therapy is mostly used to generate cell type releasing the neurotrophic factors that improve the survival of degenerating neurons. Stem cells could be differentiated into motor neurons in vitro, but whether these motor neurons (when transplanted in vivo) would be able to reinnervate the appropriate target, establish physiologically functional synapses, send axons through inhibitory white matter and direct axons to target muscles to retain neuromuscular function remains a challenge and requires further research [188].

7.5. Extracellular vesicles

In multicellular organisms, intercellular communication plays a key role. The communication could be direct or mediated via chemical

Table 2

Clinical and preclinical status of antioxidant therapy (natural/semi-synthetic) for ALS.

S. no	Antioxidant	Structure	Source	Experimental models	Pharmacological effect	Reference
1	Vitamin E or alpha-tocopherol PubChem ID: 14985		Pomegranate, wheat germ and raspberry seed oils, plant oils PMID: 31202300	SOD1G93A transgenic mice	Disease progression: slow Disease onset: delayed	[157]
2	N-Acetyl-L-cysteine (NAC) or Acetylcysteine PubChem ID: 12035		Onion (<i>Allium cepa</i>) PMID: 31035402	SH-SY5Y cells with SOD1G93	Survival time: unaffected mROS: reduced	[158]
				SOD1G93A transgenic mice	ATP levels: increased Viability: increased Survival time: prolonged	[159]
3	2-[mesityl(methylamino)-N-[4-(pyridin-2-yl)-1H-imidazol-2-yl]acetamide trihydrochloride (WN1316) PubChem ID: 87057556		Acylaminimidazole derivative PMID: 24498180	SOD1H46R and SOD1G93A transgenic mice	Motor performance: improved Disease progression: alleviated	[160]
4	Dimethoxycurcumin PubChem ID: 9952605		A synthetic analog of curcumin PMID: 27550987	NSC-34 cell lines transfected with M337V or Q331K mutant TDP-43	Mitochondrial dysfunction: improved	[161]
5	CDDO-EA or (2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid) ethylamide PubChem ID: 44159258		Synthetic triterpenoid PMID: 26443840 PMID: 20809391	SOD1G93A transgenic mice	At presymptomatic age Motor performance: enhanced	[162]
6	CDDO-TFEA or (2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid) trifluoroethylamide PubChem ID: 17756663		Derivative of morphine PMID: 27757015	Fibroblasts from ALS patients	Survival time: prolonged At symptomatic age Disease progression: slow	[163]
7	S (+)-apomorphine or (6As)-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[e,g]quinoline-10,11-diol PubChem ID: 736083		Combination of 1-(β-D-ribofuranosyl) nicotinamide chloride & 3,5-dimethoxy-4'-hydroxy-trans-stilbene PMID: 30668199	RCT: NCT03489200	Disease progression: slow	[164]
8	EH301		Different dietary sources PMID: 28387721	SOD1G93A transgenic mice	Oxidative stress: reduced Survival: improved	[165]
9	Melatonin PubChem ID: 896		Apocynum cannabinum (Canadian hemp) PMID: 10599557	MO59J glial cells and SH-SY neuronal cells overexpressing mutant SOD1	Survival time: prolonged O2-levels: decreased Viability: increased	[166]
10	Apocynin or acetovanillone PubChem 2214					

messengers such as growth factors, chemokines, and cytokines. However, a new cellular communication mechanism has emerged which involves the intercellular transfer of extracellular vesicles (EVs). EVs act as vehicles to transfer proteins, nucleic acids, and lipids between cells. Based on their size, EVs are classified into two types, microvesicles (150 nm to 1000 nm) and exosomes (30 nm to 100 nm). Microvesicles usually contain proteins associated with lipid rafts and cholesterol, sphingomyelin, ceramide, and phosphatidylserine [189]. Exosomes usually carry proteins, lipids, and functional RNA, particularly mRNAs and microRNA (miRNAs) [190]. Exosomes are smaller in size and can cross

the blood-brain barrier (BBB). In a study, NSC-34 cells (motor neuron-like cell line) were transiently transfected with different SOD1 point mutations and H₂O₂ was also introduced (as a pathological insult). These ALS mimics were then given exosomes isolated from Adipose-derived Mesenchymal Stem Cells (ASC). These models exhibited increased ALS motor neuron survival which might be due to the counteraction of the apoptotic pathway [191]. Another study showed that the ASC-derived exosomes in ALS reduced mutant SOD1 aggregation and restored mitochondrial function [192]. Neuroinflammation is a common feature underlying neurodegenerative diseases. Exosomes

Table 3

FDA-approved drug riluzole & edaravone with their respective clinical trials and their status.

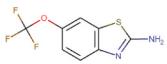
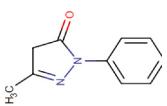
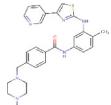
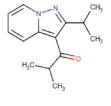
S. no	FDA approved drug	Structure	Accession ID	Pharmacology	Clinicaltrials.gov identifier	Status of clinical trial
1	Riluzole		PubChem ID: 5070 Drug Bank ID: DB00740	Glutamate antagonist (receptors, glutamate)	1) NCT00542412 2) NCT0086166 3) NCT02238626 4) NCT01378676 5) NCT01486849 6) NCT01709149 7) NCT02238626 8) NCT01378676 9) NCT01486849 10) NCT01709149 1) NCT04745299 2) NCT03127267 3) NCT03693781 1) NCT03039673 1) NCT03679975 1) NCT03457753 1) NCT01492686 2) NCT00424463 3) NCT00415519 4) NCT00330681 5) NCT04254913 6) NCT04176224 1) NCT04165824 1) NCT04577404 2) NCT04569084 3) NCT04259255 1) NCT04391361	Completed Recruiting Active not recruiting Terminated Withdrawn Completed Active not recruiting Recruiting Not yet recruiting
2	Edaravone		PubChem ID: 4021 Drug Bank ID: DB12243	Free radical scavenger	1) NCT01492686 2) NCT00424463 3) NCT00415519 4) NCT00330681 5) NCT04254913 6) NCT04176224 1) NCT04165824 1) NCT04577404 2) NCT04569084 3) NCT04259255 1) NCT04391361	Active not recruiting Recruiting Not yet recruiting

Table 4

Investigational drugs in clinical trials.

S. no	Investigational drug/therapy	Structure	Accession ID	Pharmacology	Clinicaltrials.gov identifier	Status of clinical trial
1	Masitinib		PubChem ID: 10074640 Drug Bank ID: DB11526	Tyrosine-kinase inhibitor	NCT03127267	Recruiting (phase 3)
2	Tofersen	Not available	PubChem ID: 381128150 Drug Bank ID: DB14782	Antisense Therapy (pharmacology is unknown)	NCT02623699	Active not recruiting (phase 3)
3	Ravulizumab (monoclonal antibody)	Not available	PubChem ID: 363669767 Drug Bank ID: DB11580	Long-acting complement 5 (C5) inhibitor	NCT04248465	Active not recruiting (phase 3)
4	Ibudilast (MN166)		PubChem ID: 3671 Drug Bank ID: DB05266	Phosphodiesterase (PDE) inhibitor and macrophage migration inhibitory factor (MIF) blocker	NCT02714036 NCT04057898 NCT02238626	Completed Recruiting (phase 2b/3) Completed

reduce the number of activated inflammatory microglial cells, support oligodendrocytes, and protect neurons, thus, exhibiting their anti-inflammatory role. Exosomes are transported rapidly to the brain when injected intranasally and are taken up by microglial cells [193,194]. Exosomes have one advantage over most cells and drugs, their ability to freely cross the BBB.

7.6. Physical therapy

Multidisciplinary treatment for ALS could provide a better outcome for treating ALS patients which might include a combination of pharmacology, neurorehabilitation, and symptomatic treatment [195]. Physical exercise may help in correcting the physical posture abnormalities, reduce pain & stiffness of muscles, and promote functional

independence. It also helps the patients to train so that falls could be prevented and to re-educate walking (with or without technical aid). Physical exercise should be started before the patient's muscles lose a significant amount of strength and should be practiced continuously throughout the disease. Clinical trials based on the physical exercise treatment show improvement in trained muscles of ALS patients such as lower limb training of ALS patients resulted in increased strength in action, like knee extension. Although physical exercise slowed down the muscle deterioration in ALS patients, it did not improve survival. ALS patients are recommended specific exercises with only moderate-intensity & low frequency (two sessions in a week) for the best outcome (improvement) and to prevent fatigue [196].

Table 5

List of MSCs in the clinical trials to treat ALS.

S. no	Title	Treatment	Administration	Summary	Phase/no. of participants	Identifier
1.	Effect of intrathecal administration of hematopoietic stem cells in patients with amyotrophic lateral sclerosis (ALS)	Autologous hematopoietic stem cell	Intrathecal	Autologous cell therapy in patients with ALS can stimulate neuroplasticity, modifying the neurodegenerative process and stops the clinical progression of the disease.	Phase 2 Phase 3 14 participants	NCT01933321
2	Human neural stem cell transplantation in amyotrophic lateral sclerosis (ALS)	Human neural stem cells	Intra-spinal cord	To verify the safety and tolerability of expanded human fetal neural stem cells	Phase 1 18 participants	NCT01640067
3	Clinical trial on the use of autologous bone marrow stem cells in amyotrophic lateral sclerosis (extension CMN/ELA)	Autologous bone marrow stem cells	Intraspinal and intrathecal	Safe and feasible procedure	Phase 1 Phase 2 63 participants	NCT01254539
4	Compassionate treatment: an exploratory clinical trial to assess treatment of amyotrophic lateral sclerosis	Autologous adipose-tissue derived stem cells	Brain transplantation of ADSCs combined with intravenous infusion ADSCs	The purpose of the study is to determine the safety and possible effectiveness of autologous adipose-tissue derived stem cells.	Phase 1 1 participant	NCT02383654
5	Escalated application of mesenchymal stem cells in amyotrophic lateral sclerosis patients	Autologous Mesenchymal stem cells	Intrathecal	To confirm the safety of escalated MSCs therapy in ALS patients.	Phase 1 3 participants	NCT02987413
6	A dose-escalation safety trial for intrathecal autologous mesenchymal stem cell therapy in amyotrophic lateral sclerosis	Autologous mesenchymal stem cells	Intrathecal	To determine the safety of intrathecal delivery of autologous mesenchymal stem cells (MSCs) to the cerebrospinal fluid (CSF) of patients with ALS using a dose-escalation study.	Phase 1 27 participants	NCT01609283
7	Repeated mesenchymal stem cell injections in ALS	Mesenchymal stem cells (MSC)	Intrathecal	To evaluate the safety and efficacy of repeated intrathecal administrations of autologous bone marrow-derived mesenchyme stem cells	Phase 1 Phase 2 20 participants	NCT04821479
8	Autologous cultured mesenchymal bone marrow stromal cells secreting neurotrophic factors (MSC-NTF), in ALS patients.	Mesenchymal bone marrow stromal cells	Intrathecal Intramuscular	evaluate the safety, tolerability, and therapeutic effects (preliminary efficacy) of injection of autologous cultured mesenchymal bone marrow stromal cells secreting neurotrophic factors (MSC-NTF)	Phase 1 Phase 2 12 participants	NCT01051882
9	Autologous multipotent mesenchymal stromal cells in the treatment of amyotrophic lateral sclerosis (AMSC-ALS-001)	Autologous mesenchymal stromal cell (AMSC)	Intrathecal	To assess the safety and the efficacy of autologous multipotent mesenchymal stromal cells	Phase 1 Phase 2 26 participants	NCT03828123
10	Intravenous transplantation of mesenchymal stem cell in patients with ALS	Bone marrow-derived mesenchymal stem cell	Intravenous	To evaluate the safety of intraventricular injection of bone marrow-derived mesenchymal stem cell	Phase 1 6 participants	NCT01759797
11	Intrathecal transplantation of mesenchymal stem cell in patients with ALS	Autologous bone marrow-derived mesenchymal stem cell	Intrathecal	To evaluate the safety of intraventricular injection of bone marrow-derived mesenchymal stem cell	Phase 1 8 participants	NCT01771640
12	A study to evaluate transplantation of astrocytes derived from human embryonic stem cells, in patients with amyotrophic lateral sclerosis (ALS)	Astrocytes derived from human embryonic stem cells	Intrathecal	The study hypothesis is that transplantation of astrocyte cells can compensate for the malfunctioning of patients' astrocytes by restoring physiological capabilities like the reuptake of excessive glutamate, reducing oxidative stress, reducing other toxic compounds, as well as by secreting different neuroprotective factors	Phase 1 Phase 2 16 participants	NCT03482050
13	Phase 2, randomized, double-blind, placebo-controlled multicenter study of autologous MSC-NTF cells in patients with ALS (NurOwn)	Autologous MSC-NTF cells	Intramuscular combined with intrathecal	To evaluate the safety and efficacy of a single combined intramuscular and intrathecal administration of MSC-NTF cells in early-stage of ALS	Phase 2 48 participants	NCT02017912
14	CNS10-NPC-GDNF for the treatment of ALS	Human neural progenitor cells secreting glial cell line-derived neurotrophic factor (CNS10-NPC-GDNF)	Unilateral lumbar spinal cord injections	To examine the safety of transplanting cells that have been engineered to produce a growth factor	Phase 1 18 participants	NCT02943850
15	Safety and efficacy of repeated administrations of NurOwn® in ALS patients	MSC-NTF cells	Intrathecal	To evaluate the safety and efficacy of repeated administration of NurOwn (MSC-NTF cells) therapy	Phase 3 261 participants	NCT03280056

7.7. Symptomatic therapies/treatment

Symptomatic treatments are very important in the management of ALS [28]. These treatments may not only alleviate the symptoms but

may also improve the overall quality of life and survival in ALS patients. Some of the symptomatic treatments along with their remedies include: disabled and weak patients may use orthotics, adaptive aids and undergo physiotherapy; for dysphagia patient may consult a speech

therapist/dietician, safe swallowing technique and modified diet might be adopted or gastrostomy tube might be inserted if necessary; for dyspnea and poor cough, ventilatory support, morphine or benzodiazepines, chest physiotherapy, suction machine and/or manually assisted coughing techniques might be used; to relieve pain, physiotherapy, NSAIDs, muscle relaxants (baclofen, botulinum toxin), anticonvulsants (e.g., gabapentin), re-positioning and pressure area care, opioid drugs, pressure-relieving cushions and mattress might be used; for dysarthria, speech therapist might be consulted, communication aids may be helpful; for cognitive changes, the family members & caregivers must be explained the symptomatology, antidepressant therapies may be given; for sialorrhea, anticholinergic antidepressants (e.g., amitriptyline), anti-cholinergic drugs (e.g., glycopyrronium bromide), botulinum toxin injections, radiation of salivary glands, mouth-care products, and suction may be used. For thickened saliva natural remedies (e.g., papaya), adequate hydration, saline nebulizers, suctioning of the mouth, and mouth care might be used; for emotional support and fighting depression/anxiety, ALS patients and their caregivers may be counseled, medicines such as amitriptyline, benzodiazepines, dextromethorphan hydrobromide/quinidine sulfate might be used; for constipation, dietary changes (e.g., increase fluid and fiber intake) may be advised [6].

7.8. Alternatives and off-label treatments (AOTs)

People with ALS often look for alternatives and off-label treatments (AOTs) on the internet. The evidence provided for the efficacy and safety of AOTs is mostly inaccurate and vague. To help ALS patients and their families wisely decide on these, internet-based AOTs, ALSUntangled was created in 2009. ALSUntangled systematically reviews AOTs, categorizes them based on mechanistic plausibility, preclinical models, cases, trials, and risks, and grades them from A (best) to F (worst). U means no disclosable proof was found for AOT in that category. Final grading is sourced from a team of 100 clinicians and scientists across 11 countries [197]. Details of AOTs can be found at <https://www.alsuntangled.com/>. However, as ALS is a deadly disease and there are not much therapeutic options available, therefore, AOTs may be considered after weighing their pros and cons.

8. Scope of nanoparticles in ALS

8.1. Diagnostic

Currently, the disease diagnosis is based on laboratory and neuro-imaging tests. To improve the disease outcomes, it is necessary to achieve an early diagnosis for which the drugs or immune modulators must cross the blood-brain barrier (BBB) and enter the central nervous system (CNS). BBB restricts the entry of low molecular weight compounds and biomacromolecules (such as imaging contrast agents, drugs, nucleic acids, proteins), thus posing a significant hindrance in the development of effective and safe methods of treatment and diagnosis. To overcome this, scientists have come up with strategies involving nanomedicines or nanoparticles for safe, site-specific, and effective drug delivery for therapeutic and diagnostic purposes [198]. Nanoparticles of iron oxide, cerium oxide, and zinc oxide could be developed as an imaging as well as a therapeutic tool. Due to their magnetic properties, they are very suitable for magnetic resonance imaging (MRI). The passage through BBB can be achieved by applying an external magnetic field before systemic injection. The application of a magnetic field can also guide the nanoparticles to the target site. Based on the particle size, iron oxide nanoparticles are categorized into ultrasmall superparamagnetic iron oxide (USPIO) (10–50 nm diameter) and superparamagnetic iron oxide (SPIO) (50–150 nm diameter). MRI generally uses USPIOS and modified SPIOs are generally used for regenerative functions [199]. ALS is characterized by protein misfolding and aggregation (SOD1, TDP43, FUS, etc.), thus nanoparticles could be developed to identify and quantify the ubiquitin-positive aggregates in motor neurons [200]. Quantum dots

(QDs) are fluorescent semiconductor nanocrystals, chemically as well as photo stable, that emit a fluorescent wavelength as per their size which is 2–10 nm in diameter. The metalloid crystalline core is composed of cadmium selenium (CdSe) and the shell is composed of zinc sulfide (ZnS), enhancing its solubility in water. To increase their physiological function QDs are coated with a ligand [199]. QDs are conjugated with anti SOD1 antibodies to detect SOD1 aggregates and their subcellular localization [201]. Gold nanoparticles (GNPs) are biocompatible, photostable with superior optical properties, thus could be used as excellent probes to monitor biological assembly as well as its mechanism. Free SOD1 and GNP-tethered SOD1 in aqueous solution form aggregates in response to an aggregation promoting environment, therefore, the dynamic changes in interparticle distances at different stages of protein aggregation enable GNPs to act as colorimetric reporters of SOD1 aggregates without any instrumentation. GNPs associated with protein aggregates result in decreased distance between NPs resulting in the change in color of the solution, leading to estimation of presence as well as the extent of aggregates by the naked eye [202,203]. SOD1 has a ubiquitous distribution in a cell such as a cytosol, nucleus, peroxisomes, lysosomes, and intermembrane space of mitochondria and it is evident that the SOD1 species interacts with the cell membranes. Lipid membranes are used as a model system to study the interaction of aggregated SOD1 with the membrane. Supported lipid bilayer (SLB) is prepared on a gold substrate, then, treated with SOD1 aggregate solution, and is further analyzed by surface plasmon resonance spectroscopy (SPR) and atomic force microscopy (AFM). SPR angles measure the change in surface coverage of the remaining SLB as the SPR angular shift is directly proportional to the surface coverage. AFM imaging gives the idea of defect formation in SLB by aggregated SOD1. In conclusion, lipid vesicles used as a mimicked cell system for studying protein-cell membrane interactions show that the membrane integrity and permeability were dependent on the type (normal/aggregated) of protein as well as on the amount of aggregated protein [200].

8.2. Therapeutic

Various therapeutic options have been explored to date to combat ALS. The therapies must be targeted to the CNS, however, there are major challenges faced by researchers when it comes to delivering therapeutics and diagnostics to CNS. To achieve effective delivery of therapeutics and diagnostics to CNS, it is a prerequisite to understanding the major challenges that come in the way of it [204]. The major challenges include the blood-brain barrier (BBB)/blood-spinal cord barrier (BSCB) (generally allows the nutrients and small lipid-soluble molecules and limits the pathogens or toxins) [205,206], biostability & bioavailability (might be due to low aqueous solubility, too lipophilic) [207,208], systemic distribution and clearance (due to systemic distribution or rapid clearance of the drug from system results in less drug reaching the target, therefore more dose is required, non-targeted distribution leads to systemic toxicity) [209–212].

Nanomedicine is the new emerging technology that has very promising outcomes. There are several FDA-approved nanomedicines (for different diseases) in the past few decades that have shown more efficacy as compared to their parent drug formulation. This suggests that similar approaches can be used in the treatment of ALS [213]. Nanocarriers offer a great opportunity to directly deliver drugs to CNS, overcoming the BBB along with minimizing non-targeted systemic distribution [198]. Nanomaterials that are evaluated for their potential of effective drug delivery include liposomes, nanoparticles, polymeric micelles, nanogels, nanofibers, nanotubes, and dendrimers. Few details of these are mentioned in Table 6 [198]. The three mechanisms by which nanoparticles overcome BBB/BSCB are absorptive-mediated transport, receptor-mediated transport, and cell-mediated transport [214]. Nanoparticles can be introduced non-invasively (coated with ligands) or invasively (direct intrathecal or intracranial injection). The use of nanoparticles can confer advantages such as overcoming BBB/

Table 6

List of various nanoparticles and their advantages that could be exploited for effective drug delivery in ALS.

S. no	Nanomaterial	Size	Composition	Advantages
1	Liposomes	(<10 nm)	Outer lipid bilayer Aqueous core	Extended circulation time Reduced drug side effects Enhanced therapeutic effect
2	Nanoparticles	100 to 200 nm	Insoluble polymer(s)	Efficient cell uptake Increased dispersion stability Extended circulation time
3	Polymeric micelles	10 to 100 nm	The core of hydrophobic polymer blocks The shell of hydrophilic polymer blocks	Prevents premature drug release and degradation No untargeted delivery Can incorporate solutes (different structures) High loading capacity
4	Nanogels	20 to 200 nm [215]	Cross-linked polymers that often combine ionic and non-ionic chains	Very high loading capacity Form stable dispersions Decreased uptake in liver and spleen Increased brain uptake
5	Nanofibers (NF)/ Nanotubes (NT)	100 to 300 nm for NF [216] 1 to 100 nm for NT [217]	Carbon vapor-grown from peptide amphiphiles or electrospun from polymer materials	Nanofibers are: Safer to manufacture Less risk of air pollution (Than nanotubes)
7	Dendrimers	1.1 to 12.4 nm [218]	Branched polymers	Can be formulated non-covalently with biological agents Can be used in drug therapy as well as molecular imaging

BSCB, increasing bioavailability, thus increasing drug exposure time, controlled drug release, targeted drug delivery to specific cell and specific location, real-time tracking & imaging. Some of the emerging nanotechnologies that have the potential to be utilized for ALS drug delivery are glycosylated nanocarrier, virus mimicking nanomaterials, and exosomes [204].

9. Controversies in preclinical and clinical trials in ALS

Preclinical: The most used animal model to investigate potential neuroprotectants in ALS is the SOD1 mouse model, but SOD1 mutation accounts for only 2% of ALS cases, therefore the use of this model universally is not relevant. Recent TDP43 mutant mouse models provide scientists with a better platform to identify novel treatment strategies [219,220]. The other controversy is regarding the dose and the timing of the dose. The effect of pre-symptomatic delivery of drugs on disease onset has been investigated by researchers, however, it may also contribute towards the understanding of other overlapping diseases involving motor neuron degeneration. Many preclinical studies used

ultra-high doses of drugs that might not be tolerated by human patients. Assuming, that the highest tolerable dose will produce the best outcome is not correct [221,222].

Clinical: Effective screening of the pharmacological drugs is very crucial at the phase 2 trial as phase 3 trials are decisive, as in whether to continue with confirmatory testing or to reject it considering ineffective. In the case of ALS, the preliminary evidence of drug efficacy cannot be proven in phase 2 which might be due to the absence of an effective biomarker. This ultimately led to the dilemma of whether to minimize the duration/sample size of the trial (using efficient statistical methods) so that phase 3 trials could be initiated (high false-positive rate) or to proceed with phase 3 trials designed as phase 2 trials (high false-negative rate) [223]. Phase 3 clinical trials are being carried out even if the preliminary evidence of efficacy in human patients is absent. Furthermore, the choice of primary endpoints in ALS is controversial as the success/failure of the trial depends upon the choice of primary endpoints. Trials using functional scale and strength measurements as primary endpoints dominate over the trials using improved survival of patients as the primary endpoint. The benefits of the former are that it involves a smaller sample size, lesser trial duration, clinically meaningful treatment effects. However, survival measurement is the only and most important criterion to consider that treatment is truly effective or not [6].

10. Overlap with frontotemporal dementia (FTD)

TDP43 positive ubiquitinated cytoplasmic inclusions are commonly found in ALS patients (100%) and patients with frontotemporal dementia (FTD; more than 50%), therefore suggesting an overlap between both neurological disorders [66]. ALS and FTD (independent or coincident) both have a well-recognized familial clustering present in the families. The culprit genes behind it are unknown but the linkage studies suggest a common locus at chromosome 9 [224–228]. Based on cognitive & neuropsychological assessments, 20–50% of ALS patients fulfill the criteria for probable or obvious FTD [229]. Structural abnormalities such as frontotemporal atrophy (detected by voxel-based morphometry MRI) is common in ALS and FTD-ALS, however, it is more severe in patients with FTD-ALS [226,230,231]. From a functional point of view, ALS and FTD-ALS are characterized by the frontotemporal hypometabolism as observed by ^{2-18}F luoro- 2-deoxy-D-glucose PET [225]. Furthermore, FUS-positive inclusions are also observed in ubiquitin-positive, TDP-43-negative FTD patients and fALS patients [231,232], which further suggests the pathological overlap between both the diseases. Along with TDP43, C9ORF72 is also a common genetic cause of ALS and FTD. Mutation in C9ORF72 leads to the decreased solubility of some RNA binding proteins including TDP43 resulting in the disruption of cellular processes such as alternate splicing. Reduced solubility results in the accumulation of TDP43 in the cytoplasm which is a hallmark of ALS and FTD [233,234].

11. Comorbidities

ALS patients exhibit different frequencies of specific comorbidities (Fig. 9) that may associate with the disease progression. Regular/vigorous physical activity is considered as a potential risk factor for ALS, however, it is still debatable, and more detailed studies are to be conducted to draw any conclusions regarding physical activity and ALS. Lipid metabolism-related diseases and high BMI are considered protective against ALS. Hyperlipidemia is considered as a tendency to have a lower prevalence in ALS patients due to the lower BMI of ALS patients. Therefore, hyperlipidemia and BMI are interlinked. However, this is also controversial as some studies describe higher BMI and hyperlipidemia as protective factors whereas some studies describe the opposite. Lower BMIs in ALS might be due to poor nutrition, hypermetabolism, and high resting energy expenditure. To further elucidate the effect of lower BMI and hyperlipidemia on ALS, extensive studies involving the monitoring

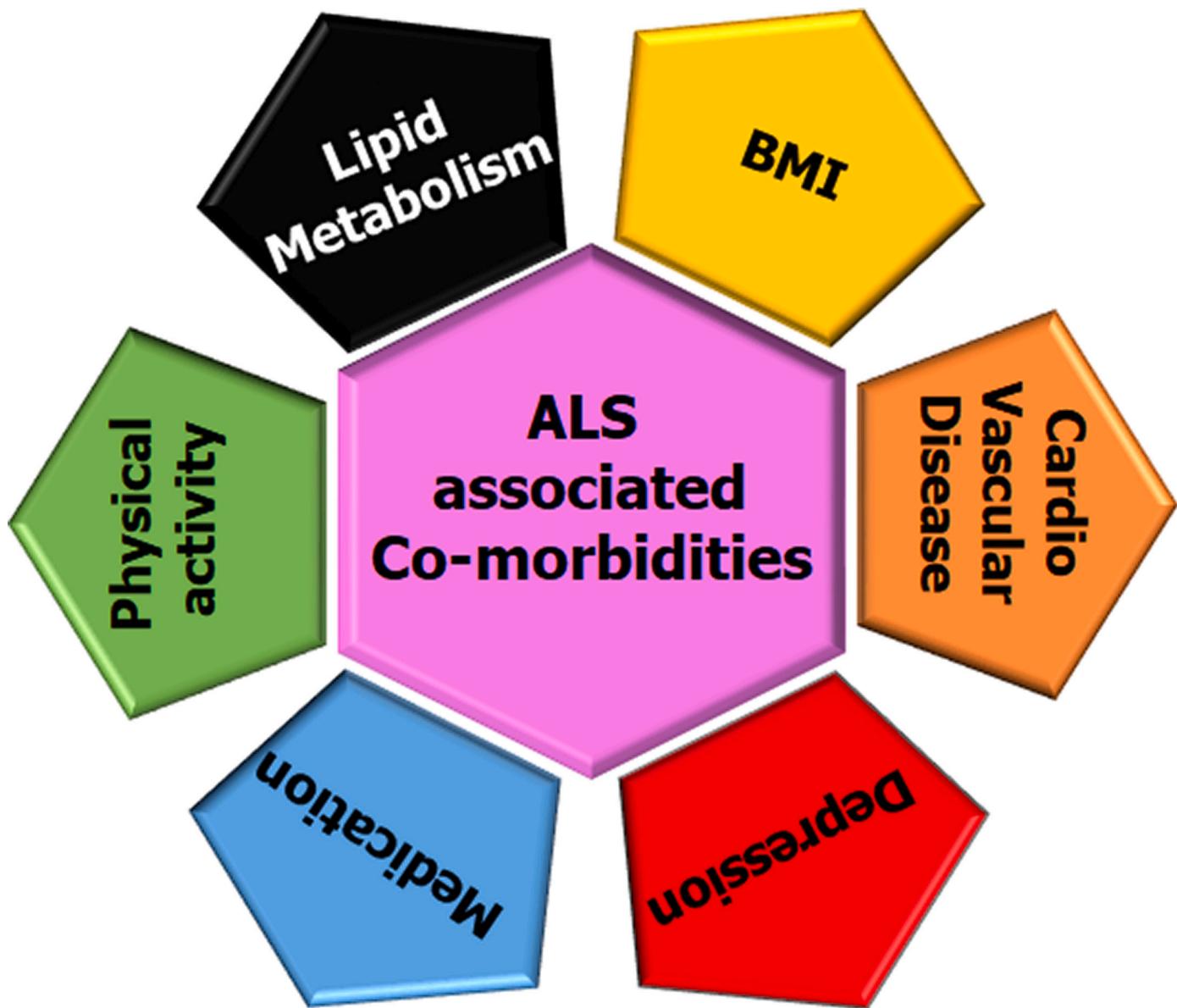


Fig. 9. ALS associated comorbidities.

of BMI over the lifetime (before and after ALS), disorders of lipid metabolism, and their correlation with various ALS phenotypes are necessary [235].

ALS patients without cardiovascular disease are considered physically fit [236–238]. However, studies reveal a positive association between stroke types/brain lesions and ALS which might be due to the increased oxidative stress, as oxidative stress plays a major role in neurodegenerative disease onset and progression [239–241]. Ischemia and ALS exhibit molecular overlap as a study reveals that ischemia induces aggregation of ALS-FTD-related proteins such as TDP43 & FUS [242]. Coronary heart disease has a significant influence on ALS progression. The faster progression of ALS in patients with coronary heart disease is due to the quick worsening of the respiratory subscale. ALS patients with cardiac diseases have impaired muscular ventilation process and perfusion which leads to poor gaseous exchange, thus, accelerating the disease progression [243]. Respiratory failure increases oxidative stress which is strongly associated with motor neuron death [244].

Many studies report a high prevalence of depression among ALS patients. Almost 22.6% of ALS patients suffer from depression. Because

of the high frequency of depression in ALS patients, they are under antidepressant treatment, and it was observed that there was a faster tendency towards disease progression in depressive patients (intaking antidepressants). This indicates that faster progression of ALS may lead to depression or vice versa [245,246].

Several medications might have an association with ALS such as contraceptives, which are thought to be protective against ALS. ALS is less common in females this could be due to the protective role of estrogen. Several in-vitro and in-vivo (in a mouse model) studies have revealed the protective role of estrogen in motor neuron diseases [247–249].

12. Future perspectives

12.1. Translation of biological processes into therapeutic approaches

Several preclinical trials have been done on rodent models and several clinical trials involving new potential drugs are in phase 1 to phase 3 trials. To date, there are only 2 FDA-approved drugs for ALS, Riluzole, and Edaravone. Apart from these drugs, symptomatic

treatments based on multidisciplinary care are used to improve the overall quality of life of ALS patients. Novel therapeutic strategies can be designed by exploiting novel biological findings such as identifying novel pathogenic pathways. Development and use of new disease models could be useful as the mutant SOD1 mouse is the mostly used model, in ALS studies, however, SOD1 mutation occurs in 20% of fALS cases and represents only 2% of all ALS cases. Motor neurons generated from iPSC's derived from patients also provide a suitable model system to study disease, however, they cannot replace the whole organism model system due to lack of anatomical and biological context. To overcome this problem, mixed (various cell types) cell cultures could be used. One of the major challenges faced by preclinical trials is that there is a lack of reproducibility of the study, therefore it is of great importance to improve the vigor of preclinical trials which could be achieved by standardizing the protocols, using proper controls, and determination of phenotypic variability, etc. Optimization of experimental design is another factor of concern when predicting the translational potential of a therapeutic. Optimization may involve estimating the number of animals required in the study sufficient to nullify the intrinsic phenotypic variability, determination of pharmacokinetics of the therapeutic compound to estimate dosing, the treatment strategy should mimic the situation in the clinic, time, and mode of drug administration. The molecular mechanism underlying the concerned therapeutic compound should be known which might give the insight to develop therapy towards another target affecting the same pathogenic process [250].

12.2. Use of genetic information

Identifying new therapeutic as well as preventive measures is a prerequisite to fully understand the underlying mechanisms of the disease. Several international groups have genotyped and sequenced millions of individuals from the distinct population and curated a comprehensive catalog of human genetic variation. These databases are

used as a worldwide reference for human genetic variation [251,252]. Detecting the genetic variants that are enriched in ALS patients as compared to unaffected controls can be a milestone towards the identification of novel biomarkers and therapeutics in the field of ALS. Next genome sequencing (NGS) is a high throughput technology that can sequence the whole genome, thus providing information about known as well as novel genetic variants. This can enable one to identify the genetic variation unique to each patient. However, there is a limitation of NGS as the fALS only accounts for 5–10% of cases, remaining 90–95% are generally from sALS. So, NGS is emerging to detect sALS too since many mutations have been identified in sALS cases [253]. The genetic information gained via NGS can also be used to understand how the genetic differences in individuals affect their response to drugs (pharmacogenetics) which could lay a base towards personalized treatment as well as minimizing the adverse reactions caused by drugs. In a clinical trial, it was observed that lithium carbonate (boosts autophagy and removes misfolded protein) was ineffective in ALS patients, however, post hoc meta-analysis across new clinical trials showed that the lithium effect was patient genotype dependent [254]. This suggests the importance of consideration of genetic information in clinical trials. Based on genetic information patients could be divided into subcategories that might be benefitted from a particular treatment. Inclusion criteria based on genetic etiology rather than clinical syndromes for a clinical trial will enable improved treatment discovery and success rates. Basket design clinical trials (Fig. 10) are new trial designs allowing the exploration of any therapeutic molecule against several clinical syndromes manifested by the same genetic/molecular anomaly. This approach has been used in neurodegenerative diseases like Alzheimer's by Tsai et al. [255]. Furthermore, genetic information can also be used to identify the most pertinent therapeutic target(s) based on the molecular profile of the disease and patient-specific etiology e.g., targeting neuroinflammation in patients exhibiting consistency in immune dysregulation. In conclusion, elucidating genetic information can be used to investigate the

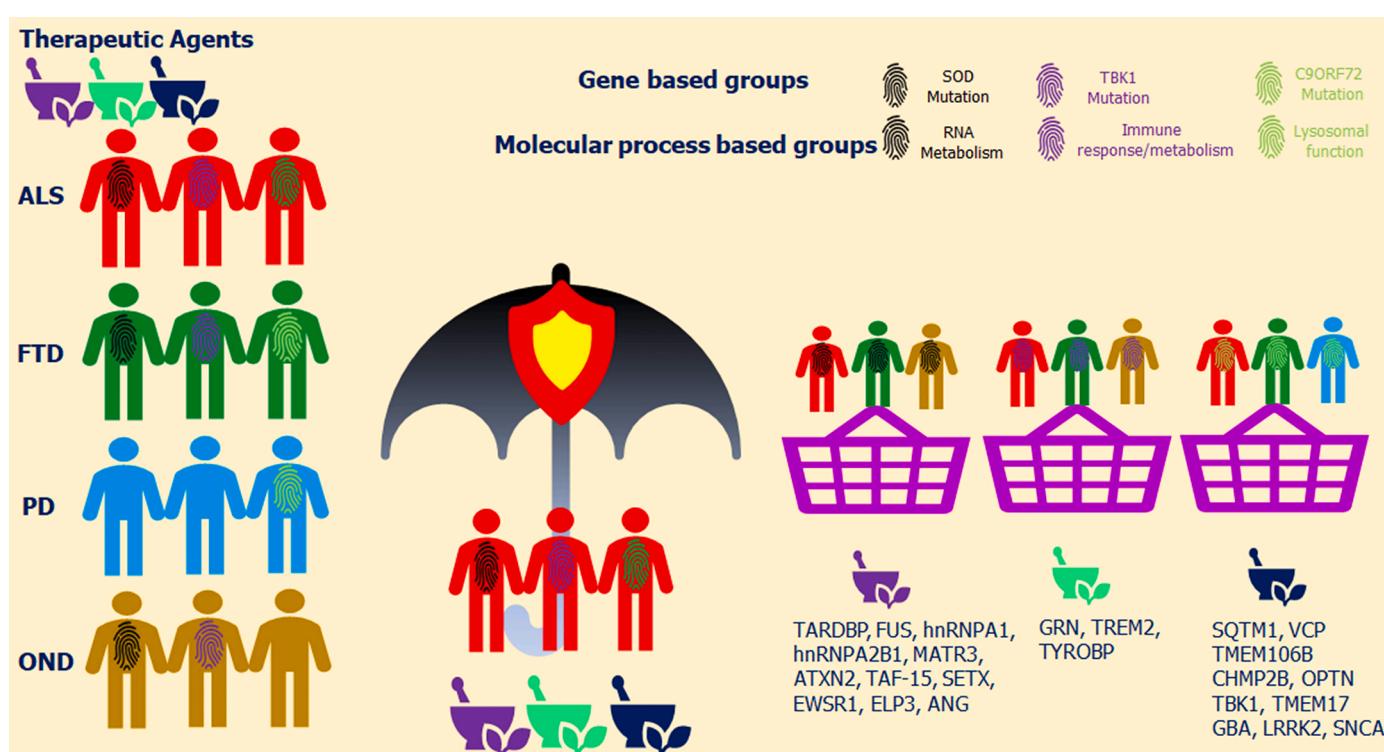


Fig. 10. Graphical depiction of umbrella and basket trials. Umbrella trials refers to the trials that involve only one disease and multiple therapies targeting it. Therefore, patients are enrolled based on single disorder and multiple targeted therapies are evaluated. Basket trial refers to the trials that involve multiple diseases with common molecular pathology therefore patients are enrolled based on different disorders for treatment targeting a common molecular alteration. Reproduced from *Frontiers in Neuroscience*, Broce et al., 15, article 639078, © 2021 Broce, Castruita and Yokoyama.

pharmacogenetic interactions in clinical trials, improve recruitment accuracy in clinical trials and accelerate the process of drug discovery.

12.3. Use of bioinformatics

Pleiotropic methods have been exploited by various scientific groups for gene discovery. Pleiotropy is the phenomenon where two or more phenotypes are influenced by one gene. Independent genome-wide association study (GWAS) has discovered pleiotropic loci between FTD and ALS beyond C9ORF72 [256]. Diseases sharing molecular pathways and genes can be treated with the same drugs which are already approved for diseases beyond ALS and FTD, and thus, could facilitate the basket trials recruiting patients with different diseases which might respond to the same treatment. Machine-learning based methods are also used for gene discovery [257] e.g. a recent study predicted novel genes associated with ALS using knowledge graph edge prediction algorithm which used information (phenotypic and biological) available from public databases [257,258]. Over 500 genes were predicted to be linked to ALS, however, they need to be validated. As the sequence datasets of more and more individuals become available, validation of such novel findings, as well as discovery of new genes, could be achieved

which in turn could be applied to a wider range of patients and detection of vulnerable individuals.

ALS is a complex disease that might be monogenic (caused by one gene), oligogenic (caused by few genes), or polygenic (caused by many genes). Polygenic risk scoring approaches would be beneficial as they could be used to create personalized diagnostic as well as a therapeutic option [259].

12.4. Scope of combination therapy

The failure of translating experimentation from animals to patients can be attributed to the fact that ALS, besides being a multifactorial disease is also a multisystemic disease affecting several cell populations. ALS is the resultant of the neurotoxic cascade that involves motoneurons' crosstalk with glial cells and muscles respectively [260,261]. This strongly suggests that the novel therapies designed against ALS should be multi-targeted. However, to develop multi-therapy, negative interactions between drugs should be taken into consideration [262]. A few of the combination therapies studied are mentioned in Table 7.

Table 7

Combination therapy could be the future of ALS as ALS is a multifactorial disease. The table lists various combinations of potential therapeutics and their outcomes.

S. no.	Therapeutic combination	Model	Observations	Prolong survival	Reference
1	Creatine + celecoxib/rofecoxib	SOD1(G93A) mouse model	1. Prolonged lifespan 2. Improved motor function 3. Neuroprotection	28.6% and 30.9% respectively	[263]
2	Creatine + minocycline	SOD1(G93A) mouse model	1. Prolonged lifespan 2. Delayed disease onset	25%	[264]
3	Riluzole + minocycline + nimodipine	SOD1(G37R) mouse model	1. Prolonged lifespan 2. Delayed disease onset 3. Delayed decline in muscle strength 4. Neuroprotection 5. Attenuation of Cdk5 Mislocalization 6. Reduced caspase-3 Activation 7. Attenuation of astrocytosis and microglial activation	12%	[265]
4	Riluzole + sodium phenylbutyrate	SOD1(G93A) mice model	1. Improved clinical and neuropathological phenotypes 2. Improved body weight loss and grip strength. 3. Improvement of gross lumbar and ventral horn atrophy 4. Decrease in lumbar ventral horn neuronal cell death 5. Reduced reactive astrogliosis	21.5%	[266]
5	Gems (fatty acids, free radical scavengers, and amino acids linked to poly-L-lysine)	SOD1(G93A) rat model	1. Prolonged survival in model rats 2. Improved electromyographic parameters 3. Delayed onset of disease 4. Prevention of early weight loss	9.5% for Gems dose 1 (1.25 mg/ml or 0.62 mg/rat/day) 18.47% for Gems dose 2 (3.75 mg/ml or 1.87 mg/rat/day)	[267]
6	hNPCs + GDNF	SOD1(G93A) rat model	1. Survival of neural stem or progenitor cells in the spinal cord 2. Production of astrocytes by NSCs 3. Release of growth factors	Unaltered	[268]
7	hNPCs + GDNF	SOD1(G93A) rat model	1. Prevention of motor neurons loss	Not Determined	[269]
8	hNPCs + GDNF	SOD1(G93A) rat model	1. Motor neurons protection 2. Delayed ALS onset 3. Extended survival time of rats	8.23%	[270]
9	NSCs + NGF	SOD1(G93A) mice model	1. Motor neurons preservation 2. Improved motor functions 3. Delayed ALS onset 4. Extended survival time of mice	16.9%	[271]
10	MSCs + GDNF; BDNF; VEGF; HGF	Patients with definite or probable ALS	1. Slower disease progression 2. Neuroprotection 3. Improved respiratory function	Not determined	[272]
11	Acamprosate + Baclofen (PXT864)	SOD1(G93A) rat model	1. Protecting NMJs and motoneurons against glutamate excitotoxicity 2. Prevent TDP43 accumulation and stress granule formation	Not Determined	[273]

12.5. Cell therapy

Cell therapy is an emerging/investigational therapeutic for ALS. Stem/progenitor cells and adult somatic cells are considered suitable candidates. Stem cells can self-renew and differentiate into different cell types. Depending on their differentiation potential, stem cells are categorized as totipotent, pluripotent, or multipotent. Totipotent cells can form the whole individual as they are zygotes. Pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can form cells from the three germ layers. Multipotent stem cells such as neural stem cells (NSCs) and mesenchymal stem cells (MSCs) form cells of a particular lineage [274].

Animal models have been extensively used for ALS-related studies, but these models only represent the familial forms of ALS which only constitutes 10%, the remaining 90% of cases are sporadic. In 2006, Japanese scientists Takahashi and Yamanaka reported a breakthrough, according to which embryonic-like pluripotent stem cells (iPSCs) were obtained from adult mice cells by genetic reprogramming [275]. Later, the same was achieved with human cells [276]. iPSCs could be derived from a patient's somatic cells such as skin/peripheral blood/urine and differentiated into motoneurons, glial cells & muscle fibers. These differentiated cells served as models to study ALS. For the past 15 years, iPSCs derived from ALS patients (both familial and sporadic) have been used to generate cell types (motoneurons, astrocytes, oligodendrocytes, and skeletal muscle cells) suitable for the study of ALS. The beauty of these cells is that they exhibit important phenotypic alterations such as reduced viability, protein aggregate formation, mitochondrial dysfunction, altered electrophysiological properties, etc. [277,278].

iPSCs can also be used for therapeutic purposes. Novel drugs/molecules and therapeutic strategies could be screened using iPSCs based on the genetic profile of patients from where the iPSCs are derived. Some examples include: iPSCs derived motoneurons exhibited reduced hyperexcitability and increased viability in response to Retigabine (anti-convulsant). This study led to the introduction of retigabine in a clinical trial for ALS [279,280]. Using high throughput screening (HTS), 1757 bioactive compounds were tested on iPSCs derived motoneurons from SALS patients. Out of these only 4 compounds (cyclin-dependent kinase inhibitors and the cardiac glycosides/Na⁺/K⁺-ATPase inhibitors digoxin, lanatoside C, and proscillarin A) had the potential to reduce TDP43 aggregates in a dose-dependent manner [281]. Similarly, many other HTS studies have been conducted on motoneurons derived from patient-derived iPSCs. These studies showed efficacy in terms of enhanced autophagy leading to misfolded protein clearance, reduced the mislocalization of FUS/TDP-43 proteins & stress granule formation, and conferred neuroprotection [277]. These studies suggest that iPSCs are best suited to serve as ALS models as well as for screening potential therapeutic compounds against ALS.

13. Conclusion

This review summarizes various aspects of ALS, thus, providing an overview of the disease. The exact cause of ALS is very hard to identify as 95% of the cases are sporadic, the remaining 5% are due to mutations in the genes such as SOD1, TARDP43, C9ORF72, and FUS. Here we discuss the various biomarkers that are used or could be further investigated for disease diagnosis. Despite having so many biomarkers available/proposed, ALS is diagnosed at later stages which suggests identifying new biomarkers as well as mechanisms that aid in easy and early detection of ALS. Also, various therapeutic strategies have been discussed that are currently under investigation or in current use against ALS. However, no potential therapy is available. There are only 2 FDA-approved drugs, Riluzole and Edaravone. ALS is a multifactorial disease which indicates that it is impossible to combat ALS using a single therapeutic intervention, therefore combination therapy is the need of the hour. Multiple research groups are working in this direction and various combination therapies are under investigation. For successful therapy, there are

certain considerations to be taken such as identification of ideal combination therapeutics, effective dose, therapy duration, administration route, designing of a clinical trial, etc. Developing a global database can be helpful so that all information regarding ALS can be available to anyone anywhere associated with ALS. Also, having the genetic information of ALS patients available worldwide could lead towards a better understanding of the disease and the generation of new therapeutic options. This information could also be used to tailor personalized medicine for ALS patients. In conclusion, early detection (in the initial stage) of ALS and identification of potential therapy that aim at the regeneration of the lost motor neurons hold the future of ALS.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Non-neuronal cells in amyotrophic lateral sclerosis — from pathogenesis to biomarkers

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Abstract | The prevailing motor neuron-centric view of amyotrophic lateral sclerosis (ALS) pathogenesis could be an important factor in the failure to identify disease-modifying therapy for this neurodegenerative disorder. Non-neuronal cells have crucial homeostatic functions within the CNS and evidence of involvement of these cells in the pathophysiology of several neurodegenerative disorders, including ALS, is accumulating. Microglia and astrocytes, in crosstalk with peripheral immune cells, can exert both neuroprotective and adverse effects, resulting in a highly nuanced range of neuronal and non-neuronal cell interactions. This Review provides an overview of the diverse roles of non-neuronal cells in relation to the pathogenesis of ALS and the emerging potential of non-neuronal cell biomarkers to advance therapeutic development.

Amyotrophic lateral sclerosis (ALS) is the third most common neurodegenerative disorder, after Alzheimer disease (AD) and Parkinson disease, and is characterized by the loss of cortical, brainstem and spinal anterior horn motor neurons. Progressive paralysis typically results in death from neuromuscular respiratory failure, with a median survival from symptom onset of 30 months. However, substantial clinical heterogeneity is observed with regard to site of onset, pattern and rate of progression, and overall survival. Taken together with the clinicopathological overlap between ALS and fronto-temporal dementia (FTD), this heterogeneity indicates that the pathophysiology of ALS is driven and modified by complex biological factors¹.

The aetiology of most cases of ALS is still unclear, although up to 15% of familial and apparently sporadic cases are attributable to single gene mutations². The most common mutation is a dynamic hexanucleotide repeat expansion in the first intron of *C9orf72*, which is found in ~40% of familial ALS (and FTD) cases^{3,4}. Mutations in the *SOD1* gene (encoding superoxide dismutase 1) are seen in ~20% of cases⁵ and mutations in *TARDBP* (encoding TAR DNA-binding protein 43 (TDP43))⁶ and *FUS* (encoding the fused in sarcoma protein) are seen in <5% of cases each⁷. In nearly all ALS cases (with the notable exception of those attributed to *SOD1* or *FUS* mutations) and 50% of FTD cases, characteristic nuclear clearing and cytoplasmic aggregation of TDP43 is observed in both neuronal and non-neuronal cells⁸. The relative selectivity and non-random pattern of

degeneration of cortical and spinal cord motor neurons suggests that ALS is a broader motor system degeneration, although the genetic heterogeneity also points towards a clinicopathological syndrome arising from a broad range of upstream converging biological processes¹.

In experimental models, multiple motor neuron-intrinsic (cell-autonomous) pathways have been implicated in ALS pathogenesis, including mitochondrial dysfunction, axonal transport, RNA metabolism and nucleocytoplasmic transport⁹. Although most of these pathways show some response to therapeutic modulation in disease models, clinical translation has proved elusive. Riluzole and the free-radical scavenger edaravone are the only licensed disease-modifying drugs for ALS, with extremely limited impact on survival and rate of progression, respectively.

One of the potential reasons for the recurrent failure of clinical trials in ALS is the focus on motor neurons in preclinical models. Non-neuronal cells exert crucial homeostatic functions in the CNS, and an increasing body of evidence implicates their involvement in ALS pathogenesis. This pathogenic involvement of non-neuronal cells in neurological disease is commonly referred to as ‘neuroinflammation’; however, the meaning of this term is context dependent and has not been clearly defined in relation to specific cell types. In rodent models of ALS, non-neuronal cells exert both harmful and protective non-cell-autonomous influences on neurons. To overcome the limitations

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Key points

- Accumulating evidence suggests that an exclusively motor neuron-centred model of pathogenesis in amyotrophic lateral sclerosis (ALS) is untenable, with important implications for therapeutic development strategies.
- Brain-resident microglia, astrocytes and oligodendrocytes as well as peripheral immune cells all have vital functions in CNS homeostasis and physiology.
- In multiple experimental models of ALS, non-neuronal cells seem to exert neurotoxic effects via both gain-of-function and loss-of-function mechanisms but also apparently show neuroprotective activity at certain disease stages.
- Many of the hypotheses surrounding the roles of non-neuronal cells in ALS pathogenesis were developed using rodent models, some of which have limited relevance to the TDP43 neuropathological hallmark of human ALS.
- Human induced pluripotent stem cells permit the investigation of non-neuronal cells carrying ALS-associated genetic mutations, but multicellular co-cultures might be needed to disentangle their nuanced interactions with motor neurons.
- Human biofluid biomarkers derived from non-neuronal cells offer an important window into the *in vivo* pathological milieu and show potential as early markers of therapeutic response.

of these models imposed by species-specific differences in cellular function, induced pluripotent stem cell (iPSC)-derived cells have the potential to provide new insights into the specific functions of human non-neuronal cells that are pertinent to neurodegenerative disorders, including ALS. A further major barrier to therapeutic development in neurodegenerative disorders such as ALS is the lack of objective human biofluid markers of disease activity, and molecules derived from non-neuronal cells are of increasing interest as potential biomarkers¹⁰.

In this Review, we outline the role of non-neuronal cells in CNS physiology, with a particular focus on microglia, astrocytes and oligodendrocytes. We consider the evidence for involvement of these cells in ALS pathogenesis and the prospects for novel biomarkers against which to assess emerging therapeutics.

Non-neuronal cells in the CNS

The non-neuronal cells of the CNS include glia, which reside in the parenchyma and are subdivided into microglia, astrocytes, oligodendrocytes and oligodendrocyte progenitors (NG2 glia), and pericytes, endothelial and ependymal cells, which reside in other cellular compartments. The prevailing dogma that glial cells substantially outnumber neurons has recently been questioned, with the glia to neuron ratio suggested to be nearer 1:1 (REF.¹¹). The perception of glia as passive ‘bystanders’ has also been overturned by research showing that all glial cell types are of high functional importance in CNS development and physiology^{12,13}. In addition, CNS-associated macrophages and peripheral non-neuronal cells, such as blood monocytes and macrophages, granulocytes and lymphocytes, and natural killer (NK) cells, reside on the borders of the CNS, and evidence is emerging that these non-resident, non-neuronal cells can also exert relevant physiological functions in the CNS¹⁴.

In the sections that follow, we introduce the different non-neuronal cell types in the CNS, including their physiological functions and the evidence from human post mortem and neuroimaging studies for their involvement in ALS.

Glia in CNS physiology and human ALS**Microglia**

Microglia, which represent ~5–10% of glial cells^{11,15}, are the resident macrophages of the CNS. They originate from yolk sac-derived precursors that migrate into the CNS during neurodevelopment^{16,17} and maintain their population by self-renewal¹⁸. Depending on the surrounding milieu, microglia can adopt either a resting or an activated state¹⁹. Although originally considered to be dormant under homeostatic conditions, microglia are in fact very active in their resting state, continually surveying and scanning the environment with their highly motile ramified processes²⁰. The detection of any disturbance to CNS homeostasis leads to microglial activation, resulting in rapid changes in their morphology, associated with alterations in their gene expression and functional behaviour^{21,22}.

Microglial activation was classically conceptualized as one of two polarized stages: a pro-inflammatory M1 and an anti-inflammatory M2 phenotype^{23–25} (for an overview on respective markers and cytokines, see REF.²⁶). Broadly speaking, M1 microglia were thought to induce or exacerbate neuron dysfunction, whereas M2 microglia were considered neuroprotective^{27,28}. However, this view of microglial physiology is now recognized to be overly simplistic, and their state of activation should be seen as a spectrum that is dependent on the location of the cell, the age of the individual and the nature of the environmental challenge^{21–23}. For example, constitutive MHC class II expression on microglia is higher in cerebellar white matter and the lumbar spinal cord than elsewhere in the brain²⁹. Using this variety of activation states and their immense capacity to interact with their environment, microglia execute crucial functions in the maintenance of CNS homeostasis by providing nurture and support to neurons and other cells via the secretion of soluble factors, synaptic refinement, clearance of dead cells or misfolded proteins, and protection from infectious agents³⁰.

PET imaging revealed widespread cerebral microglial activation in people with ALS^{31,32} and pre-symptomatic carriers of *SOD1* mutations³³. Moreover, post mortem examination of CNS tissue from patients with ALS showed elevated levels of microglial transcripts, increased microglial density and activated microglial morphology in addition to motor neuron degeneration^{34,35}. Microglial activation correlated with neuronal and axonal loss, as well as with more rapid disease progression and a greater burden of clinical upper motor neuron signs, but whether these associations are causal or a consequence of the accelerated pathology is difficult to establish^{35,36}. Carriers of the *C9orf72* expansion showed particularly extensive microglial activation in post mortem studies³⁵.

Astrocytes

Astrocytes represent ~20–40% of glial cells in the CNS^{11,37}. These cells, which have an important role in controlling homeostasis in the CNS, originate from the neuroepithelium and share a common progenitor with neurons and oligodendroglia^{37,38}. In close crosstalk with microglia, astrocytes maintain ion, neurotransmitter

and energy homeostasis, perform synaptic pruning, and provide trophic support to neurons^{37,39}. Disturbances to CNS homeostasis lead to transformation to reactive astrocytes, accompanied by substantial transcriptomic changes^{40,41}. Analogous to the M1–M2 concept that is widely used to categorize microglial phenotypes, A1 and A2 phenotypes were proposed to broadly classify the diverging reactions of astrocytes to different stimuli^{39,42}. Similar to their microglial counterparts, A1 astrocytes are suggested to have neurotoxic properties, whereas the A2 phenotype is connected to astrocyte-mediated neuroprotection^{42–45}.

In post mortem CNS tissue from people with ALS, an increased presence of reactive astrocytes has been described in addition to degenerating motor neurons^{46–50}. This finding was corroborated indirectly in vivo using PET, which demonstrated cerebral white matter and pontine astrocytosis in patients with ALS⁵¹.

Oligodendrocytes and NG2 glia

Oligodendrocytes, which represent ~50–75% of glial cells^{51,52}, are myelin-producing cells in the CNS that originate from precursor cells known as NG2 glia^{53,54}. In addition to their canonical function in neuronal action potential propagation, key roles of oligodendrocytes in providing metabolic support to neurons have been described⁵⁴. In concert with astrocytes, oligodendrocytes maintain axonal integrity, survival and adaptation^{54,55}. Interestingly, NG2 glia have recently been implicated in the modulation of microglial function⁵⁶.

Oligodendrocyte pathology has been found in multiple areas in post mortem CNS tissue from people with ALS^{46,47,57} and its relevance is underscored by the consistent presence of TDP43 inclusions in oligodendrocytes⁵⁷.

Non-glial non-neuronal cells

CNS-associated macrophages. In addition to microglia, a distinct population of CNS-associated meningeal, perivascular and choroid plexus macrophages has been recognized^{58,59}. Under normal physiological conditions, these cells form a stable population that resides at the CNS interface and does not enter the CNS parenchyma^{14,59}. The detailed physiological function of these cells is only just beginning to emerge; they are thought to be important for controlling the permeability of the blood–brain barrier (BBB) and the transport of metabolites to and from the CNS⁵⁸. To the best of our knowledge, these non-parenchymal macrophages have not yet been linked to ALS in humans.

Peripheral immune cells. The entry of peripheral immune cells into the CNS parenchyma is highly restricted in the healthy state. Activated CD4⁺ T cells — but not CD8⁺ T cells — traffic into the CNS regardless of their antigen specificity^{60,61}, whereas neutrophils and B cells are rarely, if ever, found in the healthy brain parenchyma^{14,62}. Why only certain leukocyte populations infiltrate the CNS remains unclear, although this finding, for T cells at least, is likely to be related to the low basal expression of MHC class I and the lack of expression of MHC class II antigens on brain endothelial cells^{60,63}.

Microvascular endothelial cells in non-CNS tissue in humans, and in most other mammals, basally express both class I and class II MHC molecules in vivo, and T cell recruitment to target tissue is induced by cognate recognition of antigen presented by endothelial cell MHC classes I and II^{64,65}. However, activated CD4⁺ T cells do seem to be able to cross the BBB in a T cell receptor-independent manner⁶¹.

Following injury to the CNS, multiple types of innate and adaptive peripheral immune cells, including granulocytes, monocyte-derived macrophages, lymphocytes and NK cells, can be recruited from the blood circulation across the BBB^{14,62}. Evidence is emerging for close crosstalk and communication between these infiltrating cells and resident glial cells, which could modulate glial function in both protective and harmful ways¹⁴.

In post mortem tissue from patients with ALS, peripheral cells, such as CD4⁺ and CD8⁺ T cells, macrophages, and NK cells, were shown to infiltrate multiple areas of the CNS^{34,46,66}, suggesting that immune-mediated events contribute to the pathogenesis of ALS.

Non-neuronal cells in ALS models

The findings from post mortem and neuroimaging studies in humans indicate a key role for CNS and peripheral non-neuronal cells in ALS and also raise a number of questions. First, is the observed involvement of non-neuronal cells beneficial or detrimental? Second, do different cell types have divergent roles in ALS pathophysiology and do these roles change over the ALS disease course? Last, is non-neuronal cell involvement a primary driver of neurodegeneration or a secondary reaction to tissue injury?

To attempt to answer these key questions, researchers have employed various experimental models of ALS (TABLE 1). In this section, we examine how *in vitro* and *in vivo* models for the most common mutations in *SOD1*, *TARDBP* and *C9orf72* have improved our understanding of the functional roles of different non-neuronal cell types in ALS pathogenesis.

Microglia

***SOD1* models.** Transgenic animals expressing mutant forms of human SOD1 (mSOD1) are widely used as disease models as they recapitulate some of the key clinical features of human ALS, such as progressive motor abnormalities and paralysis, and show pathological evidence of protein aggregation, motor neuron degeneration and gliosis^{67–69}. Analogous to the results obtained from human post mortem tissue, microglial activation is reported consistently in SOD1 models and, in some studies, occurs well before the onset of clinical disease^{70–72}. In some studies, selective expression of various forms of mSOD1 in motor neurons was found to be insufficient to induce motor neuron degeneration^{73,74}, although other studies indicated that mild ALS phenotypes could be generated by increasing the levels of transgene expression in these cells^{75,76}. It is difficult to compare these results with the phenotypes found in animals that ubiquitously express mSOD1 but the data suggest a role for non-cell-autonomous factors, possibly from non-neuronal cells, in ALS pathogenesis.

Table 1 | Techniques to study non-neuronal cell involvement in ALS

Technique	Main features	Advantages	Limitations
Neuropathology	Morphology; immunostaining; molecular phenotyping, including spatial transcriptomics and proteomics	High-resolution characterization of human disease-specific tissue; retention of cell relationships	Static, descriptive and biased towards phenomena associated with end-stage processes
Human CNS imaging	MRI	In vivo analysis of structure and some aspects of function; captures longitudinal changes, including at pre-symptomatic stages	Low resolution does not allow the analysis of individual cells
	PET	Relatively cell-specific tracers can be used to assess the activation of specific non-neuronal cells	
Rodent models	Mouse and rat transgenic models expressing human complementary DNA or genomic constructs with specific genetic mutations; primary cell lines	Mammalian system with retention of the complex multicellular environment; can be genetically manipulated to allow cell-specific expression; enable spatial and temporal analysis of pathophysiology	Species differences (immune, microbiome, divergence in protein functions) between humans and rodents may be a barrier to translation; phenotypes might depend on non-physiological overexpression; modelling of age-related neurodegeneration is challenging
Human primary cell lines	Fibroblasts or iPSC-derived glia	Allow analysis of human cells on a genetic background that includes disease-causing mutations; avoid non-physiological overexpression and interspecies differences in the transcriptome; useful to study cell-autonomous and non-cell-autonomous effects	Cells might be arrested in early developmental stages; expensive and labour intensive; isogenic controls required owing to considerable interindividual variance
Human iPSC models	Complex multicellular cultures derived from iPSCs	Multiple cell–cell interactions; experimental control of microenvironment	The relationship to normal tissue architecture is imperfect; cells are at an immature stage of development

ALS, amyotrophic lateral sclerosis; iPSC, induced pluripotent stem cell.

In a mouse model of familial ALS, expression of the mutant protein SOD1^{G37R} in motor neurons was found to determine disease onset and early disease progression⁷⁷. Selective expression of a different mutant form of SOD1, SOD1^{G93A}, in microglia was not sufficient to cause motor neuron degeneration, although wild-type microglia seemed to have a neuroprotective effect in SOD1^{G93A} mice. In another study, the silencing of SOD1^{G93A} by short hairpin RNA in motor neurons delayed disease onset but had no effect on progression once the disease had commenced⁷⁸, whereas the deletion of SOD1^{G37R} in microglia substantially slowed later disease progression but did not substantially alter the earlier phases of the disease⁷⁹.

These data indicate that the observed activation of microglia, in SOD1 models at least, seems to be a secondary mechanism in response to neuronal damage, which leads to disease exacerbation rather than being a primary disease driver. This notion was further corroborated by an assessment of pro-inflammatory and anti-inflammatory markers at early and late disease stages. Microglia isolated from SOD1^{G93A} mice at disease onset showed higher expression of M2-associated and lower levels of M1-associated markers than those from mice with end-stage disease⁸⁰. Furthermore, SOD1^{G93A} M2 microglia isolated at disease onset were neuroprotective in co-culture with motor neurons, whereas SOD1^{G93A} M1 microglia from end-stage disease were neurotoxic.

In another study using mSOD1-overexpressing transgenic mice, the anti-inflammatory cytokine

IL-10 was found to be upregulated in microglia at pre-symptomatic stages⁸¹. Disease onset was delayed by targeted overexpression of IL-10 in microglia and precipitated by antibody-mediated IL-10 blockade. However, several studies have demonstrated increased production of neurotoxic factors in mSOD1-expressing microglia. Compared with wild-type microglia, cultured primary SOD1^{G93A} microglia showed increased release of reactive oxygen species (such as superoxide) and nitric oxide (NO) and reduced release of the neuroprotective protein insulin-like growth factor 1 (IGF1), thereby reducing the survival of co-cultured primary neurons, both in the naive state and under lipopolysaccharide-stimulated conditions^{77,82} (FIG. 1). As a proof of principle, pre-treatment with L-NIL, an inhibitor of inducible NO synthase (iNOS), increased motor neuron survival in co-culture⁸².

In line with these results, treatment of SOD1^{G93A} microglia with SOD1^{G93A} recombinant protein, in comparison with wild-type SOD1, led to a strong increase in tumour necrosis factor (TNF), IL-1 β and superoxide release as well as iNOS expression, whereas IGF1 production declined⁸³. These effects resulted in motor neuron toxicity in co-culture, which was counteracted by concomitant application of L-NIL in combination with the NADPH oxidase inhibitor apocynin⁸³. In addition, following lipopolysaccharide stimulation, cultured primary SOD1^{G93A} microglia showed increased TNF release compared with wild-type microglia⁸⁴. However, TNF knockdown in SOD1^{G93A} and SOD1^{G37R} mice did not affect the lifespan or motor neuron loss⁸⁵, indicating

that TNF is unlikely to be a direct mediator of neurotoxicity in SOD1-associated ALS. IL-1 β knockdown slowed disease progression in SOD1^{G93A} mice⁸⁶ but did not alter the disease course in SOD1^{G37R} mice⁸⁷, suggesting that its neurotoxic effect is model dependent.

Overall, the evidence from SOD1 models currently points to early neuroprotective effects followed by a transition to toxic properties for microglia over the disease course. This transition is thought to involve both loss of neurotrophic support and gain-of-function toxicity. However, this scenario is likely to be an oversimplification of the true microglial disease phenotype. Many studies focused on specific M1–M2-associated markers, although one study demonstrated by RNA sequencing that microglia isolated from SOD1^{G93A} mice showed concurrent expression of both neuroprotective and toxic factors at different time points during disease progression, thereby diverging from the M1–M2 paradigm and suggesting the existence of a neurodegeneration-specific microglial phenotype⁸⁸. Future studies analysing microglial gene and protein expression using unbiased ‘omics’ approaches in pre-symptomatic, early and late stages of the disease will be necessary to shed light on the detailed functional role of microglia in SOD1 models.

TDP43 models. Although the human clinical syndromes of SOD1-associated and non-SOD1-associated ALS have many features in common, the molecular underpinnings are strikingly different. In particular, the cytoplasmic aggregates of TDP43 that are observed in 97% of all ALS cases are notably absent in SOD1-associated ALS, prompting a shift away from the use of SOD1 transgenic models to study ALS.

In TDP43 models of ALS, cellular dysfunction is thought to arise from a combination of cytoplasmic mislocalization-induced toxic gain of function and loss of nuclear TDP43. Most studies have focused on the cell-autonomous effects of neuronal expression of mutant TDP43 or TDP43 aggregates but some have also analysed the microglial response to mutant TDP43 expression or exogenous treatment with TDP43. In post mortem cortical tissue from patients with ALS and mice expressing TDP43^{A315T}, the latter of which develop time-dependent gait abnormalities, motor neuron degeneration and premature death⁸⁹, microgliosis and rod-shaped microglia — a reactive microglial phenotype described in many encephalopathies, including neurosyphilis — were found in addition to degenerating upper motor neurons⁹⁰.

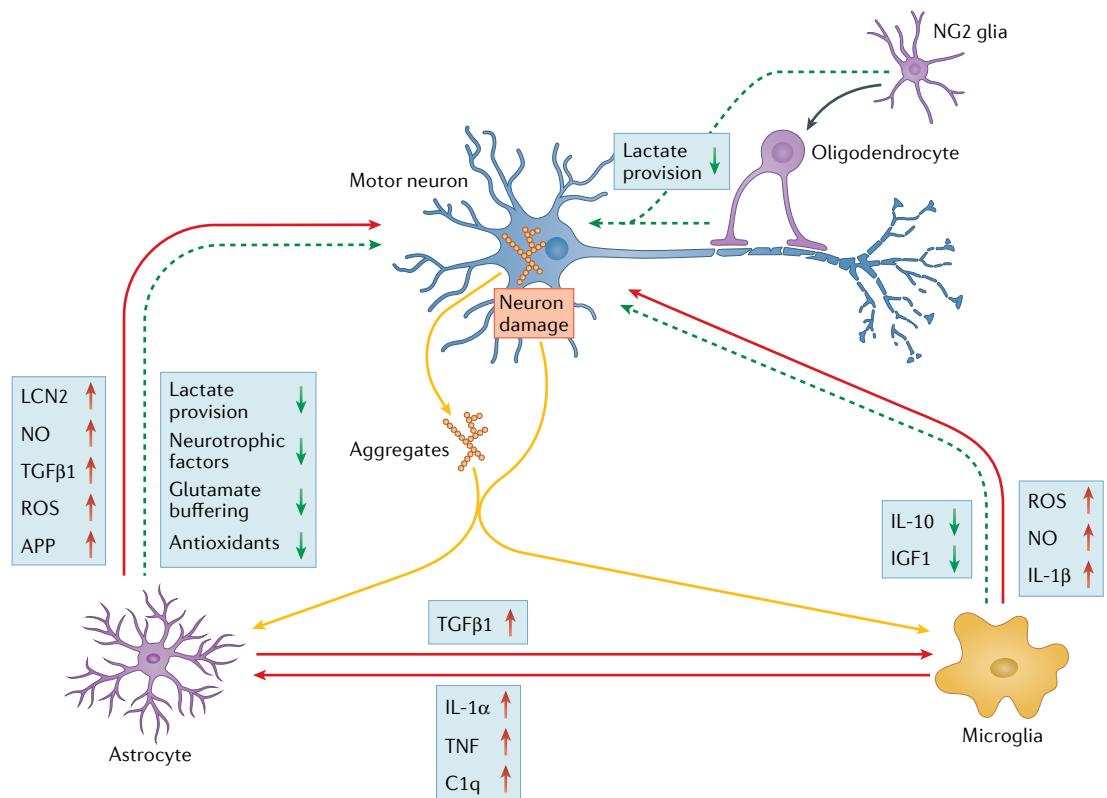


Fig. 1 | Pathophysiological roles of microglia, astrocytes and oligodendrocytes in amyotrophic lateral sclerosis. Neuronal damage associated with dysfunction and aggregation of proteins, such as superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (encoded by TARDBP) and dipeptide repeat proteins, as well as with aggregate release has been implicated in the activation of astrocytes and microglia (yellow arrows). Glial cells are thought to acquire neurotoxic properties, comprising both gain-of-function toxicity (red arrows) and loss of supportive functions (green dashed arrows), in response to the expression of amyotrophic lateral sclerosis-associated mutations in genes such as SOD1, TARDBP and C9orf72. The neurotoxicity is exacerbated by crosstalk between different cell types. The mechanisms depicted are derived from the different models discussed in this Review and have not all been identified in every model. APP, amyloid precursor protein; IGF1, insulin-like growth factor 1; LCN2, lipocalin 2; NO, nitric oxide; ROS, reactive oxygen species; TGF β 1, transforming growth factor β 1; TNF, tumour necrosis factor.

These findings do not resolve the issue of whether microglia are actively involved in disease pathogenesis in TDP43 models or whether they merely show a response, either harmful or protective, to neuronal damage. Cell culture experiments have provided further insights into the functional role of microglia in this context. The addition of wild-type and various forms of mutant TDP43 to primary microglial cultures led to microglial activation and upregulation of pro-inflammatory markers, such as TNF and IL-1 β (FIG. 1), with mutant TDP43 eliciting a more pronounced response^{91,92}. Treatment of motor neurons with TDP43^{A315T} caused no toxicity in the absence of microglia but neurotoxic effects were observed in co-culture⁹¹. TDP43 aggregates added to primary microglial cultures were internalized, also leading to the release of pro-inflammatory factors, including IL-1 β ⁹³.

Although these results are in line with the neurotoxic microglial response observed in SOD1 models of ALS, other TDP43 models have produced conflicting evidence. In a mouse model in which neuronal cytoplasmic TDP43 aggregation could be reversibly induced through the expression of TDP43 lacking the nuclear localization sequence, progressive motor dysfunction and motor neuron loss was associated with only subtle microglial changes⁹⁴. However, subsequent suppression of neuronal mutant TDP43 expression caused drastic proliferation of microglia, leading to clearance of neuronal TDP43 aggregates and functional recovery. Why the microglia were only able to proliferate once transgene expression was halted is unclear although possible mechanisms include direct inhibition of microglial activity by mutant TDP43 or effects of TDP43 on the expression of factors that drive neuronal–microglial crosstalk. The application of PLX3397, a CSF1R/kit inhibitor that depletes microglia, resulted in impaired motor function in these mice, indicating that microglia have a neuroprotective effect in this model.

Depletion of microglia eliminates the parallel neurotoxic and neuroprotective contributions of this cell population, making it difficult to evaluate the relative contributions of microglia to death and survival. A neuroprotective role for microglia was supported by a study in zebrafish that expressed human TDP43 in motor neurons⁹⁵. In these animals, ultraviolet radiation-induced neuronal injury led to neurodegeneration and TDP43 redistribution and release, which was ameliorated by microglial phagocytosis of dying neurons. However, such experiments cannot clarify whether microglia expressing mutant TDP43 or TDP43 aggregates can exert similar neuroprotective effects.

Aggregated TDP43 has been found in microglia in post mortem cortical tissue from people with ALS⁵⁷. Indirect evidence that TDP43 may be a regulator of microglial phagocytosis via the modulation of lysosomal function came from a mouse model of AD, in which conditional knockout of *Tardbp* in microglia was associated with increased phagocytic clearance of amyloid- β but enhanced synapse loss⁹⁶. Conversely, the increased microglial activation found in ALS post mortem tissue in relation to TDP43 pathology is associated with reduced levels of incidental amyloid- β ^{90,96}. Although the evidence is indirect, using amyloid clearance as a proxy

measure of microglial function, these studies support a model in which mutant TDP43 or TDP43 aggregates in human microglia might skew microglial properties towards neurotoxicity, either through gain-of-function toxicity due to cytoplasmic mislocalization or through loss-of-function toxicity due to loss of nuclear TDP43.

C9orf72 models. Microglia have the highest *C9orf72* expression of any cell type in the brain, and analysis of post mortem tissue from patients with ALS carrying the *C9orf72* expansion has confirmed microglial activation and upregulation of inflammatory pathways⁹⁷. Three hypotheses have been put forward to explain the pathophysiological effects of the hexanucleotide expansion in *C9orf72*: loss of function through reduced expression of *C9orf72* (haploinsufficiency) and gain-of-function toxicity due to the formation of RNA foci and/or through repeat-associated non-ATG-translated dipeptide repeat proteins (DPRs)⁹⁸. Animal models have been used to examine the role of microglia in the context of loss-of-function versus gain-of-function toxicity.

Most *C9orf72* transgenic mouse lines show widespread production of both DPRs and RNA foci and therefore demonstrate key gain-of-function mechanisms of the *C9orf72* mutation^{99–101}. However, these animals show no overt ALS motor phenotype, motor neuron degeneration or inflammatory response. Other studies have focused on the effects of selective expression of specific DPRs. Poly(GA) inclusions are the most abundant DPRs in patients with *C9orf72*-associated ALS¹⁰², and mouse models with the overexpression of poly(GA) under neuronal drivers demonstrate motor deficits as well as microglial and cytokine pathway activation towards a pro-inflammatory phenotype with pronounced activation of interferon-associated genes^{103–105}. Treatment of mice expressing poly(GA) under a *Thy1* promotor with an anti-GA antibody at the pre-symptomatic stage was reported to reduce poly(GA) inclusions, motor deficits, levels of neurofilament light chain (a marker for axonal damage), cytoplasmic TDP43 mislocalization and microglial activation¹⁰³. The authors hypothesized that anti-GA antibody treatment might neutralize secreted poly(GA) or enhance its degradation, thereby attenuating a harmful microglial response.

Mice expressing poly(PR), the most neurotoxic DPR according to in vitro analyses, showed no clear ALS phenotype or microglial activation in one study¹⁰⁵, but another study demonstrated motor neuron loss and microglial activation in these mice¹⁰⁶. Poly(GR)-expressing mice show neuronal cell loss and microgliosis^{107,108}. Findings from mouse models expressing other DPRs are yet to be published but the consensus seems to be that neuronal DPR inclusions induce microglial activation. Whether this activation leads to neuroprotection or enhanced toxicity remains unresolved.

The possible consequences of C9ORF72 protein loss of function for microglia have also been investigated. *C9orf72*^{+/−} and *C9orf72*^{−/−} mice failed to develop clinical ALS or to show evidence of neurodegeneration^{97,100,109}. However, *C9orf72*^{−/−} mice showed progressive splenomegaly, lymphadenopathy and upregulation of

pro-inflammatory cytokines in the periphery, suggesting a crucial role for *C9orf72* in the immune system^{97,100,110}. The reduced expression of *C9orf72* led to upregulation of microglial activation genes, increased levels of the pro-inflammatory cytokines IL-1 β and IL-6, and accumulation of lysosomal markers^{97,111}.

Increasing neuronal dysfunction and DPR release from diseased *C9orf72* neurons combined with *C9orf72* loss of function in microglia might skew the microglia towards a neurotoxic phenotype. This notion was underscored by a study in which the most severe neurotoxicity and the strongest microglial response were observed after hexanucleotide repeat expression on a homozygous *C9orf72* knockout background¹¹². However, as only a mild reduction in *C9ORF72* levels is observed in human *C9orf72* expansion carriers, the relevance of these findings to human ALS is unclear. Although *C9orf72* mutation-dependent formation of RNA foci has been described in microglia¹¹¹, the potential impact of these foci on microglial function has not been evaluated to date.

DPR inclusions have not yet been demonstrated in microglia in human post mortem tissue or in *C9orf72*-based animal models¹¹³. More authentic disease models, in which gain-of-function and loss-of-function toxicity can be concomitantly modelled in both neurons and microglia, will be needed to disentangle the effects of *C9orf72* mutations on microglia and the pathophysiological role of microglia in crosstalk with diseased neurons.

Summary. Clear evidence of microglial activation has been found in SOD1, TDP43 and *C9orf72* models of ALS. Neuroprotective microglial properties have been found in some experimental paradigms, in particular in early disease phases in SOD1 animals. However, the current consensus across these different models is that the expression of ALS-associated mutations in microglia, together with primary motor neuron damage and release of protein aggregates, generally results in a pro-inflammatory microglial phenotype. Evidence from SOD1 models indicates that this pro-inflammatory phenotype causes neurotoxicity via the release of soluble factors. However, in TDP43 and *C9orf72* models, direct neurotoxic effects of pro-inflammatory microglia have not yet been conclusively demonstrated. Further research is needed to refine our understanding of whether microglial activation can be targeted to combat neurodegeneration during ALS pathophysiology.

Astrocytes

SOD1 models. Astrocytosis is found in SOD1^{G37R} mice before the onset of a motor phenotype and increases during disease progression^{79,114}. Whether the astrocytosis precedes motor neuron degeneration is currently unclear. Astrocyte-restricted expression of SOD1^{G86R} in transgenic mice resulted in astrocytosis, but no ALS disease phenotype was evident¹¹⁵. The selective deletion of SOD1^{G85R} from astrocytes delayed disease onset and slowed early disease progression¹¹⁶. By contrast, SOD1^{G37R} deletion in astrocytes did not affect disease onset but substantially slowed later disease

progression¹¹⁴. Consistent with these findings, transplantation of wild type glial precursors that differentiated into astrocytes in the spinal cord of SOD1^{G93A} rats prolonged survival and disease duration and attenuated motor neuron degeneration¹¹⁷, whereas transplanted SOD1^{G93A} astrocyte precursors induced motor neuron death and motor dysfunction in wild-type rats¹¹⁸. These data indicate that astrocyte activation contributes to neurotoxicity during ALS pathogenesis in SOD1 models.

In co-culture experiments, the expression of various forms of mSOD1 in astrocytes led to the death of primary and mouse stem cell-derived motor neurons via the release of soluble factors, the identity of which has yet to be determined^{119–123}. The effect was more severe when the astrocytes were co-cultured with mSOD1-expressing motor neurons. By contrast, knockdown of mSOD1 attenuated astrocyte-mediated toxicity towards motor neurons¹²³. Pathways that have been implicated in the neurotoxicity of mSOD1-expressing astrocytes include dysregulated pro-nerve growth factor release, increased expression and secretion of molecules related to oxidative stress, dysregulated transforming growth factor- β (TGF β) signalling and elevated pro-inflammatory gene expression^{122,124–130} (FIG. 1).

According to a study that used a combination of bioinformatic prediction models and experimental validation, SOD1^{G93A}-expressing astrocytes can kill wild-type motor neurons through amyloid precursor protein (APP) fragment release and activation of death receptor 6 (DR6) on motor neurons — an effect that is prevented by DR6 knockdown or gene knockout¹³¹. These findings suggest that expression of mSOD1 in astrocytes leads to gain-of-function, non-cell-autonomous neurotoxicity. Loss-of-function effects, including a reduction in homeostatic function with regard to lactate provision, altered release of neurotrophic factors and attenuated protection of neurons from glutamate-induced excitotoxicity, have also been described in mSOD1-expressing astrocytes^{72,125,128,132,133} (FIG. 1). Restoring these functions improved motor neuron survival⁷².

Together, these results indicate that similarly to microglia, astrocytes can adopt neurotoxic properties in SOD1 models, which, in concert with mSOD1-induced primary neuronal damage, actively contribute to disease pathogenesis. In recent years, the crosstalk between astrocytes and other non-neuronal cells has been studied in more detail. Microglia were found to induce the conversion of quiescent astrocytes into a pro-inflammatory A1 phenotype through the release of IL-1 α , TNF and complement component C1q⁴² (FIG. 1). A triple knockout of these factors in SOD1^{G93A} mice led to a drastic reduction in the number of reactive astrocytes, thereby improving lifespan and delaying motor neuron loss and disease progression¹³⁰. Conversely, upregulation of TGF β in astrocytes was reported to interfere with the neuroprotective function of microglia and T cells in SOD1^{G93A} mice, thereby accelerating disease progression¹²⁹. Transplantation of SOD1^{G93A} astrocytes into the spinal cord of wild-type rats led to activation of microglia, and blockade of microglial activation in this model using minocycline partially rescued motor neuron loss¹¹⁸. In other studies, microglial activation was shown to be

delayed by the deletion of SOD1^{G37R} or SOD1^{G85R} from astrocytes^{114,116}.

Emerging evidence from experimental models indicates that astrocytes adopt a pro-inflammatory phenotype in SOD1-associated ALS, influenced by reciprocal interaction with other non-neuronal cells. However, a detailed analysis of astrocyte activation over the disease course remains to be performed, particularly with regard to crosstalk with microglia. Bearing in mind the expression of anti-inflammatory or neuroprotective markers in microglia reported at early disease stages in SOD1 models, future studies should also evaluate the potential neuroprotective properties of astrocytes.

TDP43 models. Experiments in transgenic animals expressing mutant forms of TDP43, such as TDP43^{Q331K}, TDP43^{M337V} or TDP43^{A315T}, have provided insights into the role of astrocytes in ALS pathogenesis. In TDP43^{Q331K} transgenic mice, selective downregulation of this protein in motor neurons delayed disease onset but did not alter disease progression or prevent astrogliosis¹³⁴, suggesting that astrocytes were contributing to pathogenesis. The astrocyte-restricted expression of TDP43^{M337V} in rats led to astrogliosis, which caused progressive non-cell-autonomous motor neuron loss and paralysis, accompanied by depletion of glutamate transporters on astrocytes, induction of the neurotoxic factor lipocalin 2 and microglial activation¹³⁵ (FIG. 1). Dysregulated astrocytic metabolism, possibly leading to impaired provision of lactate to neurons, was observed in primary rat astrocytes following the expression of inclusion-forming carboxy-terminal fragments of TDP43 (REF.¹³⁶). In addition, primary astrocytes from TDP43^{Q331K}-expressing mice showed decreased levels of the antioxidant glutathione, and astrocyte-conditioned medium from TDP43^{A315T}-expressing mice caused primary rat motor neuron death in a nitrooxidative stress-dependent manner^{137,138} (FIG. 1). A detailed assessment of TDP43^{M337V}-expressing primary rat astrocytes and spinal cord tissue from TDP43^{M337V} transgenic rats revealed downregulation of neurotrophic genes and upregulation of neurotoxic genes such as those encoding lipocalin 2 and chitinase-3-like protein 1 (CHI3L1)¹³⁹. Lipocalin 2 was also released from astrocytes in organotypic cultures of brain slices from rats with selective TDP43^{M337V} expression in forebrain neurons and in the spinal cord of transgenic rats with motor neuron-specific TDP43^{M337V} expression¹⁴⁰. Levels of this neurotoxic factor increased with progressive neurodegeneration.

These findings were all associated with overexpression of TDP43; however, similar results have been observed with reduced TDP43 levels. Conditional motor neuron-specific *Tardbp* knockout mice developed age-dependent progressive motor impairment and motor neuron degeneration and showed progressive astrogliosis in the spinal cord^{141,142}. In transgenic mice with RNA interference-mediated TDP43 knockdown, in which TDP43 levels seemed to be preferentially reduced in astrocytes instead of in motor neurons in the spinal cord, motor neuron degeneration, motor weakness and paralysis were observed¹⁴³. Similarly, knockdown of TDP43 in primary rat astrocytes led to the activation

of pro-inflammatory mediators, including lipocalin 2 (REF.¹⁴⁴). Collectively, these findings point to a crucial effect of TDP43 dysfunction in astrocytes, with both TDP43 mutations and depletion leading to neurotoxic properties. However, TDP43 knockdown is unlikely to accurately model every aspect of TDP43 dysfunction in ALS. TDP43 deletion is embryonic lethal¹⁴⁵ and postnatal TDP43 deletion also results in rapid death¹⁴⁶, indicating that TDP43 levels are tightly regulated and that the protein has an essential cellular role. Therefore, phenotypes resulting from TDP43 knockdown might merely be the consequence of altering the levels of a crucial constitutively expressed protein.

Co-culture of iPSC-derived astrocytes expressing TDP43^{M337V} with wild-type or TDP43^{M337V}-expressing neurons did not affect neuronal survival¹⁴⁷. Similarly, primary astrocytes overexpressing TDP43^{A315T} or lacking TDP43 were not toxic to wild-type motor neurons in co-culture or after transplantation of their precursors into the spinal cord of wild-type mice¹⁴⁸. In co-culture and conditioned media paradigms, iPSC-derived control astrocytes had a protective effect against seeded aggregation of TDP43 in iPSC-derived control motor neurons by reducing TDP43 mislocalization and cell death¹⁴⁹. However, whether control astrocytes that do not express mutant TDP43 or TDP43 aggregates represent an accurate disease model is questionable.

The currently available evidence points towards both gain-of-function and loss-of-function toxicity of astrocytes towards neurons with alterations in TDP43, in line with the results from SOD1 models. Divergent findings might be attributable to different expression levels of TDP43 (REF.¹²⁸), a lack of modelling of crosstalk with microglia and other non-neuronal cells, or differences between rodent and human microglia. Further studies using mutant TDP43-expressing iPSC-derived astrocytes are warranted to provide clarification.

C9orf72 models. Expression of G₄C₂ repeats and poly(GA) in the mouse CNS leads to astrogliosis, which is suggested to precede neurodegeneration^{150–152}. Induced astrocytes (i-astrocytes) directly differentiated from *C9orf72* expansion-expressing fibroblasts caused death of co-cultured mouse embryonic stem cell-derived motor neurons, probably through a toxic gain-of-function mechanism^{153,154}. Depletion of SRSF1, a nuclear export adaptor for the transport of *C9orf72* transcripts, led to a reduction in cytoplasmic RNA foci in these i-astrocytes and rescued motor neuron death, thereby directly linking the *C9orf72* expansion with the adoption of neurotoxic properties by astrocytes¹⁵⁴. In another study, iPSC-derived *C9orf72* astrocytes showed increased oxidative stress, and reduced release of antioxidants, and their conditioned media reduced the viability of mouse cortical neurons and human embryonic stem cell-derived motor neurons, possibly via increased neuronal oxidative stress¹⁵⁵ (FIG. 1).

In contrast to direct toxicity, dysfunctional metabolism of adenosine, fructose and glycogen was reported in *C9orf72* i-astrocytes and was associated with increased death of mouse motor neurons in co-culture^{156,157}. This neurotoxicity could be ameliorated by rescuing deficits

in adenosine metabolism¹⁵⁶. Altered glutamate content was found in iPSC-derived astrocytes carrying a *C9orf72* expansion, probably through a C9ORF72 loss-of-function effect on astrocytes¹⁵⁸. Extracellular vesicles also seem to play a role in the neurotoxic phenotype of *C9orf72* i-astrocytes. The microRNA miR-494-3p, which acts as a negative regulator of motor neuronal semaphorin 3A, among other targets, and is secreted via extracellular vesicles, was found to be downregulated in conditioned medium from these cells, and restoring its levels in conditioned medium improved motor neuron survival and neurite outgrowth¹⁵⁹. Broader roles for extracellular vesicles in reflecting the pathology and potentially influencing the propagation of neurodegenerative disorders are recognized¹⁶⁰. In a further study, wild-type iPSC-derived motor neurons exhibited electrophysiological dysfunction when co-cultured with iPSC-derived *C9orf72* astrocytes but the detailed mechanisms mediating this toxicity were not explored¹⁶¹.

The evidence suggests that astrocytes adopt neurotoxic properties in *C9orf72* models. Further studies are needed to shed light on the detailed mechanisms through which gain-of-function and loss-of-function mechanisms act in combination and should focus on the identification of secreted cytotoxic factors and contact-dependent mechanisms of astrocyte-dependent cytotoxicity.

Summary. Clear evidence of astrocytic activation has been found in SOD1, TDP43 and *C9orf72* models of ALS, and most studies across the three model systems support a neurotoxic astrocyte phenotype. This neurotoxicity seems to be mediated by the release of neurotoxic factors and a failure of astrocytes in their supportive functions to neurons. In contrast to microglia, astrocytes have no apparent neuroprotective role in the early stages of the disease. The neurotoxic effects of astrocytes could be mediated by crosstalk with other non-neuronal cells; preliminary evidence suggests that astrocytes are skewed towards neurotoxicity by pro-inflammatory microglia. Additional studies that model multicellular interactions will be needed to disentangle the direct effect of ALS-associated mutations on astrocytes and the pro-inflammatory contribution of other cells to astrocyte toxicity.

Oligodendrocytes and NG2 glia

SOD1 models. In SOD1^{G93A} transgenic mice, degeneration and loss of oligodendrocytes was observed in the spinal cord before disease onset^{162,163}. Overall oligodendrocyte numbers remained constant owing to increased proliferation of NG2 glia, although incomplete and failed differentiation of these cells resulted in progressive demyelination. Selective Cre-mediated removal of SOD1^{G37R} from NG2 glia delayed disease onset and prolonged survival, indicating a detrimental role of oligodendroglia in SOD1 models¹⁶². Interestingly, this intervention also led to delayed astrocytic and microglial activation. In mice expressing SOD1^{G37R} in motor neurons and oligodendrocytes, motor neuron degeneration could be mitigated by the proximity of other cells not expressing this mutant form of SOD1, suggesting

that cells other than oligodendrocytes contribute to neurodegeneration¹⁶⁴. However, primary mouse oligodendrocytes expressing SOD1^{G93A} were found to directly cause wild-type mouse motor neuron hyperexcitability and death, and similar motor neuron toxicity was observed in co-culture with iPSC-derived and induced neural precursor cell-derived oligodendrocytes from patients with sporadic or familial ALS¹⁶⁵.

Reduced levels of monocarboxylate transporter 1 (MCT1) have been found in the spinal cord of SOD1^{G93A} transgenic mice and zebrafish and in post mortem tissue from patients with ALS^{163,166,167}. Deletion of SOD1^{G93A} from NG2 glia restored MCT1 levels, indicating that this mutant protein causes neurotoxicity by impairing oligodendroglial support mechanisms¹⁶² (FIG. 1). Furthermore, impaired lactate production and release as well as reduced levels of MCT1 were found in fibroblast-derived oligodendrocytes from SOD1^{G93A} mice and patients carrying the recessive SOD1^{D90A} ALS-associated mutation¹⁶⁵. Restoration of lactate levels fully rescued the toxicity resulting from the addition of SOD1^{G93A} oligodendrocyte-conditioned medium to motor neurons, but only partly rescued decreased motor neuron survival in co-culture, implying the existence of additional close contact-dependent mechanisms¹⁶⁵. Although SOD1^{G93A} knockdown in NG2 glia efficiently rescued motor neuron death and elevated lactate levels, it did not modulate the toxicity exerted by differentiated oligodendrocytes, reinforcing a role for early dysfunction in the oligodendroglial lineage in SOD1-associated ALS¹⁶⁵.

Translating ribosome affinity purification and high-throughput RNA sequencing of SOD1^{G37R} transgenic mice identified early gene dysregulation in motor neurons, followed by changes in astrocytes and oligodendrocytes¹⁶⁸. In oligodendrocytes, changes became obvious at an early symptomatic disease stage. No changes in *MCT1* gene expression were found at this stage, but membrane and lipid signalling defects and upregulation of ETS transcription factors were observed.

Collectively, the evidence from SOD1 models implicates early oligodendrocyte dysfunction in ALS pathogenesis, primarily via reduced metabolic support to neurons. Additional mechanisms of toxicity are likely to have additional roles and should be investigated in future studies. In particular, the interplay between oligodendrocytes and other glial cells remains to be characterized in more detail.

TDP43 models. Research into the role of oligodendrocytes in TDP43 models is limited. Similarly to mutant SOD1-derived oligodendrocytes, TDP43^{G298S} fibroblast-derived oligodendrocytes caused motor neuron death in co-culture via reduced release of lactate¹⁶⁵. Further studies will be required to elucidate the effects of TDP43 aggregates and mutations on oligodendrocyte function.

C9orf72 models. Oligodendrocytes have also been successfully derived from patients carrying the *C9orf72* expansion^{165,169} but knowledge of their functional role in this context is limited. In iPSC-derived

oligodendrocytes, RNA foci were observed but no DPR formation or defects in maturation were apparent¹⁶⁹. Induced neural precursor cell-derived oligodendrocytes from patients with *C9orf72* expansion-associated ALS caused motor neuron toxicity in co-culture¹⁶⁵. However, in contrast to mutations in *SOD1* and *TARDBP*, this toxicity was not mediated via decreased lactate release, although soluble factors were probably involved. Further studies are needed to fully characterize the effects of the *C9orf72* expansion on oligodendrocytes and the non-cell-autonomous influence of these cells on motor neurons.

Summary. Most of the evidence for a role for oligodendrocytes in experimental models of ALS has come from *SOD1* models, and additional studies on their role in TDP43 and *C9orf72* models are needed. The currently available data support a neurotoxic effect of oligodendrocytes, which occurs early in the disease course and is connected to a failure in maturation of NG2 glial precursors. Reduced metabolic support of neurons through reduced release of lactate seems to be a key mechanism of oligodendroglial toxicity, but the possibility of additional means of toxicity in crosstalk with other non-neuronal cells also warrants investigation.

Non-glial non-neuronal cells

Monocytes and macrophages. In *SOD1*^{G93A} mice, microglia, neurons and other cells showed increased expression of genes encoding chemotactic proteins such as C-C motif chemokine 2 (CCL2), which was thought to lead to the recruitment of peripheral monocytes to the spinal cord¹⁷⁰ (FIG. 2). The monocytes showed a pro-inflammatory profile before disease onset, and targeting of these cells using intraperitoneal Ly6C antibody treatment skewed them towards an anti-inflammatory phenotype, resulting in delayed disease onset, extended survival and attenuated neuronal loss¹⁷⁰. However, as the antibody was injected peripherally in this model, it is difficult to determine whether these beneficial effects were mainly due to reduced monocyte infiltration or to a general immunosuppressive effect at the periphery, as blood monocytes from patients with ALS and monocyte-derived macrophages have a pro-inflammatory profile^{170–172}.

Conversely, a neuroprotective role for monocyte-derived cells was indicated by a correlation between the number of surviving motor neurons and the presence of peripheral myeloid cells in the spinal cord at early disease stages in *SOD1*^{G93A} transgenic mice¹⁷³. Furthermore, promotion of monocyte invasion using human immunoglobulins or fusion proteins was associated with increased motor neuron survival and delayed disease onset. These apparently contradictory findings might be resolved by a model similar to the early neuroprotective but late neurotoxic properties reported for microglia in *SOD1* models; co-culture studies of neurons and macrophages would help to address this issue.

Numerous infiltrating monocytes were observed within and around the blood vessels in the brain in a TDP43^{A315T} mouse model and in patients with ALS who had TDP43 pathology⁹⁰. However, the assessment of

monocyte and macrophage function in TDP43 models remains incomplete.

In models based on *C9orf72*, loss of function of the C9ORF72 protein resulted in a pro-inflammatory state in peripheral monocytes and macrophages, along with dysfunctional lysosomal trafficking and impaired autophagy^{97,174}. These effects led to STING-mediated hyperactivation of interferon signalling¹⁷⁴, indicating potential neurotoxic properties. The results of experiments investigating *C9orf72* gain-of-function toxicity are yet to be published.

In summary, important physiological roles of CNS-associated macrophages are beginning to emerge, including evidence for a pathophysiological role in AD¹⁷⁵. To shed further light on the pathophysiological role of peripheral monocytes and macrophages in ALS and their effects on neurons, iPSC-derived macrophages might prove to be a useful tool.

T cells. In *SOD1*^{G93A} mice, both CD4⁺ and CD8⁺ T cells infiltrate the spinal cord during disease progression^{66,176,177}. A lack of functional T cells or CD4⁺ T cells increased pro-inflammatory mediator release from microglia and astrocytes, decreased the levels of anti-inflammatory and trophic factors, and accelerated disease progression^{176,177}. Reconstitution with bone marrow transplants rescued these deficits and shifted microglia and astrocytes towards a neuroprotective phenotype¹⁷⁷, indicating a neuroprotective role for T cells in *SOD1*-associated ALS.

Closer evaluation of the CD4⁺ T cell population revealed that, at early, slowly progressing disease stages, the numbers of *SOD1*^{G93A}-expressing regulatory T (T_{reg}) cells increased, inducing a neuroprotective M2 microglial phenotype via IL-4 release and suppression of pro-inflammatory T helper 1 cells^{178–180}. At later, fast-progressing stages, the T_{reg} cells become depleted or dysfunctional and T helper 1 cells and M1 microglia predominate (FIG. 2). In agreement with these findings, expansion of T_{reg} cells in *SOD1*^{G93A}-expressing mice reduced microglial and astrocytic activation, preserved motor neuron size and prolonged survival. Decreased T_{reg} numbers or greater dysfunction of these cells in the blood of patients with ALS were both associated with faster rates of disease progression^{178,180–182}. We do not yet know how this switch in T cell properties between early and later stages is mediated.

CD8⁺ T cells have been relatively understudied despite being one of the main infiltrating populations in the spinal cord in *SOD1*^{G93A} mice¹⁸³. β2-Microglobulin-deficient *SOD1*^{G93A} mice, which lack MHC class I expression and are deficient in mature CD8⁺ T cells, showed delayed forelimb paralysis and prolonged survival¹⁸⁴. In *SOD1*^{G93A} mice, a *Cd8a* knockout, which prevented the generation of functional CD8⁺ T cells, improved spinal motor neuron survival but did not alter disease onset or motor performance¹⁸⁵. In other studies, intraperitoneal injection of an anti-CD8 antibody in *SOD1*^{G93A} mice partially eliminated CD8⁺ T cell infiltration but had no effect on animal survival¹⁸³ or had a less pronounced effect on counteracting CD8⁺ T cell infiltration and improving motor neuron survival¹⁸⁵ compared

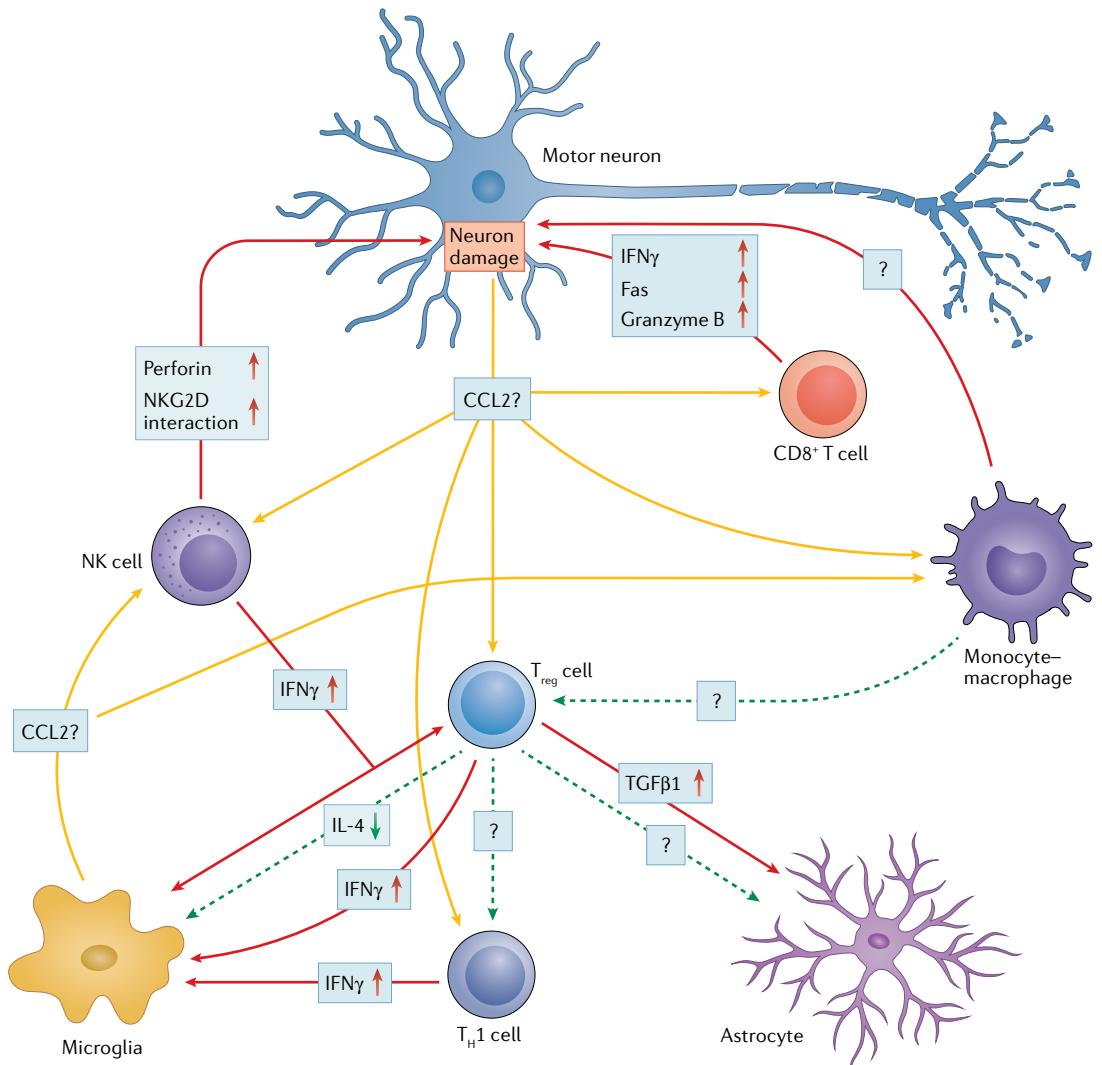


Fig. 2 | Pathophysiological roles of peripheral non-neuronal cells in amyotrophic lateral sclerosis. Peripheral non-neuronal cells, including T cells, natural killer (NK) cells and cells of the monocyte–macrophage lineage, are recruited to the CNS in response to neuronal damage associated with dysfunction and aggregation of proteins such as superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (encoded by *TARDBP*) and dipeptide repeat proteins. Chemotactic factors, such as C–C motif chemokine 2 (CCL2), have been implicated in this process (yellow arrows). The expression of mutations in genes associated with amyotrophic lateral sclerosis, such as *SOD1*, *TARDBP* and *C9orf72*, is thought to cause these non-neuronal cells to adopt neurotoxic properties, which manifest as both gain-of-function toxicity (red arrows) and loss of supportive functions (green dashed arrows). Crosstalk between different cell types exacerbates this neurotoxicity. The mechanisms depicted are derived from various models discussed in this Review and have not all been identified in every model. TGF β 1, transforming growth factor β 1; T_H1 cell, T helper 1 cell; T_{reg} cell, regulatory T cell.

with SOD1^{G93A} *Cd8a*-knockout mice. The authors of the latter study hypothesized that the lower protective effect of the antibody treatment might be attributable to the preferential action of the antibody on naive rather than infiltrating CD8⁺ T cells. Importantly, in co-culture, SOD1^{G93A}-expressing CD8⁺ T cells caused the death of wild-type and SOD1^{G93A}-expressing motor neurons through IFN γ release alongside Fas and granzyme death pathway-mediated mechanisms¹⁸⁵ (FIG. 2).

In summary, the findings from SOD1 models suggest a neuroprotective role for CD4⁺ T cells in cross-talk with microglia and astrocytes, the failure of which, together with CD8⁺ T cell activation, leads to neurotoxicity. Evidence regarding the role of T cells

in other experimental models of ALS is scarce. In *C9orf72*-deficient mice, T cell activation has been demonstrated^{174,186} but the functional role of these cells remains to be determined, particularly in the context of gain-of-toxicity hypotheses. In a study published in 2020, iPSC-derived M2 macrophages from patients with sporadic or *C9orf72*-associated ALS induced and sustained anti-inflammatory T_{reg} function, whereas pro-inflammatory M1 macrophages and cytotoxic T cells were suppressed¹⁸⁷. Hence, an interesting approach would be to explore the effects of these cells on microglial and astrocytic function, in particular in crosstalk with motor neurons, and to see whether the neuroprotective effects are also observed *in vivo*. Further

studies in multicellular models using patient-derived T cells are warranted to shed more light on the functional role of these cells in ALS pathogenesis.

NK cells. Aberrant numbers of NK cells have been found in the peripheral blood and CNS of patients with ALS, and SOD1^{G93A}-expressing mice display large numbers of NK cells in the spinal cord^{66,176,188,189}. Recruitment of NK cells to the CNS was demonstrated to be dependent on CCL2 (REF.⁶⁶) (FIG. 2). In SOD1^{G93A} mice, downregulation of NK cell activity led to the recruitment of T cells into the spinal cord before the onset of clinical symptoms, and attenuated motor neuron loss, reduced astrogliosis, delayed disease onset and improved lifespan¹⁸⁸. Anti-NK1.1 antibody-mediated reduction of NK cell infiltration into the spinal cord delayed motor neuron impairment and increased survival in TDP43^{A315T}-expressing mice. This intervention also attenuated early motor neuron loss in SOD1^{G93A} transgenic mice⁶⁶ but had no effect on survival when administered at later disease stages^{66,183}, indicating an early role for NK cells in the pathophysiology of ALS in SOD1 models. Both wild-type and SOD1^{G93A}-expressing NK cells were found to be neurotoxic in co-culture but only to SOD1^{G93A}-expressing motor neurons⁶⁶. This effect was dependent on the release of toxic factors, including perforin, and on contact-dependent mechanisms such as the expression of NKG2D ligands on motor neurons (FIG. 2). Furthermore, NK cell depletion induced an anti-inflammatory microglial phenotype and increased the number of T_{reg} cells in the spinal cord of SOD1^{G93A} mice via reduced IFN γ release⁶⁶ (FIG. 2).

Together, these data provide initial evidence for a neurotoxic role for NK cells, in crosstalk with T cells and microglia, in SOD1-associated ALS. Additional studies, also including mutant TDP43 and C9orf72 expansion-based models of ALS, will be required to provide further insight into their functional role in ALS pathogenesis.

Biomarkers from non-neuronal cells

Non-neuronal cell-derived molecules can be detected and measured in the cerebrospinal fluid (CSF) or peripheral blood of patients with ALS. Levels of these molecules, particularly if measured longitudinally, provide a window into the pathogenic role of non-neuronal cells in humans, and their prognostic and pharmacodynamic biomarker potential is increasingly being evaluated. Although the list of candidates continues to grow (reviewed in more detail elsewhere^{190,191}), we focus on CSF biochemical markers that were consistently shown to be upregulated in patients with ALS across multiple different studies and laboratories.

Multiple studies have demonstrated an increased abundance of chitotriosidase 1 (CHIT1), CHI3L1 and chitinase-3-like protein 2 (CHI3L2) in the CSF of patients with ALS^{10,192–200}. The CSF levels of these proteins correlated with disease progression rate and with levels of phosphorylated neurofilament heavy chain — a proxy for axonal damage¹⁰. CHIT1 is an enzymatically active hydrolase but CHI3L1 and CHI3L2 possess no catalytic motif²⁰¹. The release of these three proteins

could be related to the presence of chitin-like polymers in the ALS brain, as observed in amyloid plaques in AD²⁰², or to a newly acquired but thus far unknown role in the human immune system.

CHIT1 is expressed in macrophages and microglia^{192,193}. CSF levels of this protein are higher in ALS than in other neurodegenerative disorders and are associated with shortened survival and a faster disease progression rate^{10,193,195,198,203}. In a longitudinal assessment of phenoconverters, CSF CHIT1 levels and activity were relatively low in the pre-symptomatic period but showed a sharp increase between the late pre-symptomatic and early symptomatic phases^{196,197}. In a study published in 2020, intrathecal injection of CHIT1 into rats was associated with increased microglial numbers and activation, astrogliosis, release of pro-inflammatory cytokines and motor neuron loss¹⁹⁴. These studies provide emerging evidence of an important role for microglia and macrophages in the ALS disease course in humans, probably as a secondary toxic response to damage rather than as a primary disease driver. The studies also underscore the usefulness of CHIT1 as a biomarker best suited to disease monitoring and, potentially, for the evaluation of treatment response. Further studies will be required to uncover the trigger for CHIT1 release, its cellular and molecular targets, and its cellular expression at different stages of ALS.

CHI3L1 seems to be predominantly expressed in astrocytes in patients with ALS^{196,203,204}. In such individuals, CSF CHI3L1 levels increased over time, correlated with upper motor neuron symptoms and cognitive impairment, and showed an inverse correlation with patient survival^{10,195,198,199,204}. In asymptomatic mutation carriers, CHI3L1 levels were unchanged compared with healthy control individuals and, in contrast to CHIT1, longitudinal assessment revealed no striking difference between the late pre-symptomatic and early symptomatic phases^{196,197}. Overexpression of TDP43^{M337V} in primary astrocytes led to the upregulation of CHI3L1, and administration of synthetic CHI3L1 induced primary neuronal death¹⁹⁹. Although this evidence for CHI3L1 release corroborates the activation and putative neurotoxic role of astrocytes observed in experimental models of ALS, it argues against a pronounced pathogenic function during the early disease course. Future studies will need to elucidate the trigger for CHI3L1 release and its molecular targets in more detail.

IL-6 has been identified as another astrocyte-dependent biomarker that is associated with early pathology in patients with ALS²⁰⁵. In the blood of people with sporadic ALS, levels of IL-6 in astrocyte-derived exosomes were increased and positively associated with the rate of disease progression but only in individuals with a disease duration of <12 months.

CHI3L2 has not been extensively studied but its expression has been observed in macrophages^{10,201}, and CSF levels correlate with the ALS progression rate^{10,195}. Similar to CHI3L1, CHI3L2 levels were not increased in asymptomatic mutation carriers, and longitudinal assessment showed no striking difference between the late pre-symptomatic and early symptomatic phases¹⁹⁶. Owing to a lack of mechanistic studies, the

biological relevance of CHI3L2 in crosstalk with neurons is currently uncertain.

CCL2 has also been studied as a marker of non-neuronal cell involvement in ALS. In experimental models, expression of CCL2 in cells involved in ALS pathogenesis, including in motor neurons, astrocytes and microglia, is thought to result in the recruitment of CCL2-expressing cells such as microglia, peripheral macrophages, T cells and NK cells^{34,66,90,206} (FIG. 2). Elevated levels of CCL2 have been reported in the CSF of patients with ALS and were inversely correlated with survival, but no consistent correlation with the disease progression rate was found^{198,207–212}. The upregulation of CCL2 in patients with ALS probably reflects the glial activation and recruitment of peripheral immune cells into the CNS that is observed in experimental models. However, its biological relevance is unclear as both neurotoxic and neuroprotective properties of CCL2-dependent cell infiltration have been demonstrated.

Conclusions and future directions

An important role for non-neuronal cells in ALS pathogenesis is now clear, and evidence from experimental models largely replicates the activation of glial cells and infiltration of peripheral immune cells found in neuroimaging, post mortem and biomarker studies in humans. Some inconsistency has been observed across the different experimental models, and SOD1 model-based evidence might have limited relevance to the majority of ALS cases owing to the lack of TDP43 aggregates in these models. Nevertheless, a common theme is emerging, whereby cell-autonomous neuronal dysfunction is exacerbated by non-cell-autonomous toxicity.

Initial analyses of cell–cell interactions in iPSC-derived human cells have confirmed some of the key findings from rodent models. Primary neuronal

damage is likely to initiate the onset of disease and, over the disease course, the combination of a dysregulated and pro-inflammatory non-neuronal cell response to neuronal damage, together with primary failure of the supportive roles of non-neuronal cells, influences progression. However, at early disease stages, microglia and regulatory T cells seem to exert some neuroprotection in experimental models.

Differences in the specific mechanisms of non-neuronal toxicity in SOD1, TDP43 and *C9orf72* experimental models support the idea of therapeutic approaches targeted according to genotype and might partially explain why multiple clinical trials involving non-specific targeting of the immune system were unsuccessful. However, several new therapeutics targeting non-neuronal cells are currently in clinical trials (reviewed elsewhere²¹³). For instance, masitinib, a tyrosine kinase inhibitor that targets microglial, macrophage and mast cell activation, slowed disease progression in patients with ALS in a phase II/III trial when used as an add-on to riluzole²¹⁴.

Future studies should include the differentiation of further non-neuronal cell types and focus on the creation and usage of human-derived multicellular models to more accurately model the disease as well as the crosstalk between cells found in vivo. The generation of accurate disease models should facilitate drug screening in a more physiological environment. In addition, an improved understanding of the nuanced pathophysiological roles of non-neuronal cells will guide the repurposing of licensed drugs as well as novel drug development. Biomarkers derived from non-neuronal cells show promise for the future assessment of emerging therapeutic candidates.

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B. F. V. researched data for the article and drafted the manuscript. B. F. V., E. G., S. A. C., K. T. and M. R. T. made substantial contributions to discussions of the content. E. G., A. G. T., O. A., D. C. A., S. A. C., K. T. and M. R. T. reviewed and edited the manuscript before submission.

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Recent advances in understanding amyotrophic lateral sclerosis and emerging therapies

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterized by degeneration of both upper and lower motor neurons and subsequent progressive loss of muscle function. Within the last decade, significant progress has been made in the understanding of the etiology and pathobiology of the disease; however, treatment options remain limited and only two drugs, which exert a modest effect on survival, are approved for ALS treatment in the US. Therefore, the search for effective ALS therapies continues, and over 60 clinical trials are in progress for patients with ALS and other therapeutics are at the pre-clinical stage of development. Recent advances in understanding the genetics, pathology, and molecular mechanisms of ALS have led to the identification of novel targets and strategies that are being used in emerging ALS therapeutic interventions. Here, we review the current status and mechanisms of action of a selection of emerging ALS therapies in pre-clinical or early clinical development, including gene therapy, immunotherapy, and strategies that target neuroinflammation, phase separation, and protein clearance.

Keywords

ALS, Amyotrophic lateral sclerosis, ALS therapeutics, clinical trials, gene therapy, immunotherapy, platform trials

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive loss of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and anterior horn of the spinal cord. Clinically, this manifests as weakness and atrophy of voluntary muscles, resulting in the loss of the ability to walk, speak, and swallow as the disease progresses¹. Additionally, it is estimated that 20 to 50% of patients with ALS present with cognitive impairments that would meet the diagnostic criteria for probable or definite frontotemporal dementia (FTD)². Disease prognosis is poor; most patients with ALS die within 3 to 5 years after the diagnosis; this is typically due to respiratory failure resulting from the progressive weakening of respiratory muscles¹. Pathologically, more than 97% of ALS cases are characterized by inclusions of the nuclear RNA-binding protein (RBP) TAR DNA-binding protein 43 (TDP-43) in the cytoplasm of neurons and glia. The few cases that do not exhibit TDP-43 pathology present with pathological inclusions of either the superoxide dismutase (SOD1) or fused in sarcoma (FUS) protein³.

ALS can be broadly separated into two categories—familial ALS (fALS) (10–15%) and sporadic ALS (sALS) (85–90%)—depending on whether there is a family history of ALS⁴. At least 50 potential causative or disease-modifying genes have been linked to ALS⁵, but a G₄C₂ hexanucleotide repeat expansion in the *C9orf72* gene is the most common ALS-causing mutation identified to date, accounting for about 40% of fALS cases and 6 to 8% of sALS cases in Caucasian populations⁶. Other commonly mutated genes in ALS include *SOD1*, *TARDBP*, or *FUS*, and variants in other disease-causing ALS-linked genes are relatively uncommon^{4,7}. ALS linked to mutations in these genes is hypothesized to be caused by loss-of-function or toxic gain-of-function of the protein products of these genes and subsequent downstream cellular consequences⁸. In contrast, three non-mutually exclusive mechanisms have been proposed as a potential cause of *C9orf72*-linked ALS: loss-of-function of the *C9orf72* protein, sequestration of essential RBPs by foci containing G₄C₂-containing RNA, and toxicity induced by one or more dipeptide repeat (DPR) proteins^{9,10}. These DPR proteins are produced as a result of bidirectional repeat-associated non-AUG initiated (RAN) translation of the G₄C₂ hexanucleotide repeat expansion and accumulate in p62-positive, TDP-43-negative pathological aggregates in *C9orf72* repeat expansion carriers^{11–13}.

Although research into genetic forms of ALS has implicated several dysregulated biological pathways in ALS pathogenesis, the precise mechanism of disease is unknown. The lack of a defined cause of disease is reflected in the number of currently available therapeutics for ALS. Despite many previous and ongoing clinical trials of various drugs targeting different biological mechanisms (comprehensively reviewed in 14 and 15), only two approved treatments are currently in widespread use for ALS: the anti-excitotoxic drug riluzole and edaravone, whose mechanism of action is unknown but presumed to be through its antioxidant properties^{16,17}.

Here, we present a collection of emerging therapeutic approaches based on a selection of recent novel pre-clinical discoveries covering a broad range of ALS disease mechanisms or genetics (or both). A summary of the therapeutic approaches that are discussed in this review and have entered clinical trials is provided in Table 1.

Gene therapies

The heritability of fALS makes the disease a promising candidate for gene therapy; as such, clinical trials that use the principles of gene therapy are under way in fALS patients carrying *SOD1* (NCT02623699) and *C9orf72* (NCT03626012) mutations. Gene therapy is a broad term that can refer to reducing the expression of an RNA of the disease-causing gene, delivering a “normal” copy of a mutated gene to replace its expression, or modifying the mutant genome to “correct” a genetic defect¹⁸. All three of these genetic therapeutics have shown efficacy in experimental models, raising the possibility of successful gene therapy trials for fALS.

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short (13–25 nucleotides), synthetic, and single-stranded oligonucleotides that are designed to bind to specific sequences of RNA to reduce, restore, or modify RNA or protein expression¹⁹. The oligonucleotides typically contain chemical modifications that act to enhance pharmacokinetic properties and target binding affinity and the tolerability profile of the ASOs^{20,21}. Depending on their target, binding sequence, and chemistry, single-stranded ASOs can modulate gene expression or modify pre-mRNA splicing through several distinct mechanisms of action, including target degradation, translational arrest, inhibition of RBP binding, splicing modulation, and altering translational activity^{19,21}. Although ASOs do not cross the blood-brain barrier, they are effectively distributed throughout the central nervous system (CNS) when delivered into cerebrospinal fluid and have demonstrated promising results in the treatment of other neurological diseases^{22–24}.

The first demonstration of a therapeutic potential in ALS was a 20-nucleotide ASO-targeting *SOD1*. Intra-cerebroventricular injections of this ASO in mutant *SOD1* rats resulted in the reduction of SOD1 mRNA and protein levels throughout the brain and spinal cord and slowed disease progression²⁵. This ASO strategy of targeting SOD1 has since been tested in patients with SOD1 ALS in clinical trials. Intrathecal administration of the ASO (BIIB067, tofersen) was found to be safe and well tolerated and caused a significant reduction in SOD1 protein levels in the CNS in addition to slowing clinical decline in patients with SOD1 ALS²⁶. Following these promising results, the ASO is being tested in patients with SOD1 ALS in a multicenter phase III placebo-controlled trial (NCT02623699).

For *C9orf72*-linked FTD/ALS, several ASO molecules have been designed and their beneficial effects have been demonstrated in numerous *C9orf72* cellular and animal models, including *C9orf72* patient induced pluripotent stem cell (iPSC)-derived

Table 1. Summary of therapeutic approaches that have entered clinical trials for the treatment of amyotrophic lateral sclerosis.

Therapeutic	Therapeutic approach	Therapeutic target	Class of drug	Eligible ALS population	Trial phase	ClinicalTrials.gov Identifier
Tofersen (BIIB067)	Gene therapy: antisense oligonucleotide	SOD1 transcript	Antisense oligonucleotide	SOD1 ALS	III	NCT02623699
BIIB078	Gene therapy: antisense oligonucleotide	C9orf72 repeat expansion	Antisense oligonucleotide	C9orf72 ALS	I	NCT03626012
Masitinib	Modulating neuroinflammation	Tyrosine kinase inhibitor	Small molecule	Familial or sporadic ALS	III	NCT03127267
Ibudilast (MN-166)	Modulating neuroinflammation	Phosphodiesterase inhibitor	Small molecule	Familial or sporadic ALS	II / III	NCT04057898
Fasudil	Modulating neuroinflammation	Rho kinase inhibitor	Small molecule	Not specified	II	NCT03792490
Ravulizumab	Modulating neuroinflammation	Complement component 5 inhibitor	Monoclonal antibody	Familial or sporadic ALS	III	NCT04248465
Zilucoplan	Modulating neuroinflammation	Complement component 5 inhibitor	Small molecule	Familial or sporadic ALS	II / III	NCT04297683
Anakinra	Modulating neuroinflammation	IL-1 receptor antagonist	Monoclonal antibody	Familial or sporadic ALS	II	NCT01277315
Tocilizumab	Modulating neuroinflammation	IL-1 receptor antagonist	Monoclonal antibody	Not specified	II	NCT02469896
Arimoclomol	Clearance of protein aggregates	Upregulates heat shock proteins	Small molecule	Not specified	III	NCT03491462
Colchicine	Clearance of protein aggregates	Enhances expression of HSPB8	Small molecule	Sporadic ALS	II	NCT03693781
Rapamycin	Clearance of protein aggregates	Stimulate autophagy	Small molecule	Familial or sporadic ALS	II	NCT03359538
BIIB100/KPT-350	Targeting nucleocytoplasmic transport	XPO1/CRM1 inhibitor	Small molecule	Not specified	I	NCT03945279
HEALEY platform trial	Various	Various	Various	Familial or sporadic ALS	II / III	NCT04297683

ALS, amyotrophic lateral sclerosis; IL-1, interleukin 1; SOD1, superoxide dismutase; XPO1, exportin 1.

neurons and *C9orf72* BAC transgenic mouse models^{27–34}. Following the success of *C9orf72*-targeting ASOs in pre-clinical models, a phase I clinical trial of the first ASO (BIIB078) targeting *C9orf72* mRNA in *C9orf72* ALS patients was initiated in September 2018 and is ongoing (NCT03626012).

In addition to directly targeting ALS-causing mutations, pre-clinical experiments have tested ASOs targeting mRNAs that encode disease-modifying proteins, such as ataxin-2. Intermediate numbers (22–33 repeats) of the poly-glutamine repeat in *ATXN2* are associated with an increased risk of ALS^{35,36}. Genetic knock-out or heterozygous deletion of *ATXN2* homologs can rescue TDP-43 toxicity in yeast, *Drosophila*, and a

TDP-43 transgenic mouse, and a single administration of an ASO targeting ataxin-2 into the CNS of TDP-43 transgenic mice resulted in improved motor function and survival^{35,37}. Given that accumulations of TDP-43 are by far the most common pathology seen in patients with ALS, these studies indicate that an ataxin-2-targeting ASO, if effective, has the potential to be therapeutically beneficial to large cohorts of patients with ALS, including patients with sALS.

Adeno-associated viral vectors

The use of viral vectors to deliver genetic material required for gene replacement or knock-down is another therapy being explored in the treatment of ALS. Currently, the most frequently

used viral vectors for neurodegenerative and other diseases of the CNS are adeno-associated viral (AAV) vectors³⁸. AAV recombinant vectors are non-enveloped, single-stranded DNA-containing viruses that are modified such that the viral genome contains the desired therapeutic gene to be delivered along with only the necessary endogenous viral genes required for packaging³⁸. AAV9 is the principal serotype used for the development of therapeutics in neurological diseases, including ALS, because of its high transduction efficiency in neurons, ability to spread broadly throughout the CNS, and its ability to cross the blood–brain barrier, enabling intravenous delivery³⁹. AAV vectors can be used to deliver a “normal” copy of a disease-causing gene or to deliver RNA interference (RNAi) molecules—including small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), and microRNAs (miRNAs)—to downregulate and degrade mRNA transcripts of the targeted gene³⁸.

Thus far, most ALS AAV vector therapies tested pre-clinically target mutant *SOD1*. Several groups have demonstrated the effective therapeutic potential of using AAV vectors to deliver miRNA or shRNA targeting mutant *SOD1* in SOD1^{G93A} rat and mouse models^{40–44}. These studies all successfully demonstrated that AAV vector treatment resulted in knock-down of mutant SOD1 mRNA or protein expression (or both), slowed disease progression, and extended life span; one more recent study reported an increased median survival of 50%⁴⁰. The safety and efficacy of intrathecal delivery of AAV vectors targeting mutant SOD1 have also been demonstrated in non-human primate models^{41,44} and more recently in two familial ALS patients carrying *SOD1* gene mutations⁴⁵. Following these successes, an AAV9 viral vector containing an shRNA targeting mutant *SOD1* (AVXS-301) is under pre-clinical development and is expected to be tested in patients with SOD1 ALS in a phase I clinical trial in the near future¹⁴. Although AAV gene therapy is moving forward for ALS caused by *SOD1* mutations, it is important to note that *SOD1* mutations account for a small percentage of ALS cases, and it remains to be seen whether this therapy would also be beneficial to patients with sALS, as has been previously proposed⁴⁶.

Targeting the more common genetic cause of ALS, *C9orf72*, with an AAV vector would be of benefit to a larger cohort of patients with ALS. The principle of using AAV9-miRNA to silence the *C9orf72* transcript was successfully demonstrated in cultures of primary cortical neurons derived from *C9orf72* BAC transgenic mice⁴⁷. In addition, a more recent study has shown that an AAV5 vector containing miRNAs targeting repeat-containing *C9orf72* transcripts was able to reduce the accumulation of repeat-containing *C9orf72* transcripts in both the nucleus and cytoplasm of iPSC-derived frontal brain-like neurons⁴⁸. Furthermore, intra-striatal delivery of these AAV5-miRNAs into 90-day-old *C9orf72* BAC transgenic mice lowered the expression of total and repeat-containing *C9orf72* mRNA transcripts, although no behavioral changes were observed in the mice following treatment⁴⁸. Although reducing the levels of *C9orf72* transcripts has shown some promise in these experimental models, some concerns have

been raised regarding the potential adverse effects caused by a reduction in the endogenous levels of the *C9orf72* protein, and a recent study demonstrated that reducing *C9orf72* function in a mouse model exacerbated phenotypes induced by the presence of the *C9orf72* repeat expansion⁴⁹.

CRISPR-Cas9 genome editing

CRISPR-Cas9 genome editing technology has rapidly advanced within the last decade, making it a potential therapeutic option for human diseases arising from genetic mutations. This technology makes use of a naturally occurring prokaryotic defense mechanism to insert, modify, or remove specific sequences of DNA using a targeting guide RNA, a Cas9 DNA endonuclease, and the cells’ natural DNA repair mechanisms⁵⁰. In a therapeutic setting, these components would most likely be delivered via an AAV vector, adopting the principles learnt from AAV-mediated gene therapies⁵¹.

For ALS cases caused by the *C9orf72* mutation, the most likely CRISPR-Cas9-mediated approach would be to remove the hexanucleotide repeat expansion sequence. As proof of concept, this approach has been used successfully to generate isogenic *C9orf72* patient-derived iPSCs^{52–54}. CRISPR-Cas9 excision of the repeat sequence reduces some of the pathological hallmarks of the diseases, including repeat RNA foci and DPR proteins. A recent study has also demonstrated that a similar reduction in DPR proteins and a rescue of neurodegeneration can be achieved by using CRISPR to selectively delete the *C9orf72* promoter region⁵⁵. However, before CRISPR-Cas9 technology may be of therapeutic use in *C9orf72*-linked ALS, it will be important to address the problem of potentially introducing insertion/deletion events into the wild-type allele, which may have functional consequences to the *C9orf72* gene⁵⁶. Finally, a recent study successfully targeted the Cas9 protein to the repeat RNA instead of the DNA in an attempt to circumvent permanent genomic changes⁵⁷.

For SOD1 ALS, it may be possible to use CRISPR-Cas9 genome editing to correct specific disease-causing mutations, delete the mutant *SOD1* gene, or introduce strategic mutations to disable the mutant *SOD1* function. This approach has been successful in the SOD1^{G93A} mouse model where CRISPR/Cas9 editing *in vivo* reduced expression of mutant SOD1, delayed disease onset, and increased survival^{58,59}. AAV-delivered CRISPR-Cas9 technology has also been tested in combination with a cytidine base editor to introduce a nonsense coding mutation into the mutant *SOD1* gene to permanently disable SOD1 expression in the SOD1^{G93A} mouse model. This treatment slowed disease progression, prolonged survival, and caused 40% fewer SOD1 inclusions in end-stage mice compared with control⁶⁰.

Further *in vitro* and *in vivo* validation work is required to establish the feasibility and tolerability CRISPR-Cas9-mediated therapeutics in fALS as a number of challenges associated with genome editing technology—such as target specificity, off-target genome editing, and immunogenicity—would need to be overcome before it could be a viable therapeutic. Additionally,

there would need to be significant ethical and regulatory changes put in place before this technology enters the clinic.

Modulating neuroinflammation

In addition to motor neuron death and muscle denervation, a characteristic feature of ALS pathology is neuroinflammation^{61,62}. Increasing evidence from ALS patient tissue and animal studies has implicated activation of astrocytes, microglia, and the complement system, T-lymphocyte infiltration and production of inflammatory cytokines in ALS pathogenesis^{61–64}. Furthermore, numerous *in vitro* studies of motor neurons co-cultured with astrocytes, microglia, or T cells derived from iPSCs carrying an ALS-causing mutation or ALS mouse models have demonstrated that these cells have a toxic effect on motor neurons^{46,65–69}. Given the increased activation of the immune system in ALS, several clinical trials have investigated using various classes of anti-inflammatory drugs as an ALS therapeutic. Although many anti-inflammatory drugs have failed to demonstrate clinical efficacy, a number of trials remain ongoing and different aspects of the immune system are being targeted^{61,70}.

Modulating aberrant activation of microglia is the target of many of the drugs currently in trials. Microglia are the resident immune cells of the CNS and are classically described to exist in two different states: resting and activated. Whereas resting microglia survey their microenvironment and perform crucial roles to maintain homeostasis, activated microglia react rapidly to environmental abnormalities and can be both protective and detrimental to the surrounding cellular environment, a phenomenon that is sometimes referred to as M1 or M2 phenotypes⁷¹. Microglia are considered to have a “toxic” phenotype when they are responsible for the release of pro-inflammatory cytokines and are now often referred to as disease-associated microglia^{72,73}. At the same time, microglia show neuroprotective properties when they release anti-inflammatory cytokines and remove cellular debris by phagocytosis⁷². Aberrant activation and an imbalance of toxic and protective microglia states are thought to promote inflammation and motor neuron degeneration in ALS; thus, anti-inflammatory drugs that reduce microglia activation or induce a protective microglia phenotype are being explored as therapeutic options^{61,71}.

Masitinib is a tyrosine kinase inhibitor that reduces microglia proliferation and activation. It has shown promising results in both pre-clinical and clinical trials. Oral administration of masitinib decreased microgliosis, reduced motor neuron pathology, and prolonged post-paralysis survival in SOD1^{G93A} mice⁷⁴. When translated into a phase II/III trial (NCT02588677), masitinib in combination with riluzole demonstrated a slowed decline of the Revised ALS Functional Rating Scale (ALS-FRS-R) in patients with “normal progressor” ALS⁷⁵. A further phase III (NCT03127267) trial of masitinib in combination with riluzole is due to commence shortly.

Ibudilast has also recently gained approval to be tested in a phase IIb/III trial (NCT04057898) in patients with ALS after a phase II trial demonstrated that the drug was safe and had

potential benefits on survival⁷⁶. Ibudilast is a small-molecule inhibitor of phosphodiesterase 4 and 10 and Toll-like receptor 4 and is thought to promote an anti-inflammatory effect. Interestingly, ibudilast has also been shown to significantly enhance the clearance of TDP-43 and SOD1 protein aggregates and protect against TDP-43-mediated toxicity in cell culture models, suggesting that this drug may exhibit more than anti-inflammatory properties⁷⁷.

Fasudil is another drug that acts to modulate microglia activation and phenotype. It is a Rho kinase (ROCK) inhibitor that reduces the release of pro-inflammatory cytokines and has been shown to promote expression of neuroprotective microglia markers upon stimulation in cellular models and significantly prolong survival and motor function by modulation of microglial activity in SOD1^{G93A} mice^{78–80}. Fasudil is being investigated in a phase IIa clinical trial (NCT03792490) in patients with early-stage ALS⁸¹. A recent case study of three patients who were granted compassionate treatment of fasudil before the trial began has reported that the drug was well tolerated; however, no conclusions with regard to drug efficacy could be drawn⁸². This will be assessed via several secondary endpoint measures in the ongoing phase IIa trial⁸¹.

In addition to targeting microglia, anti-inflammatory drugs that target the complement system are being tested in patients with ALS. The complement system is part of the innate immune system which acts to enhance the immune response. The system consists of several small proteins that circulate in the blood as inactive precursors which become activated by proteases upon stimulation by one of several triggers⁸³. In ALS, there is increasing evidence of aberrant activation of various components of the complement system in the onset and progression of motor phenotypes^{83,84}. The terminal protein of the complement system, complement component 5 (C5), has been identified as a potential therapeutic target in ALS on the basis of evidence of C5a receptor upregulation in post-mortem ALS tissue and SOD1^{G93A} animal models and the fact that pharmacological inhibition of the C5a receptor improved symptoms and prolonged survival in SOD1^{G93A} mice^{85,86}. Clinical trials in patients with ALS are planned for two drugs acting on C5. A phase III trial (NCT04248465) is under way in both sALS and fALS patients to test the efficacy and safety of ravulizumab, a humanized monoclonal antibody designed to bind to and inhibit the activation of C5. Similarly, a phase II trial (NCT04297683) in both sALS and fALS patients is planned for a synthetic peptide inhibitor of C5 activation, zilucoplan.

A third intervention targeting neuroinflammation in ALS is suppression of the effects of pro-inflammatory cytokines by antagonizing cytokine receptors. This is an effective strategy already used in the treatment of inflammatory diseases such as rheumatoid arthritis, and several of these anti-inflammatory drugs are being trialed for repurpose in ALS. Examples include the interleukin-1 (IL-1) receptor antagonist, anakinra (NCT01277315), and tocilizumab (NCT02469896), a monoclonal antibody targeting the IL-6 receptor. Pilot studies in a small number of patients

with sALS indicate that anakinra and tocilizumab can lower cytokine levels and induce a down-regulation of inflammatory genes, respectively^{87,88}.

Immunotherapy

Protein aggregates are a pathological hallmark of all cases of ALS regardless of disease etiology and include, but are not exclusive to, SOD1, FUS, TDP-43, but also C9orf72 repeat translated DPR proteins. In experimental models of several other neurodegenerative disorders, antibody-based therapies have been effective in reducing the cell-to-cell transmission of toxic proteins—such as tau, amyloid- β , and α -synuclein—and removal of pathological aggregates of these proteins^{89,90}. A similar immunotherapy approach has been explored to reduce protein aggregation in models of ALS. This either involves direct injection of a purified antibody into the target organism (passive immunity) or can involve a vaccination approach whereby the target organism is injected with a recombinant form of the toxic protein in order to stimulate *in vivo* antibody production against the target protein (active immunity).

For SOD1 ALS, several studies have demonstrated the benefits of both active and passive immunotherapy in mutant SOD1 models, although the success of injecting recombinant SOD1 mutant protein to induce immunity seems to be dependent on the *SOD1* mutation. Although vaccination against SOD1 has been shown to delay disease onset, increase life span, and enhance the clearance of SOD1 aggregates in the SOD1^{G37R} model^{91–93}, this immunization strategy has not been successful in extending life span in the SOD1^{G93A} mouse model, and some studies have reported a worsening of disease phenotype, likely due to adverse immune responses^{91,94,95}. In contrast, studies that used a passive immunity approach by treating mutant SOD1 mouse models with various types of antibodies specific for misfolded SOD1 all reported delayed disease onset, increased life span, and a reduction in mutant SOD1 protein levels^{96–98}.

In *C9orf72*-linked ALS, antibodies targeting DPR proteins, in particular poly-glycine-alanine (GA), have shown promising effects in experimental models. Antibodies raised against the poly-GA protein are able to reduce intracellular poly-GA aggregation in primary neuron cultures and blocked the seeding activity of brain lysates extracted from *C9orf72* patient brain⁹⁹. Recent studies have further demonstrated beneficial effects of anti-GA antibodies in two *C9orf72* mouse models, as shown by reduced behavioral deficits, decreased neuroinflammation, and prolonged survival¹⁰⁰, as well as reduced TDP-43 cytoplasmic mislocalization, suggesting that the immunotherapy was able to elicit effects downstream of poly-GA aggregation¹⁰¹. These studies support the concept that poly-GA immunotherapy could be a viable therapeutic approach in reducing some aspects of *C9orf72*-linked ALS; however, given the multi-factorial disease pathogenesis of *C9orf72*, it is likely that treatment will require a synergistic approach when targeting downstream mechanisms.

Given that most ALS cases present with TDP-43 pathology, antibodies targeting this protein would be of therapeutic benefit

to a larger population of patients with ALS, including sALS. Indeed, single-chain antibodies that recognize different regions of TDP-43 have been investigated as potential therapeutics^{102,103}. In mutant TDP-43 transfected cell lines, an antibody targeting the nuclear export signal of TDP-43 was found to have high affinity for and accelerated proteasome-mediated degradation of aggregated TDP-43. Furthermore, following *in utero* electroporation in embryonic mouse brain, the antibody caused a marked reduction in the number and size of mutant TDP-43 aggregates¹⁰². Similarly, an antibody targeting the RNA recognition motif of TDP-43 significantly reduced TDP-43 proteinopathy, motor defects, and neuroinflammation in transgenic mice expressing ALS-linked TDP-43 mutations¹⁰³. It is important to note that mutant TDP-43 was studied in both cases. Given that mutations in TDP-43 account for only 5% of fALS cases, it will be important to assess whether antibody-based therapy is able to reduce aggregation of the wild-type protein, as this is the more common TDP-43 protein species found in ALS patients with TDP-43 proteinopathy.

Stimulating clearance of protein aggregates

An alternative therapeutic strategy aimed at the disassembly of protein aggregates is based on enhancing protein quality control systems via heat shock proteins. These proteins are essential to intracellular protein quality control and function as molecular chaperones to correctly fold, stabilize, and prevent the unwanted aggregation of proteins¹⁰⁴. Arimoclomol, a compound that induces the upregulation of several heat shock proteins, has shown therapeutic benefit in the SOD1^{G93A} mouse model of ALS¹⁰⁵. Administration of arimoclomol significantly improved muscle strength, motor neuron survival, and prolonged life span compared with controls when treated from the time of symptom onset^{106,107}. Histopathological analysis of arimoclomol-treated mice revealed a reduction in the abundance of ubiquitin-positive aggregates in motor neurons compared with untreated mice, suggesting that arimoclomol was acting as an anti-aggregation drug in this model of ALS¹⁰⁷. In patients with rapidly progressing SOD1 ALS, arimoclomol was found to be safe and well tolerated and provided therapeutic benefit across a range of efficacy outcome measures in a randomized, double-blind, placebo-controlled trial¹⁰⁸. A larger randomized phase 3 clinical trial to evaluate the efficacy and safety of arimoclomol in patients with sporadic and familial ALS is under way (NCT03491462). Whilst arimoclomol has demonstrated therapeutic benefit in SOD1 ALS, it will be particularly interesting to see the effect of arimoclomol on non-SOD1 ALS patients given that pre-clinical research on arimoclomol in non-SOD1 ALS is currently limited.

One molecular chaperone that is of particular interest with regard to ALS therapeutics is HSPB8. This protein has been shown to recognize and promote the autophagy-mediated removal of misfolded TDP-43 fragments, mutant SOD1, and C9orf72 DPR proteins^{109–111}. Furthermore, HSPB8 can form a chaperone complex with BAG3 and HSP70, the latter of which acts as a key regulator of stress granule surveillance to help prevent the conversion of dynamic stress granules into more solid aggregates¹¹². HSPB8 in increasing levels is being explored as an ALS

therapeutic in a phase II clinical trial of colchicine (NCT03693781), which is known to enhance the expression of HSPB8 and several other proteins involved in autophagy as well as exhibiting anti-inflammatory properties¹¹³.

An additional molecular chaperone of therapeutic interest is Hsp104, a heat shock protein with disaggregase activity that is naturally found in *Saccharomyces cerevisiae*, where it functions to regulate the construction and disassembly of yeast prion proteins^{114,115}. Interestingly, despite Hsp104 being conserved between bacteria and many eukaryotes, no homolog of Hsp104 exists within the animal kingdom, making it an attractive candidate as an exogenous therapeutic^{116,117}. Although wild-type Hsp104 has only moderate effects on the disaggregation of human proteins associated with neurodegenerative diseases, variants of Hsp104 have been engineered to suppress TDP-43 and FUS aggregation and toxicity in yeast models^{118–120}. Furthermore, co-expression of Hsp104 variants with ALS-linked mutant FUS in mammalian cells promoted the dissolution of FUS inclusions¹²¹. Further investigation is required to determine whether Hsp104 variants have the same effect on TDP-43 aggregates in mammalian cells and whether this extends to *in vivo* ALS models. It will also be necessary to determine whether expression of Hsp104 in mammalian cells has any detrimental effects on the normal protein folding process before this can be pursued as a therapeutic¹⁵.

A less targeted approach to stimulating the disassembly of pathological protein aggregates would be to increase the activity of specific cellular protein quality control pathways. Several genes linked to ALS are known to play a role in autophagy, which has led to the suggestion that dysfunctional autophagy may influence disease pathogenesis and thus therapeutic strategies aimed at enhancing autophagy could be an effective treatment for the disease. Several studies have demonstrated that stimulating autophagy can enhance the clearance of mutant SOD1, TDP-43, or FUS *in vitro*; however, results in *in vivo* models have been mixed^{122–128}. Despite these conflicting pre-clinical studies, a placebo-controlled phase 2 clinical trial to assess the biological and clinical effect of the potent autophagy-inducing drug rapamycin is ongoing in patients with ALS (NCT03359538).

Targeting phase separation

In addition to stimulating the disassembly of protein aggregates, another therapeutic strategy could be to prevent the initial formation of these structures by interfering with the process of liquid–liquid phase separation (LLPS). This is a naturally occurring phenomenon that underlies the formation of various membrane-less organelles, such as the nucleolus and stress granules, and occurs when proteins demix from an aqueous solution and form dynamic liquid-like droplets. This process is believed to be mediated by low-complexity domains within the phase-separating protein, a property that is common among many of the RBPs implicated in ALS, including TDP-43, FUS, and hnRNPA1^{129–131}. While LLPS is essential to maintain cellular function, recent *in vitro* and cellular studies have demonstrated that liquid-like droplets are capable of solidifying over time, resulting in the formation of insoluble aggregates, which can

have toxic consequences for the cell^{132,133}. Given that many of aggregated proteins detected in patients with ALS are capable of this phenomenon, a therapeutic strategy being explored in pre-clinical experiments is interfering with LLPS to prevent the deleterious process of liquid-like droplets, particularly stress granules, from forming insoluble aggregates. Although no clinical translation of these studies is planned at present, these pre-clinical experiments demonstrate how an understanding of the basic biology and biochemistry of the disease can lead to the emergence of novel therapeutic strategies.

The use of RNA bait oligonucleotides is one strategy being explored after a study demonstrated that binding of RNA to TDP-43 suppresses LLPS and inclusion formation in a light-inducible model of TDP-43 LLPS¹³⁴. Treatment of cortical-like neuronal cells with a bait RNA oligonucleotide that exhibited high affinity for the RNA recognition motif of TDP-43 suppressed phase transition of TDP-43 and reduced neurotoxicity in a dose-dependent manner. The use of such RNA oligonucleotides could be a viable therapeutic treatment as they could be readily administered to patients in a similar manner to the ASO therapies currently being trialed. Another therapeutic option targeting phase separation would be using small molecules. A number of studies have identified compounds that show anti-TDP-43 aggregation properties^{135–137}. A recent small-molecule screen identified several planar compounds capable of modulating stress granule size, number, and dynamics and preventing the accumulation of TDP-43 within persistent cytoplasmic puncta in mutant TDP-43 iPSC-derived motor neurons¹³⁸. Although both strategies are effective in reducing the deleterious accumulation of TDP-43, caution must be taken with this strategy not to interfere with the normal physiological function of phase separation and formation of essential membrane-less organelles.

Targeting post-translational modifications (PTMs) of phase-separating proteins may also be a therapeutic strategy as some modifications have been found to influence the aggregation propensity of ALS-linked proteins. Poly(ADP-ribosylation) is an important PTM in the regulation of stress granule dynamics, and it was recently discovered that TDP-43 and hnRNPA1 are targets of poly(ADP-ribose) polymerases (PARPs)^{139,140}. The addition of poly(ADP-ribose) units to these proteins enhances their phase separation, while PARP inhibition is able to reduce accumulation of cytoplasmic TDP-43 foci and rescue TDP-43-associated neurotoxicity in NSC-34 cells, rat primary spinal cord neurons, and *Drosophila* models of ALS^{139–141}. These studies suggest that inhibition of PARPs could be an effective strategy to regulate aberrant phase separation in ALS, and given that PARP inhibitors are already safely used clinically for the treatment of various cancers, this could be a viable therapeutic option¹⁴². Arginine methylation is another PTM known to regulate the phase separation of the ALS-linked protein FUS. The addition of methyl groups to arginine residues within FUS by protein arginine methyltransferase (PRMT) enzymes reduces the ability of FUS to phase-separate and form hydrogels *in vitro*^{143,144}. Treatment with select concentrations of a global methyltransferase inhibitor has been shown to mitigate the cytoplasmic mislocalization and aggregation of mutant FUS in a cell culture

system¹⁴⁵. However, caution is warranted with increasing PRMT activity as these enzymes target a large number of essential proteins that would likely be affected by this treatment. However, the discovery that PTMs can have a profound effect on the behavior of aggregation-prone proteins opens up the possibility of therapeutically manipulating a range of different enzymes to reduce the aberrant phase separation of aggregation-prone proteins. Thus, PTMs of ALS-associated proteins should be further explored to uncover potential therapeutic targets.

Targeting nucleocytoplasmic transport

Deficits in nucleocytoplasmic transport have been widely reported in several models of ALS as well as other neurodegenerative disorders¹⁴⁶. The most common defect seen in ALS patients with TDP-43 pathology is the depletion of TDP-43 from the nucleus, resulting in loss of essential nuclear functions of the RBP. In a healthy neuron, TDP-43 can rapidly traffic between the nucleus and cytoplasm; however, pathological depletion of TDP-43 from the nucleus is toxic to neurons, both *in vitro* and *in vivo*^{147,148}. A similar phenomenon is observed in patients with ALS-FUS, where the predominantly nuclear FUS protein is mislocalized from the nucleus to the cytoplasm¹⁴⁹. Thus, preventing the nuclear export or enhancing nuclear import of these proteins has been investigated as a therapeutic strategy for ALS.

TDP-43 contains a canonical nuclear export signal (NES) that is predicted to be recognized by the nuclear export factor exportin 1 (XPO1/CRM1), a nuclear export pathway that can be inhibited by selective inhibitors of nuclear export (SINE) compounds. Inhibition of XPO1 by SINE compounds has been shown to reduce TDP-43-induced cortical neuron death, rescue larval locomotor defects, and partially restore motor function in a TDP-43 overexpressing rat model^{150,151}. Additionally, genetic knockdown of XPO1 in a *Drosophila* can rescue disease phenotypes associated with FUS or G₄C₂ repeat-induced neurotoxicity^{33,152}. Interestingly, treatment with SINE compounds or siRNA depletion of nuclear export proteins had limited effects on the nuclear localization of TDP-43 in rat primary cortical neurons at concentrations shown to rescue TDP-43-induced toxicity¹⁵⁰. This observation is in line with recent studies that have suggested that the NES of TDP-43 is not functional and most TDP-43 export from the nucleus is likely a result of passive diffusion^{153,154}. Therefore, it appears that the protective effects of SINES may be mediated through other unknown mechanisms that are independent of TDP-43 localization. Despite currently lacking a clear mechanism of action, a small-molecule inhibitor of XPO1, BIIB100/KPT-350, is being tested in a placebo-controlled phase 1 clinical trial in patients with ALS (NCT03945279).

Recently, it has been shown that select nuclear import receptors are capable of antagonizing aberrant phase separation and stimulating the disassembly of protein aggregates. Several groups have demonstrated that, in addition to its nuclear import function, karyopherin β2/importin 1 functions as a molecular chaperone to prevent FUS aggregation by interfering with its ability to phase-separate and can disassemble preformed fibrils

and hydrogels of FUS *in vitro*^{143,144,155–157}. Overexpression of karyopherin β2 also reduced toxicity in both human cells expressing mutant FUS and mutant FUS *Drosophila* models¹⁵⁵. Additionally, karyopherin β2 has been shown to inhibit and reverse the fibrillization of several other RBPs linked to ALS/FTD, including TAF15, EWSR1, hnRNPA1, and hnRNAP2¹⁵⁵. Although this strategy is far from any clinical use, the utilization of nuclear importers as an antagonist for aberrant phase separation demonstrates the innovative potential of therapeutic strategies that have emerged from recent advances in our understanding of ALS biology.

Emergence of new clinical trial design for ALS therapies

The search for effective ALS therapies is not only dependent on advances in understanding disease biology and the emergence of novel targets, it is also reliant on well-designed and executed clinical trials. The majority of ALS clinical trials to date have followed the often costly and time-consuming, traditional randomized clinical trial design of testing a single treatment in a homogenous group of patients. As it has become clear that ALS is a multi-faceted and heterogeneous disease, at both the clinical and molecular levels, a need for a new trial design is warranted. Trials for other diseases, particularly cancers, have benefitted from the use of a “platform trial” design^{158–161}. A platform trial is a clinical trial with a single master protocol in which multiple treatments are evaluated simultaneously across one or more types of patients and allows for further additions or exclusions of new therapies or patient populations during the trial^{158,162}. This model accelerates the development of therapies by rapidly evaluating the effectiveness of multiple therapies simultaneously, increasing patient access, and lowering the cost of trials. Furthermore, data from all patients receiving a placebo in each group can be combined, enhancing the statistical power of the trial. In January 2020, the US Food and Drug Administration granted approval for the first platform trial in patients with ALS. Initially, the HEALEY ALS platform trial (NCT04297683) will involve simultaneously trialing three potential therapeutic drugs, and new treatments and additional participants will be added as they become available. This trial design is estimated to decrease the trial time by 50%, increase patient participation by 67%, and reduce the cost of research by 30%¹⁶³. It is hoped that use of the platform trial model will help accelerate the development of effective ALS therapies.

Summary

It is clear from the number of identified therapeutic targets discussed here that ALS is a complex and heterogeneous disease for which the development of therapies has been a challenging process. Nevertheless, significant progress made within the last few years has increased understanding of the genetics, pathology, and mechanisms of disease and led to the emergence of a range of novel therapeutic strategies, many of which are now entering clinical trials. As ALS research advances, it is becoming clear that the different forms of ALS exhibit unique molecular characteristics and pathologies—for example, DPR proteins in *C9orf72*-ALS and FUS aggregates in *FUS*-ALS—yet they all share common motor neuron neurodegeneration and

inflammatory features that make them clinically indistinguishable. These similarities and differences have given rise to the development of therapeutic strategies that are highly specific, targeted to a discrete pathological mechanism, as well as strategies that affect features common to all patients with ALS. As understanding of ALS pathobiology increases, it is becoming more unlikely that a single therapeutic will prove to be effective in treating all forms of ALS caused by different underlying molecular differences. Instead, a more appropriate approach may be to use a combination of synergistic therapeutics to target different aspects of disease pathogenesis. However, therapies that target common features of the disease, such as inflammation and protein aggregate clearance, could prove to be useful adjunct therapies that modify disease progression. To complement

this approach, the introduction of a platform trial design to ALS trials will hopefully accelerate the search to find effective therapies as it allows fast assessment of multiple therapeutic strategies. As many of the therapies discussed here enter or continue through clinical trials within the next few years, it will become clearer which therapeutic strategies, or potential combinations, are likely to make the most significant impact on disease outcomes.

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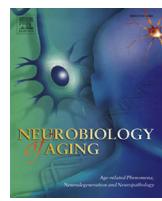


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Review

Genetics and molecular mechanisms of frontotemporal lobar degeneration: an update and future avenues

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ABSTRACT

Frontotemporal lobar degeneration (FTLD) is the second most common form of dementia after Alzheimer's disease. The study and the dissection of FTLD is complex due to its clinical, pathological, and genetic heterogeneity. In this review, we survey the state-of-the-art genetics of familial FTLD and recapitulate our current understanding of the genetic architecture of sporadic FTLD by summarizing results of genome-wide association studies performed in FTLD to date. We then discuss the challenges of translating these heterogeneous genetic features into the understanding of the molecular underpinnings of FTLD pathogenesis. We particularly highlight a number of susceptibility processes that appear to be conserved across familial and sporadic cases (e.g., and the cellular waste disposal pathways, and immune system signaling) and finally describe cutting-edge approaches, based on mathematical prediction tools, highlighting novel intriguing risk pathways such as DNA damage response as an emerging theme in FTLD.

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1. Introduction

Frontotemporal dementia (FTD)—with onset age that can vary from ≥ 45 to ≤ 70 years of age—is the second most common early-onset form of dementia after Alzheimer's disease (AD) affecting about 3–15/100,000 individuals, based on North American and European epidemiological studies (Rabinovici and Miller, 2010).

FTD is clinically heterogeneous and comprises behavioral FTD (Rascovsky et al., 2011) and language (primary progressive aphasia [PPA]) impairments. The behavioral FTD syndrome is characterized by cognitive decline and behavioral dysfunctions, while the PPA syndrome is further subdivided into semantic dementia (SD or semantic variant PPA) and progressive nonfluent aphasia (PNA or nonfluent/agrammatic variant PPA) (Gorno-Tempini et al., 2011; Neary et al., 1998). Atypical forms of FTD present an overlap with motoneuron disease (FTD-MND), mainly summarized by the FTD–amyotrophic lateral sclerosis (ALS) condition or with parkinsonian features (FTDP-17) (Rohrer and Warren, 2011).

Macropathological assessments have indicated that the major brain areas affected in patients with FTD are the frontal and

temporal lobes. On these bases, FTD is also referred to as frontotemporal lobar degeneration (FTLD) (Mackenzie and Neumann, 2016). More generally, FTLD is used as an umbrella term to define clinically and pathologically diagnosed cases (Rabinovici and Miller, 2010). We will use the abbreviation FTLD throughout the rest of this review article.

Micropathological studies suggest glia hyperproliferation and aberrant protein inclusions in the cytoplasm and nucleus of neurons as the major pathological hallmarks of FTLD (Mackenzie and Neumann, 2016). Tau, the protein product of the MAPT gene, and TDP-43, the protein product of the TARDBP gene, are the most frequent protein aggregates ($\sim 45\%$ of FTLD-tau, $\leq 50\%$ FTLD-TDP) identified in FTLD brains (Halliday et al., 2012). Rarely ($\sim 10\%$), other proteins are found in the inclusion bodies: these comprise p62 (the protein product of the SQSTM1 gene) that define the FTLD–ubiquitin proteasome category, and fused in sarcoma (the protein product of the FUS gene), Ewing's sarcoma (the protein product of the EWS gene), and TATA-binding protein associated factor 15 (the protein product of the TAF15 gene) that are collectively referred to as FTLD-FET (Halliday et al., 2012; DeLeon and Miller, 2018; Mackenzie and Neumann, 2016).

FTLD's complex clinical and pathological landscape is mirrored by heterogeneous genetic features. Despite enormous advances in FTLD genetics over the past 20 years, clearly, the substantial lack of understanding of how genetics, phenotypic, and pathological

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features are wired by underlying molecular mechanisms represents to date the major gap to the dissection of FTLD pathogenesis.

In this review, we will survey the state of the art of FTLD genetics and discuss their biological implications (i.e., FTLD risk pathways) and touch on next steps to be taken in the field to increase our genetic and functional understanding of FTLD pathogenesis. Clearly, these 2 subjects will need to jointly advance to accelerate and support the implementation of programs to identify biomarkers and drug targets, design clinical trials, and develop strategies for early diagnosis, disease prevention/monitoring, and cure.

2. FTLD genetics

Neurodegenerative disorders are characterized by complex genetic features, and, generally, a minority of familial cases are outnumbered by a large population of sporadic cases.

Mendelian genes have been classically isolated in familial studies through linkage analysis and, more recently, whole-exome sequencing studies of trios, first-degree relatives, or large (well phenotyped) pedigrees (Hardy and Singleton, 2009).

Sporadic forms of disease are investigated through case/control association studies: for example, genome-wide association studies (GWASs) allow to assess whether allele frequencies of common genetic markers significantly differ across large cohorts of patients and population-matched controls and thus isolate disease-associated genetic risk factors (Manolio et al., 2009).

2.1. Familial FTLD—mendelian genetics

Familial forms of FTLD account for up to 30%–40% of all cases and are associated with mutations in a handful of genes that are usually referred to as Mendelian FTLD genes (Seelaar et al., 2011; Snowden et al., 2002; Turner et al., 2017).

The microtubule (MT)-associated protein tau [MAPT, ≥44 pathogenic mutations (Ghetti et al., 2015)] and progranulin (GRN, ≥70 pathogenic mutations (Gijsselinck et al., 2008)) are classical familial FTLD genes (Table 1). Truncation mutations in the charged multivesicular body protein 2B (CHMP2B) gene have been linked to a large Danish family with FTLD and a Belgian patient with FTLD (Skibinski et al., 2005; van der Zee et al., 2008); this genetic form of FTLD is extremely rare and nomenclated as FTLD-3 (i.e., FTLD linked to chromosome 3) (Brown et al., 1995; van der Zee et al., 2008) (Table 1). An abnormal GGGGCC expansion in the chromosome 9 open reading frame 72 gene (C9orf72) has also been suggested as a frequent genetic cause of familial FTLD (DeJesus-Hernandez et al., 2011; van der Zee et al., 2013). The expansion has however been reported with variable prevalence across multiple neurodegenerative conditions: with higher prevalence in ALS and FTLD-ALS cases (Hardy and Rogeava, 2014; Nguyen et al., 2018) as well as, although with lower prevalence, in AD, Parkinsonian, corticobasal (CBS) and ataxia cases (Al-Chalabi et al., 2017; Cooper-Knock et al., 2014; Ferrari et al., 2012; Ferrari and Momeni, 2013; Galimberti et al., 2014; Hensman Moss et al., 2014; Lindquist et al., 2013; Majounie et al., 2012; Simon-Sanchez et al., 2012; Smith et al., 2013; van der Zee et al., 2013) (Table 1). Interestingly, the expansion has also been reported in neurologically normal elderly individuals (Galimberti et al., 2014).

Very rare mutations in other genes such as sequestosome 1 (SQSTM1) (Le Ber et al., 2013), ubiquilin 2 (UBQLN2) (Synofzik et al., 2012), optineurin (OPTN) (Pottier et al., 2015), coiled-coil-helix-coiled-coil-helix domain containing 10 (CHCHD10) (Bannwarth et al., 2014), TANK binding kinase 1 (TBK1) (Gijsselinck et al., 2015; Pottier et al., 2015), dynactin-1 (DCTN1) (Munch et al., 2005), and cyclin-F (CCNF) (Williams et al., 2016) have been associated with FTLD-ALS (Table 1); interestingly, kindred of individuals carrying

mutations in these genes comprise variable FTLD and/or ALS features within the same pedigree (Hardy and Rogeava, 2014; Van Mossevelde et al., 2018). Mutations in the valosin-containing protein (VCP) have been identified in few cases carrying a combination of conditions such as inclusion body myopathy (IBM) with Paget disease of the bone (PDB) and FTD (IBMPFD) (Watts et al., 2004), and in FTLD-ALS cases (Koppers et al., 2012), while mutations in SQSTM1 have recently also been described in families whose affected members presented various phenotypes including gait abnormalities, ataxia, dysarthria, dystonia, vertical gaze palsy, and cognitive decline (Haack et al., 2016). The TIA1 cytotoxic granule-associated RNA-binding protein gene (TIA1), originally identified in an FTLD-ALS kindred (Mackenzie et al., 2017), was not replicated in a subsequent follow-up study (Baradaran-Heravi et al., 2018) (Table 1). Finally, it is noteworthy to consider that pathogenic genetic variability in TARDBP and FUS, whose protein products clearly represent FTLD pathological hallmarks (Mackenzie and Neumann, 2016), has been reported to be extremely rare (TARDBP) (Benajiba et al., 2009; Borroni et al., 2009, 2010; Caroppo et al., 2016; Huey et al., 2012), or, in some cases, equivocal (TARDBP and FUS) (Cannas et al., 2013; Hardy and Rogeava, 2014; Pottier et al., 2016; Quadri et al., 2011).

In summary, one might categorize the Mendelian FTLD genes on the basis of their disease specificity: MAPT, GRN, and CHMP2B have mainly or exclusively been identified in FTLD cases; thus, we suggest to label them as “major-FTLD genes.” C9orf72, VCP, TARDBP, FUS, SQSTM1, UBQLN2, IFT74, OPTN, CHCHD10, TBK1, and TIA1 appear to encompass ALS and/or some heterogeneous array of disorders; thus, we suggest to label these as “spectrum-FTLD genes” (Ferrari and Momeni, 2018). Fig. 1 summarizes the global Mendelian genetics landscape of FTLD.

2.2. Sporadic FTLD

Sporadic forms of FTLD account for 60%–70% of all cases (Ferrari and Momeni, 2018; Turner et al., 2017).

The genetics of sporadic FTLD is poorly understood. Besides the reports of a few MAPT, GRN, and C9orf72 mutations (Cruts et al., 2015; Takada, 2015; Pottier et al., 2016; Rademakers et al., 2012) (Table 1), the genetic architecture of idiopathic FTLD primarily refers to genetic risk markers with small effect size likely modulated by multiple modifying factors that span from genetic to environmental (Manolio et al., 2009).

To date, the most informative studies exploring genetic risk and/or modifying factors with small effect size in FTLD have been a number of GWASs. These studies were designed to address actual sporadic cohorts [i.e., the International clinical FTLD-GWAS (Ferrari et al., 2014) and the Italian clinical FTLD-GWAS (Ferrari et al., 2015)] or more selective cohorts either characterized by TDP-43 pathology [i.e., FTLD-TDP GWAS (Van Deerlin et al., 2010)] or including GRN mutations carriers [i.e., FTLD-GRN GWASs (Pottier et al., 2018)].

2.2.1. What is a GWAS?

A GWAS is designed as a large-scale case/control study to identify common genetic variants (i.e., variants with minor allele frequencies > 1%) associating with a trait of interest. A GWAS is hypothesis free; it interrogates the genome in an unbiased manner by means of millions of evenly distributed (genotyped and imputed) single-nucleotide polymorphisms (SNPs) and assesses differences in their allelic frequencies between the 2 study groups (cases and controls). Such analysis allows for the identification of loci (not genes) that increase susceptibility for a particular trait (i.e., genetic markers within genetic regions that increase risk of developing a particular trait with small to moderate effect size). A GWAS generally consists of a discovery phase (or phase I) where

Table 1
FTLD Mendelian genetics

Major phenotype	Gene	Familial cases	Sporadic cases	Clinical Presentation(s)	Brain pathology	Reference
FTD	<i>CHMP2B</i>	<1%	NA	FTD	ubiquitin/p62	(Ferrari et al., 2011; Isaacs et al., 2011; Pottier et al., 2016)
	<i>GRN</i>	5%–20%	1%–5%		TDP43	(Chen-Plotkin et al., 2010; Pottier et al., 2016; Rohrer and Warren, 2011; Takada, 2015)
	<i>MAPT</i> (tau)	5%–20%	0%–3%		tau	(Pottier et al., 2016; Rohrer and Warren, 2011; Takada, 2015)
FTD-ALS	<i>CHCHD10</i>	<1%	NA	ALS, FTD, myopathy	NA	(Bannwarth et al., 2014)
	<i>C9orf72</i>	30%	~5%	ALS	TDP43/p62/repeat-dipeptides/ubiquitin	(Pottier et al., 2016; van der Zee et al., 2013)
		~25%	~5%	FTD, FTD-ALS		
		~1%	NA	AD, PD, CBS, A		
	<i>CCNF</i>	<1%	NA	ALS, FTD	Not reported	(Williams et al., 2016)
	<i>DCTN1</i>	<1%	NA	ALS, HMN7B, Perry syndrome, FTD	TDP43	(Munch et al., 2005)
	<i>OPTN</i>	<1%	NA	ALS, FTD	TDP43/OPTN/ubiquitin	(Pottier et al., 2015)
	<i>SQSTM1</i> (p62)	<1%	NA	ALS, FTD, IBM, Paget's disease	TDP43/p62	(Gang et al., 2016; Kovacs et al., 2016; Le Ber et al., 2013)
	<i>TBK1</i>	1%–3%	NA	ALS, FTD	TDP43/p62	(Pottier et al., 2016; Van Messemelde et al., 2016)
ALS	<i>UBQLN2</i>	<1%	NA	ALS, FTD	TDP43/p62/UBQLN2/FUS/OPTN	(Deng et al., 2011; Synofzik et al., 2012)
	<i>VCP</i>	~1%	NA	ALS, FTD, IBM, Paget's disease	TDP43/p62	(Ferrari et al., 2011; Gang et al., 2016)
	<i>KIF5A</i>	<1%	NA	SP, ALS	TDP43	(Brenner et al., 2018)
	<i>FUS</i>	~4%	NA	ALS, FTD	FUS/ubiquitin/EWS/TAF15	(Mackenzie and Neumann, 2016; Nguyen et al., 2018; Urwin et al., 2010)
	<i>MATR3</i>	<1%	NA	ALS, myopathy	MATR3	(Johnson et al., 2014)
	<i>SOD1</i>	~20%	NA	ALS	SOD1/ubiquitin	(Ferrari et al., 2011; Saberi et al., 2015)
	<i>TARDBP</i> (TDP43)	~3%–4%	NA	ALS, FTD	TDP43	(Ferrari et al., 2011; Nguyen et al., 2018)
	<i>TIA1</i> ^a	<1%	NA	ALS, myopathy, FTD	TDP43	(Baradaran-Heravi et al., 2018; Mackenzie et al., 2017)

Key: A, ataxia; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CBS, corticobasal syndrome; FTLD, frontotemporal lobar degeneration; HMN7B, hereditary motor neuropathy, type 7B; IBM, inclusion body myopathy; MS, multiple sclerosis; PD, Parkinson's disease; SP, spastic paraparesis.

^a Not replicated.

one or more genetic loci associated with the trait under study are "discovered" and a replication phase (or phase II), performed in an independent cohort, that assesses the statistically significant (and suggestive [i.e., SNPs that are close to but below statistical significance]) loci of phase I for validation. Most reported associations in GWAS are intronic or intergenic, most probably affecting DNA structure and gene expression rather than protein sequence (Manolio et al., 2009). Although GWASs identify risk loci, defined by SNPs that might be the actual reason of the signal or just in linkage disequilibrium with it, the associated variants might be informative implying to causal regulation of gene expression (e.g., expression quantitative trait loci [eQTLs]) or susceptibility functional pathways (Pearson and Manolio, 2008). The exponential growth in the number of GWASs in the past decade has led to the discovery of thousands of associations for a range of traits (over 25,000 unique SNP-trait associations from over 2500 studies [<http://www.ebi.ac.uk/gwas>]). More extensive information on the concept, study design, good practices, and results interpretation for GWASs can be found in the study by Ferrari and Momeni (2018), Hardy and Singleton (2009) and Manolio et al. (2009).

Major findings of the FTLD-GWASs are discussed in the next paragraphs and summarized in Tables 2 and 3.

2.3. FTLD-TDP GWAS

This study was published in 2010 by van Deerlin et al. (Van Deerlin et al., 2010).

It included FTLD-TDP cases diagnosed either by pathological (FTLD-TDP) or genetic (i.e., presence of pathogenic *GRN* mutations) assessments. As classical case-control studies, it was performed on 515 FTLD-TDP cases and 2509 controls (discovery phase) and 89 independent FTLD-TDP cases and 553 controls (replication phase).

Discovery phase analyses indicated 3 significant lead SNPs (rs6966915, rs1020004, and rs1990622), encompassing the transmembrane protein 106B (*TMEM106B*) gene (7p21.3). Replication analysis confirmed the association and same direction of effect for 2 lead SNPs (rs1020004 and rs1990622), yet such associations could not be replicated in additional 192 individuals with unspecified

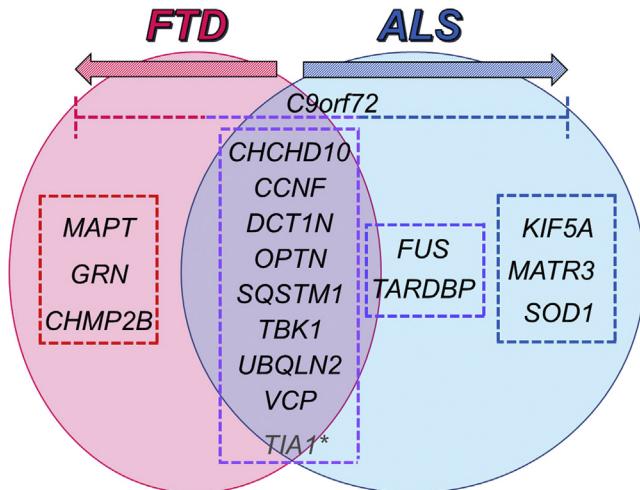


Fig. 1. Summary of Mendelian genetics associated with FTLD and the FTLD-ALS spectrum. Genes circled in red are "major-FTLD genes"; genes circled in violet are "spectrum-FTLD genes," that is, they belong to the FTLD-ALS spectrum. Genes circled in dark violet and blue are edge/borderline between FTLD and ALS and pure ALS genes, respectively. * = not replicated. Abbreviations: FTLD, frontotemporal lobar degeneration; ALS, amyotrophic lateral sclerosis.

FTLD (Van Deerlin et al., 2010). Authors also characterized the effects on *TMEM106B* expression levels in FTLD-TDP postmortem versus neurologically normal control brains (frontal cortex) for rs1020004 and rs1990622, indicating that risk allele carriers had 2.5-fold higher *TMEM106B* expression levels than controls (Van Deerlin et al., 2010). Of note, *GRN* mutation carriers showed highest increase of *TMEM106B* expression compared with non-carriers and controls (Van Deerlin et al., 2010).

Altogether, this study indicated SNPs encompassing the *TMEM106B* gene as risk factors for the FTLD-TDP subtype and that their risk alleles appeared to be particularly enriched in *GRN* mutation carriers, thus suggesting *TMEM106B* as a *GRN* modifier. In addition, not only it is relevant to note that *TMEM106B* association with FTLD has been confirmed in subsequent studies (Cruchaga et al., 2011; Finch et al., 2011; Van Deerlin et al., 2010; Vass et al., 2011) but also that *TMEM106B* is involved in endolysosomal pathways and modulates PGRN protein levels (Lattante et al., 2014).

2.3.1. International clinical FTLD-GWAS

This study was published in 2014 by Ferrari et al., 2014.

It included cases belonging to 4 clinical subgroups: behavioral frontotemporal lobar degeneration (bvFTLD), PPAs (SD and PNFA), and FTLD-MND. The discovery cohort (FTLD-GWAS phase I) consisted of 2154 cases and 4308 controls, while the replication cohort (FTLD-GWAS phase II) comprised 1372 cases and 5092 (for a total of 3526 FTLD samples and 9400 controls). Following the classical case-control strategy, analyses were performed after excluding Mendelian genes (*MAPT* and *GRN*) mutation carriers. The association analyses for the discovery phase were performed for each subtype before meta-analyzing the entire cohort: for the bvFTLD subtype (consisting of 1377 cases and 2754 controls), 2 suggestive lead SNPs—rs302652 and rs74977128—were identified, respectively, mapping to Ras-related protein Rab-38 and cathepsin C (*RAB38* and *RAB38–CTSC*) (11q14). Association analyses on the other subtypes—308 SD cases (vs. 616 controls), 269 PNFA cases (vs. 538 controls), and 200 FTLD-MND cases (vs. 400 controls)—did not indicate genetic markers reaching genome-wide significance (because of insufficient power). Conversely, the meta-analysis for all 4 subtypes indicated significant SNPs encompassing butyrophilin-like 2 (*BTNL2*; rs1980493) and major histocompatibility complex, class II, DRs (*HLA-DRA–HLA-DRB5*; rs9268877 and rs9268856) at 6p21.3. Replication (for 690 bvFTLD cases vs. 5094 controls), and joint analyses, indicated rs302668 as significant for the bvFTLD subtype (11q14; *RAB38*). Replication for the entire cohort and joint analyses confirmed strong association for 3 lead SNPs rs9268877, rs9268856, and rs1980493 at 6p21.3. From a

functional perspective, although no eQTLs in brain were evident, the top SNP for *RAB38–CTSC* (rs302652) was associated with decreased levels of *RAB38* mRNA in blood, while a significant *cis*-methylation quantitative trait loci for rs1980493 was evident for *HLA-DRA* in the frontal cortex (Ferrari et al., 2014).

Altogether, this study revealed two novel susceptibility loci for clinical FTLD: one suggestive involving lysosome-phagosome pathways (*RAB38–CTSC* locus) for the bvFTLD subtype, and one affecting immune system processes (*BTNL2* and *HLA-DRA–DRB5* locus) in global FTLD.

2.3.2. Italian clinical FTLD-GWAS

This study was published in 2015 by Ferrari et al., 2015.

It was performed on a multicenter Italian FTLD cohort of 530 samples (bvFTLD [n = 418], SD [n = 27], PNFA [n = 61], and FTLD-MND [(n = 24)]) and 926 population-matched controls. The inclusion criteria were in line with those of the clinical FTLD-GWAS (see Ferrari et al., 2014). The work used a standard cases versus controls association strategy for the discovery phase followed by replication analyses based on the implementation of 3 different tests (also called “SNPs-to-genes” analyses)—GATES, supervised PCA, and the sequential kernel machine association test—to score and prioritize genes (Ferrari et al., 2015). Discovery analyses identified 2 suggestive loci (i.e., close to significance, yet not genome-wide significant): 1 defined by 7 SNPs encompassing *LOC730100* (centromeric to neurexin 1 [*NRXN1*]) at 2p16.3 and the other also defined by 7 suggestive SNPs encompassing centrosomal protein 131 (*CEP131*), *C17orf89*, and ENTH domain containing 2 (*ENTHD2*) at 17q25.3. Interestingly, the risk alleles at 17q25.3 defined a suggestive risk haplotype causing decreased expression of the *cis*-genes RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (*RFNG*), apoptosis-associated tyrosine kinase (*AATK*), and microRNA 1250 (*MIR1250*), suggesting this as the biological mechanism underlying the association. During replication, *CEP131* and *ENTHD2* were consistently identified as the most significant genes across the 3 analysis methods (GATES, supervised PCA, or sequential kernel machine association test).

Altogether, this study indicated two novel potential loci for FTLD and that, from a functional perspective, an effect on expression of genes involved in neuronal development, differentiation, and maturation processes might drive FTLD pathogenesis in the Italian population.

2.4. FTLD-GRN GWAS

This study was published in 2018 by Pottier et al., 2018.

Table 2

Summary of association results for FTLD-TDP, the International clinical FTLD-GWAS and the FTLD GRN GWAS

Phenotype	Chr	BP	Marker	Alleles	Risk allele	p-value Discovery	OR	p-value Replication	OR	p-value Joint	OR	Reference
FTLD-TDP	7	12,283,787	rs1990622	C/T	T	1.08×10^{-11}	1.64	0.0002 ^a	1.75	NA	NA	(Van Deerlin et al., 2010)
		12,255,778	rs1020004	A/G	A	5.00×10^{-11}	1.67	0.004 ^a	1.89	NA	NA	
		12,265,988	rs6966915	A/C/T	C	1.63×10^{-11}	1.64	NA	NA	NA	NA	
International clinical bvFTD	11	87,894,831	rs302652	T/A	T	2.02×10^{-8}	1.37	NA	NA	NA	NA	(Ferrari et al., 2014)
		87,876,911	rs302668 ^{b,c}	T/C	T	NA	NA	0.041	1.14	2.44×10^{-7}	1.23	
		87,936,874	rs74977128	T/C	C	3.06×10^{-8}	1.81	NA	NA	NA	NA	
Meta International clinical FTLD	6	87,934,068	rs16913634 ^{b,c}	G/A	A	NA	NA	0.71	1.04	8.15×10^{-4}	1.25	
		32,363,215	rs1980493	T/C	T	4.94×10^{-8}	1.39	0.02	1.17	1.57×10^{-8}	1.30	
		32,431,147	rs9268877	A/G	A	1.65×10^{-10}	1.33	0.104	1.08	1.05×10^{-8}	1.20	
GRN GWAS	7	32,429,719	rs9268856	A/C	C	1.30×10^{-8}	1.33	0.014	1.14	5.51×10^{-9}	1.24	
		12,283,787	rs1990622	C/T	T	1.61×10^{-10}	1.88	4.09×10^{-7}	1.81	3.54×10^{-16}	1.85	(Pottier et al., 2018)
		21,621,247	rs36196656	A/C/G/T	A	9.44×10^{-6}	1.51	4.4×10^{-4}	1.46	1.58×10^{-8}	1.49	

^a Replication tested in 89 FTLD-TDP cases.

^b surrogate/proxy SNPs for the bvFTD subtype.

^c denotes heterogeneity p-value < 0.01 in the meta-analysis of the discovery and replication phases combined.

Table 3

Summary of association results for FTLD Italian clinical GWAS

Phenotype	Chr	BP	Marker	Alleles	Risk Allele	p-value	OR	Method	Gene	FDR	Study
						Discovery					
Italian clinical FTD	2	52,600,067	rs17042852	C/T	C	2.01×10^{-7}	2.82	NA			(Ferrari et al., 2015)
		52,635,727	rs1526678	G/A	G	2.19×10^{-7}	2.83				
		52,571,393	rs17042770	C/G	C	2.22×10^{-7}	2.83				
		52,509,876	rs12621157	T/G	T	3.73×10^{-7}	2.75				
		52,546,301	rs12622570	C/G	C	3.99×10^{-7}	2.76				
	17	52,532,874	rs12619513	A/G	A	6.39×10^{-7}	2.53				(Ferrari et al., 2015)
		52,521,716	rs12614311	T/C	T	8.83×10^{-7}	2.55				
		79,173,462	rs906175	T/C	T	1.22×10^{-7}	1.58	GATES	CEP131	0.001469	
		79,192,430	rs2725391	T/C	T	2.50×10^{-7}	1.52		ENTHD2	0.004264	
		79,195,814	rs969413	A/T	A	4.26×10^{-7}	1.52		C17orf89	0.004264	
	17	79,177,974	rs2659030	A/G	A	4.42×10^{-7}	1.56	sPCA	CEP131	0.000650	(Ferrari et al., 2015)
		79,213,562	rs2255166	C/T	C	6.19×10^{-7}	1.55		ENTHD2	0.001007	
		79,192,446	rs9319617	C/T	T	6.62×10^{-7}	1.51	SKAT	CEP131	0.000014	
		79,202,329	rs1048775	G/C	C	8.04×10^{-7}	1.51		ENTHD2	0.000651	
									C17orf89	0.000651	

Key: FTLD, Frontotemporal lobar degeneration; GWAS, genome-wide association study; SNP, single-nucleotide polymorphism.

It was performed on FTLD samples selected for the specific feature of carrying loss of function *GRN* mutations ($n = 120$ distinct mutations) (Pottier et al., 2018). The discovery phase was done using 382 patients and 1146 controls, while the replication phase included 210 patients (67 patients with *GRN* mutations and 143 confirmed FTLD-TDP type A cases). A standard case-control association study was performed to identify modifiers for *GRN* mutation carriers and/or FTLD-TDP in general. The discovery phase analyses did not reveal modifiers affecting age at onset (neither after conditioning on rs5848); yet a genome-wide significant lead SNP downstream to *TMEM106B* was evident (rs7791726; 7p21.3). Of note, rs7791726 appears to be in almost complete linkage disequilibrium with the previously indicated GWAS hits at this locus (rs1990622, rs3173615, and rs1990620); thus, it might be argued that this result further replicates the original findings by Van Deerlin et al., 2010. A total of 44 suggestive markers were carried forward for replication and, after meta-analyzing the discovery and replication phases, 2 markers resulted outstanding: rs3173615 (*TMEM106B*) and rs36196656 (*GFRA2* [GDNF family receptor alpha 2]). It was established that the effect allele for rs36196656 was a *cis*-eQTL to *GFRA2*, where homozygous carriers showed a reduced expression of *GFRA2* in cerebellar tissue and it was shown that PGRN and *GFRA2* coprecipitated in HEK293T cells, suggesting that both might be part of the GDNF signaling pathway that promotes neuronal survival.

Altogether, this study revealed *GFRA2* as a novel potential modifier in *GRN* mutation carriers. However, authors suggested that not only this genetic association needs to be replicated in follow-up studies but also that *GFRA2*'s biological implication as a PGRN modifier needs to be further assessed in ad hoc functional studies and models.

Besides the typical GWASs, recently, epigenetic findings combined with GWAS data indicated that markers sitting in the *HLA* locus (i.e., rs9357140) might contribute to the regulation of proinflammatory players' expression in the brain cortex, impacting FTLD patients' age of onset (Zhang et al., 2018). This is interesting in that it further supports that multiple risk factors and/or modifiers (i.e., genetic markers with small effect size) might in fact significantly contribute to disease endophenotypes or disease-specific features.

The associations reported in the different FTLD-GWASs hardly replicated across each other. The only hit that appears to be seen in at least 2 independent GWAS (the FTLD-TDP and the FTLD-GRN GWAS) is the *TMEM106B* locus. Both studies targeted a category of cases presenting overlapping features: on 1 hand, the presence of genetic variability in the *GRN* gene, on the other, the underlying pathology (i.e., TDP-43 pathology). Considering that the *TMEM106B*

locus was by far not a hit in the International and the Italian clinical GWASs, one might argue *TMEM106B* being an exclusive modifier of *GRN* mutation carriers and cases featuring TDP-43 pathology. The International and the Italian clinical GWASs indicated different loci and were not cross-supportive: here, the lack of replication in a bidirectional manner may underlie the fact that differences in populations might indeed play an important role in determining genetic association, even more than sample size and statistical power.

All this taken together warrants that future study designs take into account a number of points: from a statistical perspective, it is and will be fundamental to increase samples sizes to improve the power of associations. From a study design perspective, it will be fundamental to study the different FTLD subtypes (based on both clinical and pathological diagnoses) to better define their genetic architecture. This will be extremely valuable to genetically classify groups of patients both for later ad hoc inclusion in clinical trials and for the development, in the long run, of syndrome-specific prevention and treatment options.

2.4.1. Ongoing and future efforts to tackle sporadic FTLD

In comparison to other neurodegenerative disorders such as AD and Parkinson's disease (PD), FTLD is a rather rare condition with an insidious clinical presentation that embraces a spectrum of syndromes. As indicated in the previous paragraph, gathering large and phenotypically well-characterized cohorts of the various syndromes contributing to FTLD is a critical element to allow powered genetic studies of sporadic FTLD, and issues of this kind can only be overcome by large international collaborations involving multiple research centers, worldwide.

The International Frontotemporal Dementia Genomics Consortium (IFGC; <https://ifgcsite.wordpress.com/>) is among the largest consortia for the study of sporadic FTLD. The IFCG conducted the clinical FTLD-GWAS (see Ferrari et al., 2014) and is expanding its data set through the current *FTLD-GWAS phase-III* project. Through these efforts, the IFCG has been and is generating genetic data—through a mix of whole-genome array, exome chip, and NGS techniques—for about 6000 independent sporadic FTLD cases that encompass the various and major subtypes such as bvFTLD, SD, PNFA, and FTLD-MND.

The IFCG comprises research groups from Europe, North America, and Australia who share an interest in the genetics and understanding of sporadic FTLD. The IFCG's vision entails the use of genetics to expand on genes and genetic markers that cause or increase risk of developing FTLD and bioinformatics to interpret genetic data and predict risk pathways, *in silico*.

Clearly, this altogether represents a unique resource for the wider scientific community with an interest in FTLD and neurodegeneration as it helps increasing statistical power for genetic analyses and a range of cross-disciplinary studies and projects. Particularly, this resource allows well-powered studies aiming at dissecting syndrome-specific genetic fingerprints, disease-risk prediction through polygenic risk scores, genotype-phenotype correlation, defining parameters for the identification of cohorts qualifying for tailored clinical trials, as well as fostering large-scale meta-analysis and/or pleiotropy analysis with other closely related neurodegenerative conditions.

3. Molecular mechanisms of FTLD pathogenesis

The translation from genetic knowledge to functional understanding of impacted biological processes and molecular mechanisms at the basis of disease is currently among the major and most debated topics in biomedical research in the context of complex disorders (Karczewski and Snyder, 2018), including FTLD.

Genetics of FTLD clearly has and is contributing to drive research efforts aimed at better understanding its molecular mechanisms and underpinnings. Mendelian FTLD candidate genes are particularly informative to functional biologists to design experiments around their protein products and further their characterization in *in vitro* and *in vivo* models. Although it might be argued that Mendelian genes "just" account for a minority of cases (all together ~30%–40% of all FTLD), an intriguing hypothesis is that, if familial genes indicate functions and processes whose alteration is necessary and sufficient to trigger FTLD pathogenesis, they might be baits for defining the global pathogenic mechanisms leading to FTLD and thus being informative also for the vast majority, that is, the sporadic (~60%–70% of all FTLD), of cases. In the latter scenario, in fact, not only their genetic features are still understudied but also a specific link to genes is less clear than in Mendelian cases because of the nature of GWASs.

The study of protein products of Mendelian (and sporadic [or GWAS]) candidate genes and their functional characterization is thus, in the first instance, informative on the potential impacted processes. Nevertheless, this approach tends to take into consideration "one gene at a time" and might be reductionist in the long run. In addition, proteins encoded by the candidate genes are involved in multiple subcellular processes/pathways; thus, it is critical to highlight those that are truly involved in disease pathogenesis.

Innovative methods to aid in this respect rely on data integration and bioinformatics analyses. These are emerging alternatives to the classical studies in that they allow to evaluate altogether the genetic players contributing to the phenotype, to simulate different pathogenic scenarios and to isolate the most likely risk pathways to be validated and tested in the functional setting. For instance, network analyses based on gene co-expression and protein-protein interactions (PPIs) are becoming suitable methods to serve these purposes. Weighted gene co-expression network analysis (WGCNA) is a bioinformatics pipeline developed in 2008 by Langefelder (Langefelder and Horvath, 2008) that allows the generation of a network whose nodes are genes connected on the basis of their co-expression profile. This type of analysis relies on the assumption that genes that are temporospatially expressed together are likely to be involved in similar functions within the cells expanding on the functional environment of candidate genes. PPI networks are composed by proteins connected on the basis of proof of physical interaction (*as per* peer reviewed functional literature). The hypothesis, behind this second type of networks (work adopting PPI network analyses [WPPINAs]), is that proteins that interact with each other likely share the same function within a conserved pathway due

to biochemical reasons (Ferrari et al., 2017). Network analyses have been recently supported by high-throughput approaches that have the advantage of being unbiased and can take into consideration multiple genes at a time. Comparative mass spectrometry has been used to compare FTD, ALS, and controls to define common and specific molecular players and cellular pathways possibly involved in disease onset and progression (Umoh et al., 2018).

The study of the physiology of Mendelian genes has to date indicated a number of susceptibility processes that appear to be conserved across familial and sporadic cases, suggesting these processes being commonly impacted biological processes underpinning FTLD pathogenesis. This appears to be the case for cellular waste disposal pathways and immune signaling. In addition, there seem to be a number of novel intriguing processes emerging from the more holistic (network based) approaches, including DNA damage response (DDR) (Fig. 2).

3.1. Cellular waste disposal pathways (CHMP2B, C9orf72, GRN, VCP, UBQLN2, OPTN, SQSTM1, TBK1, CCNF, TMEM106, RAB38)

The cellular waste disposal pathways involve an intertwined subcellular continuum of processes that comprise: (1) the endolysosomal pathway that delivers endocytic cargoes engulfed from the extracellular environments to the lysosomes for degradation; (2) the macroautophagy and chaperone-mediated autophagy pathways that target damaged organelles and misfolded proteins for lysosomal degradation; (3) the mitophagy pathway for mitochondrial removal via autophagy, and; (4) the unfolded protein response and the ubiquitin proteasome systems pathways that are responsible for degradation of ubiquitinated proteins. Different alterations of the waste disposal process have been associated with various neurodegenerative disorders including PD, AD, prion disease and Huntington's disease (Menzies et al., 2017). There is still no unequivocal agreement on the detailed mechanisms; however, it is accepted that an alteration of the waste disposal capacity can lead to an accrual of toxic molecules within the subcellular environment that, through time, accounts for progressive neuronal damage and accumulation of misfolded and aggregated proteins. Drugs to potentiate the cell waste disposal machinery have therefore been proposed at least as coadjutant therapies in neurodegenerative conditions (Sarkar et al., 2008).

Among the "major-FTLD genes," GRN encodes for a long glycoprotein product (PGRN) that is secreted in the extracellular space. Extracellular PGRN can be taken up and subsequently cleaved into 7 units of granulins (GRNs) within the endolysosomal pathway (Holler et al., 2017). The function of PGRN and GRNs are still not completely clear, and it has been linked to growth factor like activities and modulation of the inflammatory response (Tang et al., 2011; Van Damme et al., 2008). However, it is interesting to note that homozygous mutations in GRN are causative of neuronal ceroid lipofuscinosis, a lysosomal storage disorder, whereas heterozygous mutations in GRN are associated with FTD (Almeida et al., 2016). These mutations appear to be loss of function (Cruts and Van Broeckhoven, 2008), and PGRN deficiency has been linked to defective autophagy (Chang et al., 2017) and alteration of lysosomal homeostasis (Evers et al., 2017).

CHMP2B encodes a component of the endosomal sorting complex required for transport III involved in the endosomal trafficking, concentration of ubiquitinated cargoes, and proteins/enzymes involved in the endocytic pathway as well as molding lipid bilayers (Bodon et al., 2011; Morita et al., 2010). Among the "spectrum-FTLD genes," SQSTM1, that encodes the p62 protein, is responsible for recognizing polyubiquitinated cargoes and for delivering them to the autophagy machinery for degradation (Katsuragi et al., 2015); also, it has been suggested that a dysfunctional p62 might impact

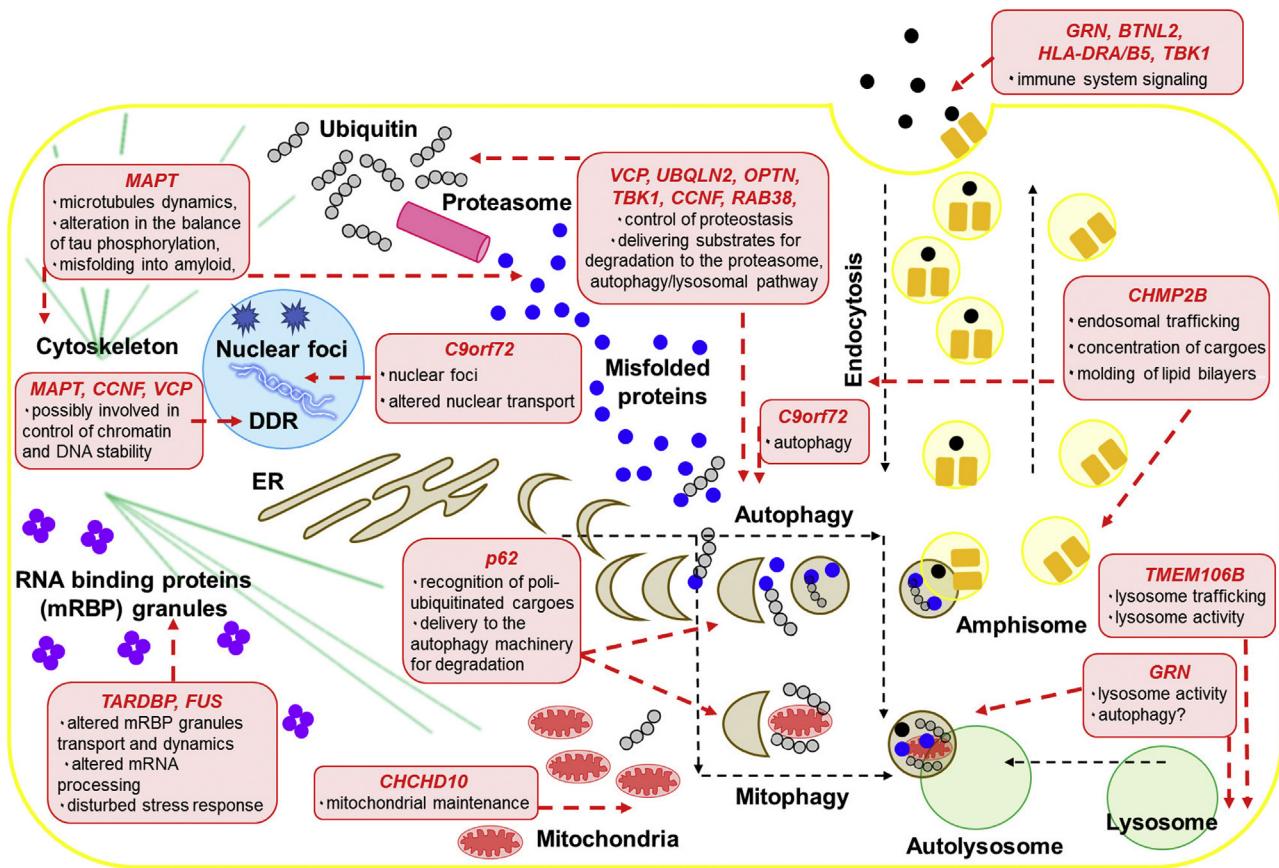


Fig. 2. Summary of all putative impacted molecular processes contributing to FTLD pathogenesis on the basis of the current global genetic characterization of FTLD including “major,” “spectrum,” and “GWAS” FTLD genes. Abbreviations: DDR, DNA damage response; ER, endoplasmic reticulum; GWAS, genome-wide association studies; FTLD, Frontotemporal lobar degeneration.

mitochondria depolarization and lead to a reduced autophagosome formation (Haack et al., 2016). *VCP* is well known for its relevant roles within the ubiquitin-mediated proteostasis (Meyer and Weihl, 2014). *UBQLN2* codes for a protein involved in the control of proteostasis by delivering substrates tagged for degradation to the proteasome (Hjerpe et al., 2016). *OPTN* has been reported to recognize protein aggregates in a ubiquitin-independent fashion and in association with the kinase *TBK1* before directing them to the autophagy/lysosomal pathway for degradation (Korac et al., 2013). *CCNF* holds a function in the E3 ubiquitin-protein ligase complex to mediate proteasomal targeting of ubiquitinated CP110 during G2 phase (Nguyen et al., 2018).

Peculiar is the case of *C9orf72*: although its physiological role is still not known, many studies have been carried out to evaluate the effect of the pathogenic hexanucleotide expansion, indicating that *C9orf72* expansions might reduce mRNA expression or generate toxic nuclear foci (Pottier et al., 2016) with a possible alteration of the nuclear transport. Nevertheless, more recently, *C9orf72* has been linked to autophagy and proteostasis (Pottier et al., 2016).

“GWAS” candidate genes also appear to play major roles within the endolysosomal trafficking and the waste disposal processes: such is the case of *TMEM106B* that is associated with maintenance of endolysosomal trafficking (Busch et al., 2016; Jun et al., 2015), while *RAB38* is responsible for vesicle trafficking, fusion, and autophagosome maturation (Wasmeier et al., 2006).

Altogether, this suggests that a number of “major,” many of the “spectrum” and some of the “GWAS” genes point toward different yet convergent components of the more broad waste disposal pathway, indicating that both dominant mutations (high

penetrance) as well as common markers with small effect size (low penetrance) support a common process involved in disease pathogenesis. Clearly, more studies will need to be performed to further characterize how genetic variability in the candidate genes affects these pathways and contributes to disease mechanisms to be able to recognize potential biomarkers and/or druggable targets within the waste disposal matrix.

CHCHD10 does not seem to fit in the waste disposal picture: *CHCHD10* codes for a mitochondrial protein that might play a role in maintaining mitochondrial physiological activity, yet further ad hoc investigations will be needed to verify whether *CHCHD10* might exert a relevant role in mitochondria quality control and mitophagy.

3.2. Immune system signaling (GRN, TBK1, BTNL2, HLA-DRA)

Immune-related processes are extremely complex and heterogeneous. A few “major,” “spectrum,” and “GWAS” genes appear to support signaling pathways involved in immune responses.

PGRN has been shown to be upregulated in activated microglial cells (Baker et al., 2006) and, among multiple processes, to be involved in wound healing and inflammation (van Swieten and Heutink, 2008). Interestingly, *PGRN* appears to act as an anti-inflammatory agent, while *GRNs*, the products of *PGRN* cleavage, have been shown to promote proinflammatory activities (He and Bateman, 2003). All the more, it was also suggested that *PGRN* is involved in inflammatory processes acting as antagonist of the tumor necrosis factor α in binding the TNF receptor (Tang et al., 2011) or by regulating innate immunity gene expression and complement production thus, in turn, controlling synaptic pruning by microglia

(Lui et al., 2016). Interestingly, TBK1, although implicated in the proteostasis processes (see “*Cellular waste disposal pathways*” section) is, primarily known for mechanisms that relate to the innate immune response (Xiao et al., 2017), suggesting a potentially ubiquitous role in FTLD pathogenesis that warrants additional focused studies. The clinical FTLD-GWAS also clearly supported the involvement of the immune system signaling and inflammatory response: BTNL2 encodes a membrane protein that is ubiquitously expressed across different tissues, including the brain, and is involved in repressing T-cells proliferation (Valentonyte et al., 2005), while HLA-DRA encodes a monomorphic class II HLA-DR transmembrane receptor that is expressed on the surface of microglia. Interestingly, increased expression of HLA-DR molecules on microglia may reflect pathological activity, as previously indicated in AD and PD (McGeer et al., 1988), suggesting that aberrant HLA-DRA levels might in fact impact modulation and regulation of immune responses in the brain. The relevance of elements of the immune system mapping to the HLA locus was further supported by a recent study, assessing genetic pleiotropy across autoimmune disorders and FTLD (Broce et al., 2018).

Clearly, these avenues need to be further explored, yet it is relevant to note that rare and common genetic variability might imply that constitutive functional alteration of innate and adaptive immune responses may contribute to disease pathogenesis.

3.3. Gene expression regulation pathways

Despite the extremely rare genetic variability of *TARDBP* and *FUS* in FTLD, yet considering TDP-43 and FUS being pathological hallmarks of FTLD, a brief note about their contribution to disease pathogenesis is warranted: TDP-43 and FUS are functionally involved in processes that control gene expression and RNA metabolism (more details can be found in Ratti and Buratti, 2016). Particularly, it is widely accepted that defective and mislocalized RNA-binding protein granules (i.e., stress granules that have been directly associated with the activity of TDP-43 and FUS) cause neuronal dysfunctions, abnormal stress response, and ultimately neurodegeneration (Bowden and Dormann, 2016).

3.4. Other (minor) processes/pathways: protein aggregation (*MAPT*) and neuronal development and homeostasis (GRN, RFNG, AATK, GFRA2)

Interesting is the case of *MAPT* that encodes the tau protein. Our current knowledge about tau’s functions and physiology points to binding and stabilization of MTs. The consequences exerted by mutations in *MAPT* have been associated with both alterations in splicing events leading to an imbalanced ratio of tau isoforms and with impairment in binding (and stabilizing) MTs (due to mutations affecting the MT-binding domain), all this leading to increased free cytosolic population of tau that favors its aggregative properties (Ferrari et al., 2011). Tau is target of multiple post-translational modifications such as phosphorylation and acetylation (Min et al., 2010), and balance in tau phosphorylation has shown to be essential for supporting tau physiological functions: hyperphosphorylation does occur in the disorder setting and increases tau’s aggregative capacity (Bodea et al., 2016). It follows that, as mentioned previously, besides the occurrence of abnormal protein aggregation, it is not fully clear what pathways and cascades become disrupted in *MAPT*-driven Mendelian cases.

Other processes that might be impacted on the basis of genetic variability affecting both “major” and “GWAS” genes are neuronal development and homeostasis. Among the (many) functions associated with PGRN and GRNs, in fact, PGRN by activating several kinase-dependant signaling cascades, stimulates the induction of

vascular endothelial growth factor (Tangkeangsirisin and Serrero, 2004), promotes endothelial cell migration during wound healing (He et al., 2003), and appears to play a role in brain development (Mackenzie and Rademakers, 2007) and neurite outgrowth (Van Damme et al., 2008). Similarly, PRGN has been shown to bind to EphA2 (a tyrosine kinase receptor) with consequent activation of mitogen-activated protein kinases and AKT, thus promoting vessel growth with capillary morphogenesis (Neill et al., 2016). Furthermore, recent GWAS (Ferrari et al., 2015; Pottier et al., 2018) indicated a number of loci including genes that, if confirmed as the real biological reason for association (provided that RFNG and AATK result from suggestive loci, i.e., close to but below genome-wide significance), support to some good extent additional processes related to neuronal development and protection (RFNG, AATK, and GFRA2).

3.5. Emerging pathways: DNA damage response (*MAPT*, VCP, CCNF)

Gene co-expression and protein interaction networks are state-of-the-art bioinformatics tools supporting fast *in silico* characterization of shared functions across groups of candidate genes/proteins. In this respect, results from extended bioinformatics work focusing on gene co-expression analyses (WGCNA) of “major,” “spectrum,” and “GWAS” genes confirmed susceptibility pathways belonging to the waste disposal processes such as autophagy, ubiquitin proteasome system, and immune response pathways (Ferrari et al., 2016). Other WPPINA further supported the waste disposal process driven by endoplasmic reticulum stress particularly referring to the ubiquitin/proteasome and unfolded protein response (Ferrari et al., 2017).

Most interestingly, these bioinformatics works jointly supported DDR as a novel potential mechanism underpinning FTLD (Ferrari et al., 2016, 2017). In the WGCNA work, it was evident that *MAPT* was a hub in modules indicating DNA protection among the most significant biological processes in frontal and temporal cortices (Ferrari et al., 2016). The WPPINA work replicated quite in detail the DDR results described previously (Ferrari et al., 2017).

This bioinformatics work globally raised the importance of DDR in FTLD. Recently, a number of studies showed indeed that alterations in the DDR are among the functional consequences of mutations in *MAPT*, suggesting that alterations of the cellular cycle, chromatin damage, and aberration of the normal DNA repairing process may be functionally linked to brain cells’ death and neurodegeneration observed in FTLD (Rossi et al., 2008, 2013). More functional characterization of this process in FTLD models is currently under way.

Interestingly, CCNF plays a role in the E3 ubiquitin-protein ligase complex and mediates proteasomal targeting of ubiquitinated CP110 during G2 phase, thereby not only acting in the cell waste disposal (see section 3.1) but also in the control of the cell cycle check points that control genome stability through ubiquitin-mediated proteolysis (Nguyen et al., 2018). As well, VCP has been shown to be part of a complex of proteins recruited to the DNA double-strand breaks (Acs et al., 2011; Meerang et al., 2011).

The DDR is the general functional term indicating the process through which a cell keeps control of alterations/mutation in the genetic code through mechanisms of damage recognition, repair, tolerance, in addition to cell-cycle checkpoint pathways and signaling events (Giglia-Mari et al., 2011). Alterations of the DDR have been associated with different severe disorders such as Xeroderma Pigmentosum, a syndrome characterized by photosensitivity, increased risk of cancer (particularly skin cancer) often presenting with neurological symptoms (Garcia-Moreno et al., 2018). In addition, it is indeed remarkable that congenital and age-related neurodegeneration has been associated with accumulation of DNA lesions (Madabhushi et al., 2014).

4. Final remarks

Genetics and cell biology work over the past two decades have tremendously contributed shedding light on genetic causes and molecular mechanisms involved in FTLD.

However, there is much more to be learned on these matters as our current understanding of FTLD pathogenesis is clearly in its infant stages and not sufficient to achieve effective preventive and therapeutic measures.

From a genetics perspective, we are at a moment in time where the available and emerging technologies (e.g., genome-wide, exome-chip arrays and NGS techniques) are becoming more cost-effective, enabling a better characterization of common and rare genetic variability contributing to disease. Much of this work will need to be aimed at fine-mapping classical GWAS loci, exploring more in depth the (likely) oligogenic nature of disease and identifying novel causative genes. This will clearly impact the way and pace at which we will fill the gap of missing heritability in FTLD, a key step in deciphering and defining the genetic risk architecture of FTLD. Of course, these approaches will be best applied to large and well-defined patients' cohorts, the gathering of which is made possible by international disease-focused working groups and/or Consortia. Applying these techniques to large cohorts representative of the different FTLD subtypes (i.e., clinical bvFTLD, SD, and PNFA cases as well as pathologically defined cohorts [e.g., FTLD-tau or FTLD-TDP]) will aid improving genotype-phenotype correlation defining syndrome- and/or subtype-specific genetic fingerprinting (important, e.g., to identify cohorts qualifying for tailored clinical trials).

The gap from genes to mRNA and proteomics shall be reduced through better study designs, where multi-omics approaches are applied to the same sample source and disease-relevant tissue(s) to avoid inter-sample variability issues and correlate data by grounds of sample and tissue specificity (Manzoni et al., 2016). From a functional perspective, we need a more time-effective way to coherently translate genetic into functional knowledge. These goals will be achieved by the use of more holistic approaches to interpret the genetic knowledge and guide functional studies investigating risk pathways. For example, *in silico* methods involving assessments of genetic variability's effect on gene-expression regulation (e.g., eQTLs, methylation quantitative trait loci, allele-specific expression, transcriptome-wide association study) (Gusev et al., 2016; Manzoni et al., 2016) and molecular interactions and functional annotation analyses of gene co-expression and PPI networks will allow to better put into perspective the biological processes and pathways that are impacted by genetic variability (Furlong, 2013). In turn, this will provide solid ground for the development of testable hypotheses and aid functional biologists designing more precise experimental models, including cell-specificity studies (Skene et al., 2018), for validating risk pathways.

Normalizing all such strategies will require some time, yet this is the paradigm shift to improve basic and translational research and pave the way for advancements in preventive, monitoring, and therapeutic measures.

Disclosure

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Multifaceted Genes in Amyotrophic Lateral Sclerosis-Frontotemporal Dementia

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Amyotrophic lateral sclerosis and frontotemporal dementia are two progressive, adult onset neurodegenerative diseases, caused by the cell death of motor neurons in the motor cortex and spinal cord and cortical neurons in the frontal and temporal lobes, respectively. Whilst these have previously appeared to be quite distinct disorders, in terms of areas affected and clinical symptoms, identification of cognitive dysfunction as a component of amyotrophic lateral sclerosis (ALS), with some patients presenting with both ALS and FTD, overlapping features of neuropathology and the ongoing discoveries that a significant proportion of the genes underlying the familial forms of the disease are the same, has led to ALS and FTD being described as a disease spectrum. Many of these genes encode proteins in common biological pathways including RNA processing, autophagy, ubiquitin proteasome system, unfolded protein response and intracellular trafficking. This article provides an overview of the ALS-FTD genes before summarizing other known ALS and FTD causing genes where mutations have been found primarily in patients of one disease and rarely in the other. In discussing these genes, the review highlights the similarity of biological pathways in which the encoded proteins function and the interactions that occur between these proteins, whilst recognizing the distinctions of *MAPT*-related FTD and *SOD1*-related ALS. However, mutations in all of these genes result in similar pathology including protein aggregation and neuroinflammation, highlighting that multiple different mechanisms lead to common downstream effects and neuronal loss. Next generation sequencing has had a significant impact on the identification of genes associated with both diseases, and has also highlighted the widening clinical phenotypes associated with variants in these ALS and FTD genes. It is hoped that the large sequencing initiatives currently underway in ALS and FTD will begin to uncover why different diseases are associated with mutations within a single gene, especially as a personalized medicine approach to therapy, based on a patient's genetics, approaches the clinic.

Keywords: ALS, FTD, C9orf72, RNA processing, autophagy, protein aggregation

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder caused by progressive loss of upper motor neurons in the motor cortex and brainstem and lower motor neurons in the spinal cord (Hardiman et al., 2017). It has an incidence of 2–3 per 100,000 and a lifetime risk of 1 per 400 individuals (Brown and Al-Chalabi, 2017). Disease onset occurs most frequently in the limbs, characterized by a loss of dexterity in the fingers or a mild limp, whilst bulbar onset, which occurs in 20–25% of cases, is characterized by a slurring of speech (dysarthria) or difficulty swallowing (dysphagia). Less than 3% of cases are due to respiratory onset, with shortness of breath (dyspnea) being the most common symptom (Gautier et al., 2010). As the disease rapidly progresses, muscle wasting, fasciculations and weight loss occur, with death usually due to respiratory failure 32 months following symptom onset (Cooper-Knock et al., 2013).

Amyotrophic lateral sclerosis clinical features may also be accompanied by cognitive impairment in up to 50% of patients, whilst up to 15% may develop symptoms which are clinically diagnosed as frontotemporal dementia (FTD), resulting in a clinical diagnosis of ALS-FTD (Nguyen et al., 2018). FTD is the second most common form of presenile dementia after Alzheimer's disease, accounting for 3–26% of cases of dementia in individuals under 65 years of age, depending on population (Bang et al., 2015). Whilst FTD is a clinical diagnosis, evidence of degeneration of the neurons in the frontal and temporal lobes upon post-mortem allows the pathological diagnosis of frontotemporal lobar degeneration (FTLD) (Mackenzie and Neumann, 2016). FTD can be divided into three different clinical subtypes. Behavioral variant FTD (bvFTD) is characterized by personality changes such as disinhibited behavior, apathy and loss of empathy. In contrast, semantic variant primary progressive aphasia (svPPA or svFTD) is characterized by individuals having difficulties understanding the meaning of words or naming objects or people, whereas non-fluent variant PPA (nfvPPA or nfvFTD) is when individuals have difficulties in pronunciation, grammar and fluency of speech (Bang et al., 2015).

Whilst the majority of ALS and FTD cases are sporadic (sALS and sFTD), with no family history of disease, in around 10% of cases ALS is inherited, usually in an autosomal dominant manner with an adult onset (fALS) (Brenner and Weishaupt, 2019). In FTD, it is estimated that 10–30% shows autosomal dominant inheritance (ffTD), though this figure may be increased to approximately 40% when a history of neurodegenerative disease is included (Sirkis et al., 2019). To date, 5 ALS-FTD genes have been recognized along with 24 ALS-associated genes and 3 FTD-associated genes (Table 1). This review will firstly summarize the genes that have been recognized as ALS-FTD genes (defined as FTDALS loci on Online Mendelian Inheritance in Man¹) before summarizing other known ALS and FTD causing genes where mutations have been found primarily in patients of one disease and rarely in the other. Finally, a brief comment on the other ALS and FTD genes allocated an ALS or FTD loci number

is provided for completeness and to highlight the similarity of biological pathways that are implicated in both disorders, supporting the proposal that these two disorders represent either end of a disease spectrum. Whilst some of these genes result in Mendelian inheritance of ALS, others act as risk factors. However, understanding why a particular mutation in a gene leads to ALS, FTD or both, currently remains unknown.

ALS-FTD GENES

Mutations in five genes have been recognized as being implicated in ALS-FTD families. These are the GGGGCC (G4C2) hexanucleotide repeat expansion (HRE) in *C9orf72* (FTDALS1), and missense and/or loss of function mutations in *CHCHD10* (FTDALS2), *SQSTM1* (FTDALS3), and *TBK1* (FTDALS4). In addition, *CCNF* has also been reported as an ALS-FTD gene and is referred in this review as FTDALS5. All of these genes encode proteins with a function in autophagy, except *CHCHD10*, which localizes to the mitochondria.

FTDALS1: Chromosome 9 Open Reading Frame 72 (*C9orf72*)

Linkage and genome wide association studies in several families presenting with ALS/FTD, ALS or FTD revealed the diseases to segregate with a locus on chromosome 9p21 (Morita et al., 2006; Vance et al., 2006; Laaksovirta et al., 2010; Shatunov et al., 2010). In 2011, the pathogenic G4C2 hexanucleotide repeat expansion (HRE) in intron 1 of chromosome 9 open reading frame 72 (*C9orf72*) (Figure 1) was found to be the most common cause of familial ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011) with ~40% fALS and 25% ffTD carrying the *C9orf72* repeat expansion (Majounie et al., 2012). However, the frequency of *C9orf72*-related ALS-FTD (and *C9orf72*-ALS and *C9orf72*-FTD) patients positive for the presence of repeat expansion in *C9orf72* varies between different populations and ethnicities; whilst the *C9orf72* G4C2 HRE is the most frequent cause of ALS-FTD, ALS and FTD in European and North American populations, it was found to be extremely rare in Asia and the Middle East (Majounie et al., 2012). Whilst it is currently unclear why some patients with the *C9orf72* expansion develop ALS or FTD while others manifest a combination of both, the discovery of *C9orf72* in causing both ALS and FTD has strengthened the genetic link between these neurodegenerative disorders. *C9orf72*-related ALS-FTD has an autosomal dominant mode of inheritance with evidence of incomplete penetrance (Majounie et al., 2012). Anticipation has been reported in some families (Gijselinck et al., 2016; Van Mossevelde et al., 2017b), however, this phenomenon has been found to be inconsistent, with other studies having reported no association between age of onset and repeat length (Dols-Icardo et al., 2014) or even an inverse correlation between expansion size and age of onset in successive generations, with contractions in the repeat size also reported in subsequent generations (Fournier et al., 2019; Jackson et al., 2020). Some of these contradictory findings may be attributed to the age at collection of sample, owing to the dynamic nature of repeat size relative to age, the methodology

¹<https://www.ncbi.nlm.nih.gov/omim>

TABLE 1 | Overview of known ALS-FTD, ALS, and FTD loci.

ALS Loci number	Chromosomal location	Gene	Onset	Inheritance	Implicated pathogenic mechanisms	Original References
ALS-FTD genes						
FTDALS1	9p21.2	<i>C9orf72</i>	Adult	AD	RNA processing; nucleocytoplasmic transport defects; proteasome impairment; autophagy; inflammation; protein aggregation (DPRs)	DeJesus-Hernandez et al., 2011; Renton et al., 2011
FTDALS2	22q11.23	<i>CHCHD10</i>	Adult	AD	Mitochondrial function, synaptic dysfunction	Bannwarth et al., 2014
FTDALS3	5q35.3	<i>SQSTM1</i>	Adult	AD	Proteasome impairment; autophagy; protein aggregation; axonal defects; oxidative stress	Fecto et al., 2011
FTDALS4	12q14.2	<i>TBK1</i>	Adult	AD	Autophagy; inflammation; mitochondrial dysfunction	Cirulli et al., 2015; Freischmidt et al., 2015
FTDALS5	16p13.3	<i>CCNF</i>	Adult	AD	Autophagy, axonal defects, protein aggregation	Williams et al., 2016
Predominantly ALS genes also found with FTD						
ALS6	16p11.2	<i>FUS</i>	Adult	AD (AR)	RNA processing; nucleocytoplasmic transport defects; stress granule function; protein aggregation	Kwiatkowski et al., 2009; Vance et al., 2009
ALS10	1p36.22	<i>TARDBP</i>	Adult	AD	RNA processing; nucleocytoplasmic transport defects; stress granule function; protein aggregation	Sreedharan et al., 2008
ALS12	10p13	<i>OPTN</i>	Adult	AD (AR)	Autophagy; protein aggregation; inflammation	Maruyama et al., 2010
ALS15	Xp11.21	<i>UBQLN2</i>	Adult	X-LD	Proteasome impairment; autophagy; protein aggregation; oxidative stress; axonal defects	Deng et al., 2011
ALS22	2q35	<i>TUBA4A</i>	Adult	AD	Cytoskeleton	Smith et al., 2014
ALS13	12q24.12	<i>ATXN2</i>	Adult	AD	RNA processing	Elden et al., 2010
Predominantly FTD genes also found with ALS						
ALS14	9p13.3	<i>VCP</i>	Adult	AD	Autophagy; proteasome impairment; defects in stress granules; protein aggregation; mitochondrial dysfunction	Johnson et al., 2010
ALS17	3p11.2	<i>CHMP2B</i>	Adult	AD	Autophagy; protein aggregation	Parkinson et al., 2006
Known ALS genes						
ALS1	21q22.11	<i>SOD1</i>	Adult	AD (AR)	Oxidative stress; protein aggregation; mitochondrial dysfunction, axonal defects, proteasome impairment apoptosis	Rosen et al., 1993
ALS2	2q33.1	<i>ALS2</i>	Juvenile	AR	Intracellular trafficking	Hadano et al., 2001; Yang et al., 2001
ALS4	9q34.13	<i>SETX</i>	Juvenile	AD	RNA processing	Chen et al., 2004
ALS5	15q21.1	<i>SPG11</i>	Juvenile	AR	Axonal defects	Orlacchio et al., 2010
ALS8	20q13.32	<i>VAPB</i>	Adult	AD	Proteasome impairment; intracellular trafficking	Nishimura et al., 2004
ALS9	14q11.2	<i>ANG</i>	Adult	AD	RNA processing	Greenway et al., 2006
ALS11	6q21	<i>FIG4</i>	Adult	AD	Intracellular trafficking	Chow et al., 2009
ALS16	9p13.3	<i>SIGMAR1</i>	Juvenile	AD and AR	Proteasome impairment; intracellular trafficking	Luty et al., 2010; AL-Saif et al., 2011
ALS18	17p13.2	<i>PFN1</i>	Adult	AD	Axonal defects	Wu et al., 2012
ALS19	2q34	<i>ERBB4</i>	Adult	AD	Neuronal development	Takahashi et al., 2013
ALS20	12q13.13	<i>hnRNPA1</i>	Adult	AD	RNA processing	Kim et al., 2013
ALS21	5q31.2	<i>MATR3</i>	Adult	AD	RNA processing	Johnson et al., 2014
ALS23	10q22.2	<i>ANXA11</i>	Adult	AD	Intracellular trafficking	Smith et al., 2014
ALS24	4q33	<i>NEK1</i>	Adult	AD	Intracellular trafficking	Brenner et al., 2016
ALS25	12q13.3	<i>KIF5A</i>	Adult	AD	Axonal defects; intracellular trafficking	Nicolas et al., 2018
ALS	3p21.1	<i>GLT8D1</i>	Adult	AD	Ganglioside synthesis	Cooper-Knock et al., 2019
Known FTD genes						
FTD	17q21.2	<i>MAPT</i>	Adult	AD	Axonal defects, protein aggregation	Hutton et al., 1998
FTD	17q21.31	<i>GRN</i>	Adult	AD	Inflammation; protein aggregation	Baker et al., 2006; Cruts et al., 2006
FTD	6q27	<i>TBP</i>	Adult	AD	RNA processing	Olszewska et al., 2019

AD, autosomal dominant; AR, autosomal recessive; X-LD, X-linked inheritance.

used and the source of samples being compared (peripheral blood versus brain autopsy tissue) (van Blitterswijk et al., 2013a; Fournier et al., 2019).

Although, the exact cut-off for repeat size that would result in pathogenicity has not been clearly defined, neurologically healthy non-carriers typically present with less than 20 repeats, while repeat expansion mutation carriers show more than 30 repeats on repeat primed PCR (Renton et al., 2011). Southern blotting of the region has estimated that repeat lengths of several hundred to several thousand are associated with *C9orf72*-related ALS-FTD, though smaller repeat sizes have been found to co-segregate with disease (Van Mossevelde et al., 2017a). Conventional techniques such as short-read next generation sequencing (NGS) limit accurate repeat sizing of the larger repeats, and somatic instability has been reported, thereby contributing to the variability in repeat size in different tissues from the same individual, such as in comparisons between blood and central nervous system (CNS) tissue (Vatsavayai et al., 2019). Studies have described an inverse relationship between repeat expansion size and disease duration with *C9orf72*-related ALS-FTD presenting an earlier age of symptom onset, a higher incidence of bulbar onset and shorter disease duration (Majounie et al., 2012; van Blitterswijk et al., 2013b; Suh et al., 2015; Trojsi et al., 2019). However, other studies have also reported disease durations between 1 and 22 years (Majounie et al., 2012; Woollacott and Mead, 2014). Sex has been reported to be a risk factor in driving phenotype with one study showing females have a higher prevalence for *C9orf72* HRE in ALS in a meta-analysis study (Trojsi et al., 2019) whilst males presented with a shorter survival time based on Cox proportional hazard regression multivariate analysis (Curtis et al., 2017). It is noteworthy that *C9orf72* repeat expansions have been shown to be associated with a number of neurodegenerative conditions including Parkinson disease, Alzheimer's disease, Huntington-disease like syndrome among others (Woollacott and Mead, 2014; Cooper-Knock et al., 2015c).

The function of endogenous *C9orf72* protein is not fully characterized although it has been identified as a guanine exchange factor (GEF), with both Rho and Rab-GTPase GEF activity (Iyer et al., 2018). It has also been shown that the *C9orf72* protein interacts with the Rab1a and Unc-51-like kinase 1 (ULK1) autophagy initiation complex, with the *C9orf72* protein regulating the trafficking of the ULK1 complex to the phagophore (Webster et al., 2016). As such, a reduction in *C9orf72* protein would lead to reduced autophagy and accumulation of p62-positive aggregates, similar to those seen upon neuropathological examination of patients.

Whilst the exact mechanism of action of how the HRE in *C9orf72* causes neurodegeneration remains to be fully elucidated, three mutually compatible mechanisms have been proposed including haploinsufficiency of endogenous *C9orf72* protein, loss of function and/or toxic gain of function following the formation of RNA foci and toxic gain of function of the dipeptide repeat (DPR) protein inclusions (**Figure 1**).

Haploinsufficiency of *C9orf72*

The *C9orf72* gene contains 12 exons (1a, 1b, 2–11) and has three well characterized transcripts which produce two

protein isoforms (**Figure 1**) (Balendra and Isaacs, 2018) though additional alternatively spliced and protein coding transcripts have been identified (see Ensembl ENSG00000147894). The HRE is located in the pre-mRNA transcript of variant 1 and 3 but in the promoter region of variant 2. It is noteworthy that variants 1 and 3 are predominantly expressed in the brain. Additionally, HRE dependent epigenetic changes such as hypermethylation of the *C9orf72* gene locus has been reported and associated with disease duration and advanced age of onset (Nordin et al., 2015; Gijselinck et al., 2016; Zhang M. et al., 2017). Studies have shown that transcript sequences upstream of the repeat are increased relative to those downstream which might imply that the transcription was aborted due to the presence of repeat. Consequently, reduced levels of the transcript variants have been reported in blood lymphocytes (Ciura et al., 2013), induced pluripotent stem cells (iPSCs)-derived neurons (Shi et al., 2018) and post mortem tissue of *C9orf72*-related ALS and FTD patients (van Blitterswijk et al., 2013b). *C9orf72* knockdown in zebrafish was shown to produce defective axon generation and motor deficits (Ciura et al., 2013) indicating that *C9orf72* protein might play a role in neuronal health. In contrast, specific knockdown of *C9orf72* in mouse brain by antisense oligonucleotides (Lagier-Tourenne et al., 2013) or full ablation of *C9orf72* (*C9orf72*^{−/−}) in neuron-specific (Atanasio et al., 2016) or all tissues (Sudria-Lopez et al., 2016) in mice models showed no neurodegenerative phenotype, although ablated mice developed an autoimmune phenotype and showed reduced survival. This suggests that haploinsufficiency alone may not be sufficient to cause disease and a combination of aberrant pathways such as gain of toxic function with loss of endogenous *C9orf72* protein function might therefore be required for disease pathogenesis.

RNA Foci Formation

The hexanucleotide repeat DNA sequences are bidirectionally transcribed resulting in the production of G4C2 sense and C2G4 antisense transcripts retaining the repeat expansions. Hexanucleotide repeat-retaining RNA forms secondary structures (such as a G-quadruplex) in which the abnormal RNA accumulate to form RNA foci. These RNA foci have been shown to be present in post-mortem brain and spinal cord tissues of *C9orf72*-related ALS-FTD patients whilst being absent in age-matched non-ALS/FTD neurologically healthy controls (Zu et al., 2013). Sense and antisense RNA transcripts get transported to the cytoplasm and have been detected in the cytoplasm of patient post mortem tissue (Mizielinska et al., 2013; Cooper-Knock et al., 2015b) and RNA foci have been found in many cell lines and patient biosamples, such as leukocytes, fibroblasts, and iPSC-derived motor neurons (Gendron and Petrucci, 2018). The aggregation of RNA foci are dynamic and result from association and dissociation of RNA binding proteins (RBPs) which results in loss of their function. Research has shown that antisense foci were observed to be higher in the Purkinje neurons in cerebellum and motor neurons whereas sense foci were significantly increased in the granule neurons in the cerebellum obtained post mortem from *C9orf72*-related ALS or *C9orf72*-related ALS-FTD patients, as detected by fluorescence

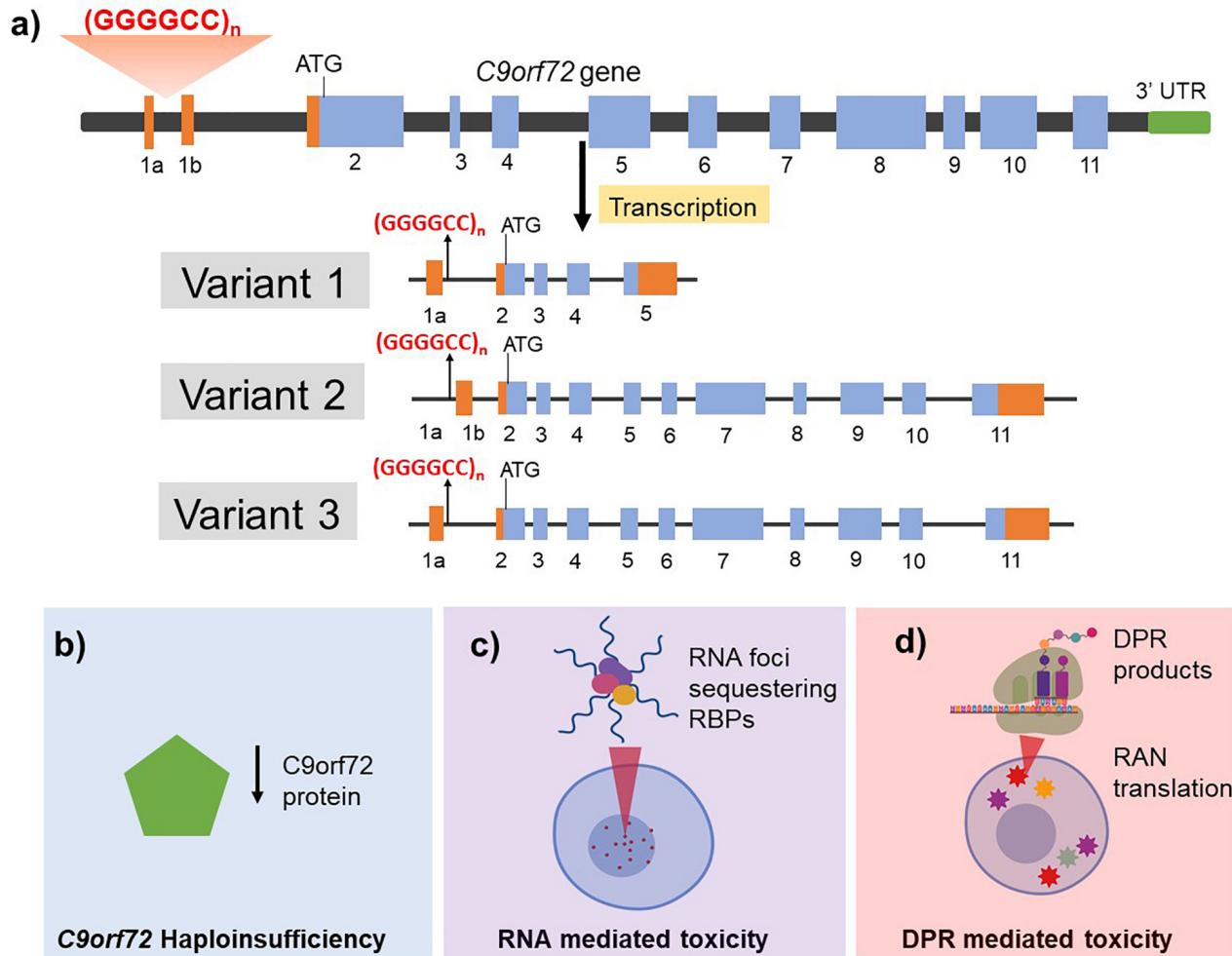


FIGURE 1 | Structure of the C9orf72 gene (a) and the proposed mechanisms of C9orf72 related toxicity in driving ALS/FTD pathogenesis (b–d). (a) C9orf72 has 11 exons and is transcribed into three different mRNA transcripts: variant 1 results in translation of the short protein isoform while variants 2 and 3 both generate the long protein isoform. The non-coding introns are represented in orange and the coding exons are shown in blue. The hexanucleotide repeat expansion is present within intron 1 region and is retained in the pre-mRNA transcript in variants 1 and 3 whereas it is present in the promoter region in variant 2. The G4C2 HRE is proposed to cause neurotoxicity by three mutually compatible mechanisms. (b) Haploinsufficiency of endogenous C9orf72 protein by incorporation of the repeat in the transcript leading to reduced production and function of normal C9orf72 protein. (c) RNA mediated toxicity by the formation of RNA foci that sequester RNA binding proteins (RBPs) and (d) DPR mediated toxicity resulting from repeat associated non-AUG (RAN) translation of mRNA transcripts retaining hexanucleotide repeats thus generating five different dipeptide repeat (DPR) products.

in situ hybridization (Cooper-Knock et al., 2014, 2015b) and RT-PCR (Zu et al., 2013).

Several studies have aimed to identify the RBPs that are sequestered within the C9orf72 RNA foci and have found hnRNPA1/3, PUR-a, ADARB2, Nucleolin, SRSF2, and ALYREF in post mortem CNS tissue, iPSCs-derived cortical neurons reprogrammed from C9orf72-related ALS-FTD patient fibroblasts, neuronal cell lines or a *Drosophila* model of C9orf72 ALS (Donnelly et al., 2013; Sareen et al., 2013; Cooper-Knock et al., 2014; Hautbergue et al., 2017). Gene ontology and transcriptomic analyses have revealed that the formation of foci result in transcriptional profiles unique to C9orf72-related ALS and FTD when compared to other genetic causes of ALS and FTD. Changes in gene expression are associated with various

cellular pathways including unfolded protein response (UPR), RNA splicing, inflammatory response, cell signaling and synaptic transmission (Cooper-Knock et al., 2015a; Prudencio et al., 2015). Taken together, these studies provide evidence for both a loss of function of the RNA binding proteins and a potential gain of toxic function of the downstream effects of the RNA foci formation in driving C9orf72-related ALS-FTD pathogenesis.

Toxicity by DPR

The C9orf72 HRE sense and antisense RNA transcripts can get translated by a non-canonical mechanism of repeat associated non-ATG (RAN) translation resulting in the production of dipeptide repeats. RAN translation occurs in the absence of an AUG start codon resulting in multiple reading frames of

a single repeat transcript. These DPRs can be generated from the sense and/or antisense transcripts resulting in the formation of five different products: poly-GA and poly-GR are translated from the sense GGGGCC strand whereas poly-PA and poly-PR are translated from the antisense CCGGGG strand; poly-GP is translated from both strands. DPRs have been shown to aggregate in the cytoplasm and appear as star-shaped inclusions in both neurons and glia (Mann et al., 2013; Schludi et al., 2015). Sense-derived poly-GA aggregated seem to be more frequent than others, however, both sense and antisense DPRs have been observed in neocortex, hippocampus, and thalamus (Cooper-Knock et al., 2012; Schludi et al., 2015). poly-GA and poly-PR inclusions were found to be more frequent in the granular layer of cerebellum and CA3/4 regions, respectively, in C9orf72-FTD compared to C9orf72-ALS and C9orf72-related ALS-FTD patients (Schludi et al., 2015). In contrast, DPRs were rarely observed in the brainstem and spinal cord. Arginine rich DPRs (poly-PR and poly-GR) have been documented to be toxic and contributory to neurodegeneration although these studies were performed on primary neuronal cultures and animal models (May et al., 2014; Mizielska et al., 2014; Moens et al., 2017). DPR inclusions stain positive for p62 (SQSTM1), which is a component of ubiquitin-proteasome system (and which is also mutated in ALS-FTD) whilst TDP-43 pathology is found to be variable, with TDP-43 inclusions not always present in the same neurons as DPR inclusions (Cooper-Knock et al., 2012, 2015a; Mann et al., 2013; Schludi et al., 2015; Hautbergue et al., 2017).

In a *Drosophila* model of C9orf72-related ALS and FTD, it was demonstrated that neurodegeneration was mediated through DPRs rather than RNA foci (Mizielska et al., 2014; Tran et al., 2015). Firstly, only pure repeats rather than stop-codon interrupted “RNA only” repeats led to a neurodegenerative-like phenotype in the flies (Mizielska et al., 2014). A second study generated several uninterrupted repeat constructs including 5, 12, 40, and 160 G4C2 repeats with flanking intron and exon sequences (Tran et al., 2015). Although the fly expressing this 160 G4C2 repeat formed abundant RNA foci in neurons and glia, no DPR were produced and it did not develop neurotoxicity (Tran et al., 2015). In contrast, BAC transgenic mice models of C9orf72-related ALS-FTD showed RNA foci and DPR inclusions without development of a neurodegenerative-like phenotype or behavioral abnormalities (O’Rourke et al., 2015; Peters et al., 2015). Recently, a DPR-only mouse expressing poly(GA)₁₄₉ conjugated to cyan fluorescent protein developed p62-positive poly-GA inclusions in motor neurons and interneurons of spinal cord, brain stem and in cerebellar nuclei, with motor deficits occurring at 4 months. However, there was no impairment to learning and memory (Schludi et al., 2017).

Expansions of G4C2 which are translated to form DPRs have been shown in *Drosophila* models and C9orf72-ALS iPSC derived neurons to disrupt nucleocytoplasmonic transport (NCT), both export of nuclear RNA and import of nuclear proteins, through binding to many of the nuclear pore complex proteins (Freibaum et al., 2015). RanGAP, a key regulator of nucleocytoplasmonic transport, was found to bind to both the G4C2 RNA and the DPR protein poly(GA) thereby causing defective nucleocytoplasmonic transport in *Drosophila* and mouse models of C9orf72-ALS and

iPSC derived neurons (Zhang et al., 2015, 2016). Poly(PR) and poly(GR) were also found to interact with RNA binding proteins and other low complexity domain proteins, including those in the nuclear pore complex (Lee et al., 2016) and using *Xenopus laevis* oocytes, poly(PR) was shown to bind and block the central channel of the pore (Shi et al., 2017). However, in the SHSY-5Y neuronal cell line and iPSC-derived neurons, it was shown that poly(GR) and poly(PR) had no effect on active nucleocytoplasmonic transport, though poly(GA) deficits were observed (Vanneste et al., 2019). Thus, there are likely to be specific pathogenic mechanisms associated with the different DPRs.

In summary, many studies have been conducted to elucidate the pathogenesis of the C9orf72 HRE in causing neurodegeneration and cognitive dysfunction without obtaining clear conclusions. Several factors including background of the animal studies, overexpression of mutation and experimental design can contribute to the variable results. It remains to be determined whether loss of C9orf72 protein function and toxic gain of function by RNA foci formation and DPR inclusion act in a concerted manner to manifest neurodegeneration in ALS and patient-derived cell models may be a more appropriate model for addressing these questions as they carry the HRE in a natural genetic context and the protein is expressed at physiological levels. However, a recent paper described that knocking down one or both endogenous C9orf72 alleles in transgenic mice expressing either 66 repeats or 450 repeats led to reduced autophagy and enhanced DPR accumulations, cognitive deficits, hippocampal neuron loss and glial activation (Zhu et al., 2020). Thus, this work demonstrated that there is a synergy between the loss of C9orf72 protein and the toxic gain of function mechanisms. In addition, these mechanisms offer targets for novel therapeutics and the antisense oligomer strategy currently in clinical trials may offer a disease-modifying therapy.

FTDALS2: Coiled-Coil Helix Coiled-Coil Helix Domain Containing Protein 10 (CHCHD10)

CHCHD10 was initially associated with ALS when it was shown to segregate with disease in a family presenting with a complex phenotype including ALS, FTD, cerebellar ataxia, and myopathy (Bannwarth et al., 2014). Subsequently, several cohorts of ALS and ALS-FTD patients were screened for *CHCHD10* mutations and a number of candidate rare, predicted deleterious mutations were identified (Johnson et al., 2014; Dols-Icardo et al., 2015). However, it has been shown more recently that many of the proposed mutations are present at similar frequency in controls (Marroquin et al., 2016), perhaps because exome sequencing studies typically give poor coverage of the *CHCHD10* gene which leads to a propensity for false positives. Whole genome sequencing, where coverage of the *CHCHD10* is complete, revealed that there was no significant burden of disease-associated mutations in sporadic ALS patients (Project MinE ALSSC, 2018). In fact, with increased coverage of control cohorts most of the ALS-associated mutations have been found at comparable frequency in controls despite *in vitro* and *in vivo* evidence for toxicity. Of the remaining mutations there is a

notable association with complex phenotypes including motor neuron degeneration but not typical ALS. The exception is c.44G > T (p.Arg15Leu) which has been identified in both sporadic and familial ALS cases with variable penetrance but is virtually absent in control databases (Project MinE ALSSC, 2018). Some of these cases have additional phenotypes such as hearing loss which may represent a distinct pathogenic process. However, assessment of TDP-43 pathology in these cases, which is arguably the molecular hallmark of ALS, is not yet available. CHCHD10 is localized to the mitochondria and patients with mutations in *CHCHD10* demonstrated abnormal mitochondrial morphology and respiratory chain deficiencies (Bannwarth et al., 2014).

FTDALS3: Sequestosome 1 (SQSTM1):

SQSTM1, also known as p62, is a ubiquitin binding protein that is present in a variety of ubiquitinated inclusions associated neurodegenerative diseases including ALS and FTD. Mutations in this gene were originally associated with Paget's disease of bone (PDB), a progressive skeletal disorder associated with an increased bone turnover producing localized lesions and bone pain (Rea et al., 2014). Missense mutations in the ubiquitin associated domain (UBA) or truncation mutations that cause partial or complete remove of the UBA account for 25–50% of familial PDB and 5–10% of sporadic PDB. However, the mutations in *SQSTM1* associated with ALS, FTD, and ALS-FTD cases are found throughout the gene, including the UBA, thereby impacting on many of the other pathways that the *SQSTM1* protein participates in Rea et al. (2014). Functional domains include a light chain 3 (LC3) interacting region (LIR) that interacts with LC3 to promote autophagy, a KEAP1 interacting region (KIR) which binds KEAP1 competitively with NRF2 to regulate oxidative stress response and the PB1 domain, that interacts with several proteins which impact on neuronal survival and inflammation (Ma et al., 2019). Whilst *SQSTM1* mutations have been found in individuals who also carry a *C9orf72* expansion (Almeida et al., 2016; Kovacs et al., 2016), the pathogenicity of *SQSTM1* mutations has been demonstrated in zebrafish, which showed behavioral abnormalities as well as disrupted autophagy and shorter axons following knockdown of the *SQSTM1* ortholog. Importantly, these features were rescued by human *SQSTM1* protein but not by the p.P392L common *SQSTM1* mutation (Lattante et al., 2015). In addition, the KEAP1-NRF2 signaling pathway and oxidative response has been shown to be disrupted by *SQSTM1* mutations (Deng et al., 2019; Foster et al., 2019), a pathway originally found to be dysregulated in SOD1-ALS (Kirby et al., 2005), which was subsequently implicated in sALS (Sarlette et al., 2008). Finally, it has recently been shown that *SQSTM1* co-localizes with misfolded MAPT and degrades the insoluble forms of the protein (Xu et al., 2019). In transgenic rTg4510 mutant MAPT mice, AAV-*SQSTM1* was administered to increase *SQSTM1* protein expression and this resulted in reduced mutant insoluble MAPT and improved pathology, including reduced astrogliosis and microgliosis.

FTDALS4: TANK-Binding Kinase (TBK1)

TBK1 mutations were first linked to ALS (Cirulli et al., 2015; Freischmidt et al., 2015), FTD (Gijselinck et al., 2015; Le Ber et al.,

2015) and ALS-FTD in 2015 (Pottier et al., 2015). Loss of function (LoF) mutations, including frameshifts, splice-site alterations, read-throughs and nonsense mutations have been reported to show definite or probable pathogenicity. The pathogenicity of missense mutations is less certain as some have also been found in controls (van der Zee et al., 2017) but such mutations in functional domains which impair target protein binding, or target or TBK1 phosphorylation, can also cause loss of function at the protein level (Freischmidt et al., 2015; Pozzi et al., 2017; van der Zee et al., 2017; de Majo et al., 2018). In addition, both LoF and missense mutations increase the risk of ALS/FTD (odds ratio 11.78 and 1.62, respectively) (Cui et al., 2018).

The mutation frequency of *TBK1* in ALS, FTD and ALS-FTD is reported to be from 0.4 to 1.7% (Gijselinck et al., 2015; van der Zee et al., 2017). More recently, a meta-analysis found LoF mutations in 1.0% and missense mutations in 1.8% of ALS and/or FTD, and suggests a higher prevalence in European populations compared to Asian populations (Cui et al., 2018). A separate paper also showed *TBK1* mutations to be the most important cause of ALS-FTD after *C9orf72* (Dols-Icardo et al., 2018).

TBK1 codes the TBK1 (TANK-binding kinase 1) protein, a kinase which binds and phosphorylates proteins involved in innate immunity (Pilli et al., 2012), autophagy (Korac et al., 2013), and mitophagy (Heo et al., 2015). Protein targets include optineurin (*OPTN*) (ALS12) and p62 (*SQSTM1*) (FTDALS3), two ALS-FTD associated genes (Maruyama et al., 2010; Rea et al., 2014) and mutations in both of these genes have been found along with *TBK1* mutations in patients (Pottier et al., 2015; Borghero et al., 2016; Dols-Icardo et al., 2018; Lattante et al., 2019).

TBK1 mutations have also been identified alongside the *C9orf72* expansion (Gijselinck et al., 2015; van der Zee et al., 2017), and mutations in *FUS* (Lattante et al., 2019), *TARDBP* (Freischmidt et al., 2015; de Majo et al., 2018), or *DCTN1* and *FUS* together (Muller et al., 2018). Interestingly, those harboring *TBK1* and *TARDBP* (de Majo et al., 2018) or *TBK1* and *FUS* (Freischmidt et al., 2015) mutations, showed earlier disease onset than those with *TBK1* alone (Freischmidt et al., 2015; Pozzi et al., 2017). One case showing *TBK1* and *C9orf72* mutations had a later disease onset; however, this was attributed to a shorter *C9orf72* expansion size of 59 repeats and variable penetrance of the *TBK1* mutation (Gijselinck et al., 2015). No further genotype-phenotype associations have been identified between *TBK1* mutation type or position, and clinical phenotype. Mutations occur throughout the *TBK1* gene, though missense variants cluster within the kinase and ubiquitin like domains (de Majo et al., 2018). *TBK1*-linked clinical phenotypes show variable age of onset, different rates of progression, and survival length (Gijselinck et al., 2015; Borghero et al., 2016; Pozzi et al., 2017; van der Zee et al., 2017; de Majo et al., 2018; Weinreich et al., 2019).

Cyclin F (CCNF)

Mutation of *CCNF* was first identified using genome-wide linkage followed by exome sequencing within a large ALS-FTD pedigree (Williams et al., 2016). Subsequently additional variants in both ALS and FTD cases were identified accounting for 0.6–3.3% of fALS-FTD patients among different populations (Williams et al., 2016; Pan et al., 2017). Cyclin F, encoded by *CCNF*, is one

of the components of an E3 ubiquitin-protein ligase complex also known as SCF^{CyclinF} (Skp1-Cull1-F-box E3 ubiquitin ligase complex) (Galper et al., 2017). Mutation of *CCNF* in neuronal cells causes errors in ubiquitination leading to ubiquitinated protein accumulation of SCF^{CyclinF} and TDP-43 as well as impairment of autophagosome-lysosome fusion (Williams et al., 2016; Lee et al., 2018). Recently, a *CCNF* mutation in a zebrafish model has been shown to have disrupted axonal outgrowth (Galper et al., 2017; Hogan et al., 2017). Further support for *CCNF* as an ALS-FTD gene comes from the finding that *CCNF* interacts with valosin containing protein (VCP) (ALS14), increasing VCP's ATPase activity, which in turn promotes TDP-43 aggregation (Yu et al., 2019). Thus, for the purposes of this review, *CCNF* is described as FTDALS5.

ALS GENES SUBSEQUENTLY ASSOCIATED WITH ALS-FTD AND FTD

There are many genes which have been characterized as causative for ALS where potentially pathogenic variants have also been described in FTD cases. Whilst this is rare, the co-occurrence of ALS and FTD being associated with mutations in these genes further strengthens the genetic linkage between these two disorders. These genes encode proteins associated with autophagy/ubiquitin proteasome system (UPS) or RNA processing. The exception is *TUBA4A*, which encodes a microtubule associated protein. In addition, intermediate CAG repeat expansions in *ATXN2* have been reported as a risk factor in ALS and ALS-FTD and a disease modifier in both ALS and FTD.

ALS6: Fused in Sarcoma (*FUS*)

Fused in sarcoma was initially identified as part of a fusion oncogene, *FUS-CHOP*, resulting from a *t*(12;16) (q13;p11) translocation event in malignant liposarcoma (Crozat et al., 1993; Robbins et al., 1993). Located at chromosome 16p11.2, *FUS* encodes a predominantly nuclear DNA/RNA binding protein which belongs to the FET protein family. As a functional component of the hnRNP complex, *FUS* is involved in many RNA processing activities, including transcription regulation, RNA transport and trafficking, pre-mRNA splicing, and miRNA processing. *FUS* consists of 15 exons which encode 526 amino acids. *FUS* has a multidomain structure consisting of an N-terminal glutamine-glycine-serine-tyrosine (QGSY) domain, three arginine-glycine-glycine rich domains (RGG1-3), an RRM, zinc finger motif (ZnF), and a highly conserved C-terminal NLS (Deng et al., 2014).

Mutations in *FUS* were first associated with autosomal recessive fALS, with additional screening revealing mutations in *FUS* to be causal in autosomal dominant ALS (Kwiatkowski et al., 2009; Vance et al., 2009). Further studies have shown that *FUS* mutations account for approximately 4% of fALS cases, and 1% of sALS cases. The vast majority of mutations are missense, with in-frame insertions and deletions occurring rarely. Although ALS-associated mutations occur throughout the whole length of the gene, most mutations are located in exons 3–6, encoding the N-terminal transcriptional activation

domain, QGSY, and the nucleic acid binding domain RGG1, or in exons 12–15 which encode C-terminal nuclear binding domains RGG2 and RGG3, a ZnF domain and an NLS domain (Deng et al., 2014). Mutations within exons 12–15 have been found to be functional, whilst those in exons 3–6, which are also more commonly found in sALS, do not always segregate with disease. This indicates the presence of non-pathogenic variations, and incomplete penetrance, highlighting the complexity of the role of *FUS* in ALS pathogenesis. Screening of FTD patients subsequently identified several *FUS* mutations in patients with bv-FTD either with or without concurrent ALS though the frequency of *FUS* mutations is much rarer in FTD than ALS cases (Ticozzi et al., 2009; Blair et al., 2010; Van Langenhouve et al., 2010; Huey, Ferrari et al., 2012).

Fused in sarcoma plays an important role in RNA processing. Therefore, mutations in *FUS* have a negative impact on RNA transcription, alternative splicing, and mRNA transport and stabilization. It is evident that this results in widespread neuronal dysfunction, contributing to the ALS phenotype, although, how this occurs is not well understood. Several theories have been proposed, including gain- and loss-of-function mechanisms (Deng et al., 2014). Wild-type *FUS* is predominantly located in the nucleus, however, disease-causing mutations in the C-terminal NLS of *FUS*, including the most common *FUS* mutation, p.R521C, leads to *FUS*-positive neuronal cytoplasmic inclusions. The accumulation of *FUS* aggregates has been found in the neuronal cytoplasm and dendrites of ALS and FTLD patients. This disruption of nuclear import may result in toxic gain of cytoplasmic function and loss of nuclear function (Deng et al., 2014; Lopez-Erauskin et al., 2018). *FUS* inclusions have also been found in atypical FTLD cases (aFTLD-U) as one of the proteins in the ubiquitinated neuronal inclusions, as well as being found in glial cells (Neumann et al., 2009). None of these cases had mutations in the *FUS* gene.

Fused in sarcoma was first found to have a role in RNA transcription when nuclear depletion of RNA polymerase II (RNAPII) resulted in an increase in cytoplasmic *FUS* (Zinszner et al., 1997). Further studies demonstrated the role of *FUS* in pre-mRNA splicing. *FUS* mediates the interaction between RNAPII and U1 snRNP, a splicing factor responsible for recognizing the 5' splice junction (Yu and Reed, 2015). Beyond this, *FUS* has been identified as component of the spliceosome, and also interacts with other important splicing factors such as hnRNPA1 (Rappsilber et al., 2002; Zhou et al., 2013; Kamelgarn et al., 2016). Loss of *FUS* functionality affects the splicing of its target genes, contributing to widespread splicing dysfunction of genes involved in neuronal functions, such as *PPP2R2C* which is required for neurogenesis and *ACTL6B* which has a role in dendritic development (Reber et al., 2016). Beyond this, *FUS* mutations also result in the mislocalisation of U1 snRNP to the cytoplasm, and the aggregation of *FUS*, hnRNPA1, hnRNPA2 and SMN1 into stress granules (Takanashi and Yamaguchi, 2014; Yu et al., 2015).

Transcriptome analysis of human MNs generated from mutant *FUS* iPSCs, identified changes in expression levels of genes involved in cellular processes which have previously been associated with neurodegenerative disease, including cell

adhesion. Also, TAF15 which is also a member of the FET family was found to be differentially expressed in FUS mutant MNs (De Santis et al., 2017). More recently, mutant FUS has been shown to affect important processes vital for neuronal functionality in mice. Studies using transgenic mice demonstrated that ALS/FTD-linked mutant FUS accumulates within axons, reducing intra-axonal translation which, in turn, causes early activation of the integrated stress response (ISR) and increased phosphorylation of eIF2 α . Ultimately, this inhibits the protein synthesis of important RNAs, including those encoding ion channels and transporters essential for synaptic function (Lopez-Erauskin et al., 2018). Furthermore, suppressed protein synthesis and disrupted regulation of nonsense mediated decay was detected in fibroblast cells derived from FUS-related ALS cases (Kamelgarn et al., 2018).

ALS10: TAR DNA Binding Protein (TARDBP)

TARDBP is located on chromosome 1p36.22 and encodes the transactive response DNA-binding protein 43 (TDP-43). Like FUS, TDP-43 is a predominantly nuclear DNA/RNA binding protein which is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family (Sreedharan et al., 2008). The *TARDBP* gene consists of 6 exons, and has a similar structure to *FUS*; an N-terminal domain (NTD), 2 RNA recognition motifs (RRM1-2) which are involved in RNA and DNA binding, a nuclear localization signal and nuclear export signal, and a C-terminal glycine-rich domain (GRD) which is responsible for protein–protein interactions (Lagier-Tourenne et al., 2010; Baralle et al., 2013). TDP-43 was initially recognized as a transcription repressor protein which binds to the TAR regulatory element of human immunodeficiency virus-1 (HIV-1) (Ou et al., 1995). Further investigations have shown that TDP-43 has other important roles in RNA processing, including RNA transcription, pre-mRNA and pre-miRNA splicing, RNA transport and mRNA stability (Scotter et al., 2015).

Mutations in *TARDBP* are responsible for 4–5% of fALS cases and 1% of sALS cases, and are inherited in an autosomal dominant manner (Millecamps et al., 2010). *TARDBP* mutations cause an ALS phenotype consisting of classic ALS symptoms. Mutations in *TARDBP* have also been reported in patients with FTD, with and without ALS (Benajiba et al., 2009; Borroni et al., 2009; Kovacs et al., 2009; Pesiridis et al., 2009). The frequency of *TARDBP* mutations in patients with FTD is estimated at 1%, the majority presenting with bvFTD, though some patients do present with svFTD or nfvFTD at onset (Caroppo et al., 2016).

The majority of mutations are located in exon 6, which encodes the aggregation-prone C-terminal GRD. These mutations increase the aggregation potential of this protein. Ubiquitinated aggregates of TDP-43 are found in the cytoplasm of MNs of ALS and FTD patients, not just patients with *TARDBP* mutations (Neumann et al., 2006; Mackenzie and Rademakers, 2008; Johnson et al., 2009; Kirby et al., 2010). Given that 97% of fALS and sALS patients are positive for TDP-43 cytoplasmic inclusions, it is evident TDP-43 plays an important role in MN

degeneration and disease pathogenesis (Sreedharan et al., 2008; Qin et al., 2014). In addition, TDP-43 positive inclusions are also found in 50% of FTLD cases (Neumann et al., 2006; Mackenzie et al., 2010). Although it is unknown how this occurs, it has been hypothesized that this may be due to toxic gain of cytoplasmic function and loss of nuclear function (Kabashi et al., 2010).

TDP-43 is functionally homologous to FUS and also has important functions in RNA metabolism. Mutations in *TARDBP* result in aberrant RNA processing on multiple levels; transcription regulation, alternative splicing and mRNA stability (Buratti and Baralle, 2008). Beyond regulating its own expression level by binding to the 3' untranslated region (3' UTR) of its mRNA, TDP-43 is also essential for maintaining normal expression levels and splicing patterns of over 1,000 mRNAs (Ayala et al., 2008; Polymenidou et al., 2011). Specifically, TDP-43 dysfunction results in the dysregulated expression of other ALS-associated proteins which also have roles in RNA metabolism, including FUS, ATXN2, and progranulin (PGRN) (Polymenidou et al., 2011; Sephton et al., 2011; Highley et al., 2014). Furthermore, dysfunction of TDP-43 results in defective alternative splicing of its target genes, including *hnRNPA1*, which negatively impacts cellular stability (Butti and Patten, 2018). Additionally, TDP-43 is involved in the splicing of cryptic exons of particular mRNAs, such as *ATG4B* (autophagy related 4B cysteine peptidase). Splicing of cryptic exons produces aberrant mRNA products. These have been observed in the CNS of ALS and FTD patients and have been recently been linked to impaired autophagy (Ling et al., 2015; Torres et al., 2018). It is known that TDP-43 is a component of stress granules, but how this contributes to the ALS phenotype is unknown (Aulas and Vande Velde, 2015).

ALS12: Optineurin (OPTN)

Amyotrophic lateral sclerosis-associated mutations in *OPTN*, which was previously implicated in glaucoma, were first identified in 2010 in six affected members of a Japanese pedigree with consanguineous marriages presenting with three different types of mutations: a homozygous deletion of exon 5, a homozygous nonsense p.Q398X mutation and a heterozygous missense p.E478G mutation (Maruyama et al., 2010). Subsequently, more than 20 mutations have been described although not all have been investigated in *in vitro* and *in vivo* disease models. The incidence of *OPTN* mutations in FTD is still under debate, as one study reported copy number variants in *OPTN* in 4.8% of FTD cases (Pottier et al., 2015) while another study, recruiting a larger cohort of 371 FTD cases, did not detect any mutations using whole exome sequencing (Rollinson et al., 2012). More recently, a patient with ALS-FTD was reported with compound heterozygous mutations, resulting in a 75–80% reduction in *OPTN* (Pottier et al., 2018).

OPTN is a highly conserved hexameric protein that is ubiquitously expressed with significantly high expression in skeletal muscles (Toth and Atkin, 2018). *OPTN* is known to interact with TBK1 (FTDALS4); in fact, a series of evolutionarily conserved serine residues precedes the hydrophobic core sequence in *OPTN* which bears homology to TBK1-binding site of TANK, another substrate of TBK1 (Wild et al., 2011).

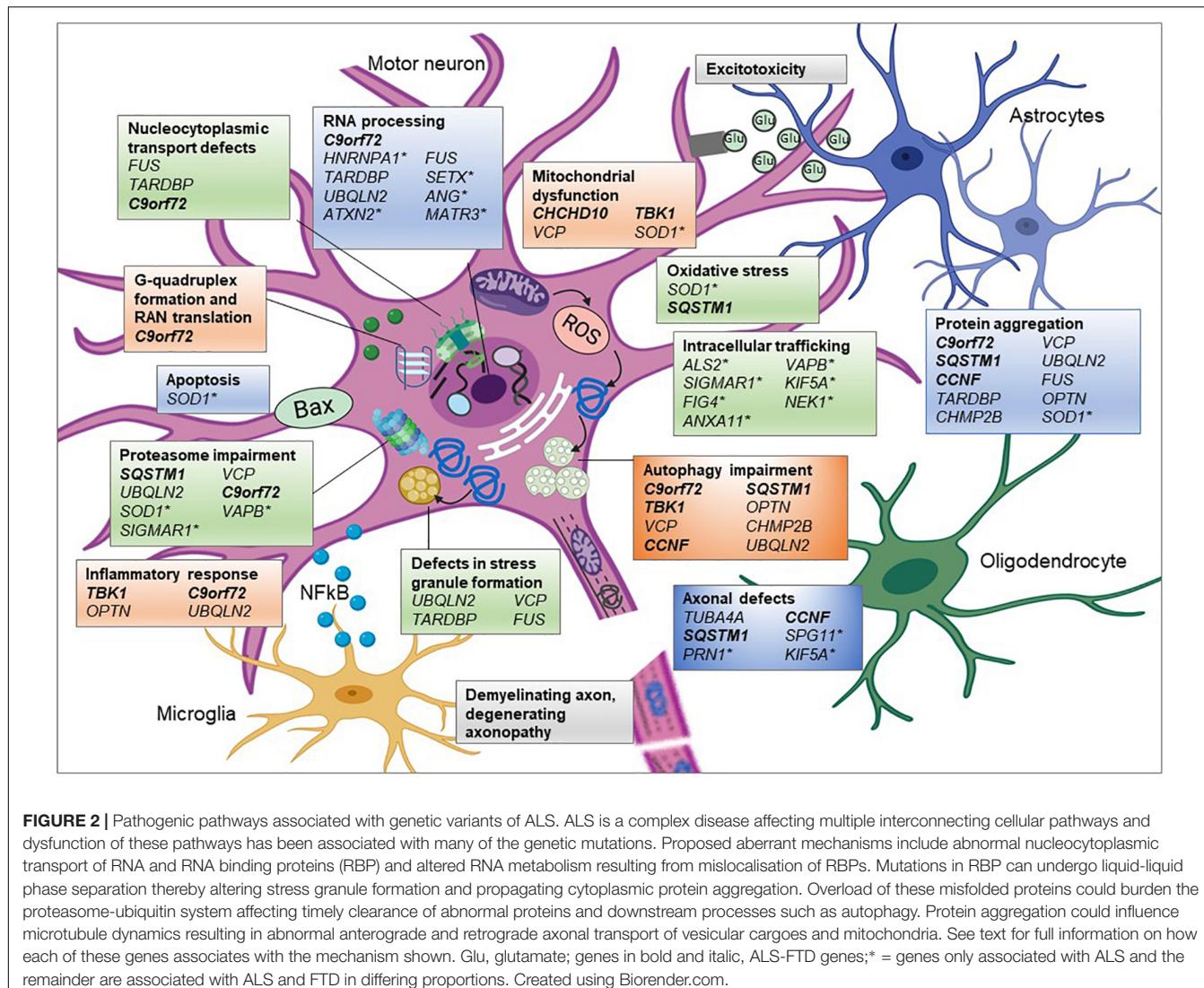


FIGURE 2 | Pathogenic pathways associated with genetic variants of ALS. ALS is a complex disease affecting multiple interconnecting cellular pathways and dysfunction of these pathways has been associated with many of the genetic mutations. Proposed aberrant mechanisms include abnormal nucleocytoplasmic transport of RNA and RNA binding proteins (RBP) and altered RNA metabolism resulting from mislocalisation of RBPs. Mutations in RBP can undergo liquid-liquid phase separation thereby altering stress granule formation and propagating cytoplasmic protein aggregation. Overload of these misfolded proteins could burden the proteasome-ubiquitin system affecting timely clearance of abnormal proteins and downstream processes such as autophagy. Protein aggregation could influence microtubule dynamics resulting in abnormal anterograde and retrograde axonal transport of vesicular cargoes and mitochondria. See text for full information on how each of these genes associates with the mechanism shown. Glu, glutamate; genes in bold and italic, ALS-FTD genes;* = genes only associated with ALS and the remainder are associated with ALS and FTD in differing proportions. Created using Biorender.com.

OPTN is involved in several cellular functions including autophagy, vesicular trafficking, Golgi maintenance [as evident from Golgi apparatus fragmentation in spinal motor neurons and glia in post mortem tissue obtained from an ALS-FTD patient (Kamada et al., 2014) and neuroinflammation (Toth and Atkin, 2018; McCauley and Baloh, 2019)]. OPTN has also been shown to regulate NF κ B signaling wherein ALS associated mutations in OPTN showed increased immunoreactivity of microglia (McCauley and Baloh, 2019). OPTN-positive cytoplasmic inclusions in the CNS are not only seen in cases with OPTN mutations, but also in *C9orf72*, *FUS*, and *SOD1*-related cases (Bury et al., 2016). Interestingly, conditional loss of OPTN by Cre-loxP system in different cell types using a murine model (*Cnp-cre*, *Lyz2-cre*, *Gfap-cre*, and *Mnx1-cre* mice) showed RIPK1-mediated necroptosis resulting in axonal myelination pathology when OPTN was depleted in oligodendrocytes and myeloid cells, whereas no pathology was observed when OPTN expression was selectively removed in astrocytes and motor neurons (Ito et al., 2016),

further confirming non-cell autonomous toxicity in driving neurodegeneration.

ALS15: Ubiquilin 2 (UBQLN2)

Mutations in *UBQLN2*, which is localized on the X chromosome, were first identified in large ALS-FTD family in 2011 (Deng et al., 2011). Four mutations located within the proline-X-X (PXX) repeat region of the protein were subsequently found through additional screening of FALS cases with no male to male transmission. Further variants within or adjacent to the PXX repeat region have been identified in ALS, FTD, or ALS-FTD patients, though at rare frequencies (Williams et al., 2012; Gellera et al., 2013; Ugwu et al., 2015). As a member of the ubiquilin family, the protein is actively associated in the degradation of misfolded and redundant proteins through macroautophagy and the ubiquitin-proteasome system (Renaud et al., 2019). Mutations cause defective binding to the proteasome leading to interruption of the protein degradation, triggering mislocalisation of OPTN from Rab-11 positive endosomal

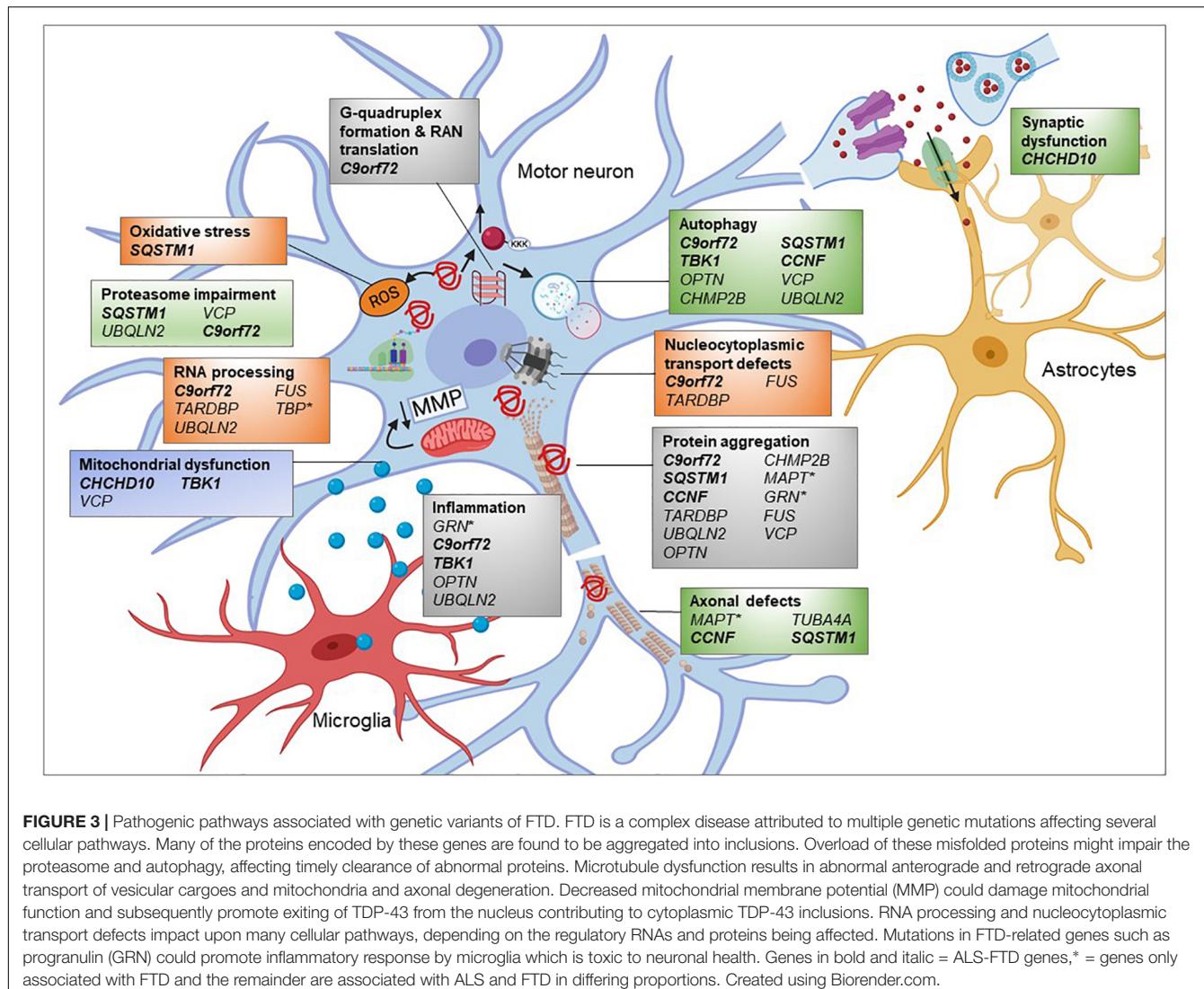


FIGURE 3 | Pathogenic pathways associated with genetic variants of FTD. FTD is a complex disease attributed to multiple genetic mutations affecting several cellular pathways. Many of the proteins encoded by these genes are found to be aggregated into inclusions. Overload of these misfolded proteins might impair the proteasome and autophagy, affecting timely clearance of abnormal proteins. Microtubule dysfunction results in abnormal anterograde and retrograde axonal transport of vesicular cargoes and mitochondria and axonal degeneration. Decreased mitochondrial membrane potential (MMP) could damage mitochondrial function and subsequently promote exiting of TDP-43 from the nucleus contributing to cytoplasmic TDP-43 inclusions. RNA processing and nucleocytoplasmic transport defects impact upon many cellular pathways, depending on the regulatory RNAs and proteins being affected. Mutations in FTD-related genes such as progranulin (GRN) could promote inflammatory response by microglia which is toxic to neuronal health. Genes in bold and italic = ALS-FTD genes, * = genes only associated with FTD and the remainder are associated with ALS and FTD in differing proportions. Created using Biorender.com.

vesicles as well as loss of binding of UBQLN2 to hnRNP proteins, including hnRNPA1 (ALS20) resulting in impaired RNA metabolism (Chang and Monteiro, 2015; Gilpin et al., 2015; Osaka et al., 2015). ALS-linked mutations in UBQLN2 gene were also found to be associated with dysfunction of autophagy, neuroinflammation, as well as the formation of stress granules, where mutations disrupted interaction with FUS (ALS6) and FUS-RNA complexes (Picher-Martel et al., 2015; Hjerpe et al., 2016; Alexander et al., 2018; Dao et al., 2018; Renaud et al., 2019).

ALS22: Tubulin Alpha 4A (TUBA4A)

Mutations in Tubulin alpha 4A (TUBA4A) were identified as a very rare cause of ALS following the discovery of non-synonymous variants during whole exome sequencing of fALS index cases (Smith et al., 2014). Whilst patients had spinal-onset ALS, two cases developed FTD, whilst a third case had FTD in a first degree relative. Additional studies identified further cases of FTD and ALS with mutations in TUBA4A, though they were exceptionally rare, whilst other papers failed to find

any TUBA4A mutations in ALS or FTD cohorts (Dols-Icardo et al., 2016; Perrone et al., 2017; Li et al., 2018). TUBA4A encodes an alpha tubulin subunit which combines with other alpha and beta tubulins to form microtubules. Mutant TUBA4A proteins showed altered incorporation into microtubules, thereby reducing the stability of the microtubule network in a dominant negative manner (Smith et al., 2014). Interestingly, a study identifying miR-1825 as decreased in both serum and plasma of sALS and fALS, was shown to directly target tubulin-folding co-factor b (TBCB) expression and this was associated with depolymerisation and degradation of TUBA4A protein in HEK293 cells (Helferich et al., 2018). Additional studies in zebrafish embryos expressing human TBCB displayed reduced levels of the TUBA4A zebrafish homolog and reduced axonal length and branching, whilst TBCB and TUBA4A proteins levels were inversely correlated in post-mortem brain cortex of fALS and sALS. Thus, TUBA4A is implicated not only through genetic mutations, but also by dysregulation of an upstream miRNA in both fALS and sALS cases.

TABLE 2 | Clinical phenotypes also associated with ALS and FTD related genes.

ALS loci	Gene name	Alternative clinical phenotypes	Inheritance
FTDALS2	<i>CHCHD1</i>	SMA, Jokela type (SMAJ); Myopathy isolated mitochondrial, autosomal dominant (IMMD)	AD AD
FTDALS3	<i>SQSTM1</i>	Paget disease of bone 3 (PDB3); Myopathy, distal, with rimmed vacuoles (DMRV); Neurodegeneration with ataxia, dystonia and gaze palsy, childhood onset (NADGP)	AD AD AR
FTDALS4	<i>TBK1</i>	Encephalopathy, acute, infection-induced 8 (IIAE8) (susceptibility to)	AD
ALS1	<i>SOD1</i>	Spastic tetraplegia and axial hypotonia, progressive (STAHP)	AR
ALS2	<i>ALS2</i>	Primary lateral sclerosis, juvenile (PLSJ); Spastic paraparesis, infantile onset ascending (IAHSP)	AR AR
ALS4	<i>SETX</i>	Spinocerebellar ataxia, with axonal neuropathy 2 (SCAN2)	AR
ALS5	<i>SPG11</i>	Spastic paraparesis 11 (SPG11); Charcot-Marie-Tooth disease, axonal, type 2X (CMT2X)	AR AR
ALS6	<i>FUS</i>	Tremor, hereditary essential 4 (ETM4)	AD
ALS8	<i>VAPB</i>	Spinal muscular atrophy (SMA), late onset, Finkel type (SMAFK)	AD
ALS11	<i>FIG4</i>	Charcot-Marie-Tooth disease, type 4J (CMT4J); Yunis-Varon syndrome Polymicrogyria, bilateral temporo-occipital (BTOP)	AR AR AR
ALS12	<i>OPTN</i>	Glaucoma, primary open angle (POAG); Glaucoma, normal tension (susceptibility to)	AD
ALS13	<i>ATXN2</i>	Spinocerebellar ataxia 2 (SCA2); Parkinson's disease, late onset (susceptibility to)	AD AD
ALS14	<i>VCP</i>	Charcot-Marie-Tooth disease, type 2Y (CMT2Y); Inclusion body myopathy with early onset Paget disease and frontotemporal dementia 1 (IBMPFD1)	AD AD
ALS16	<i>SIGMAR1</i>	SMA, distal, autosomal recessive 2 (DSMA2)	AR
ALS20	<i>hnRNPA1</i>	Inclusion body myopathy with early onset Paget disease and frontotemporal dementia 3 (IBMPFD3)	AD
ALS24	<i>NEK1</i>	Short-rib thoracic dysplasia 6, with or without polydactyly (SRTD6)	AR
ALS25	<i>KIF5A</i>	Spastic paraparesis 10 (SPG10); Myoclonus, intractable, neonatal (NEIMY)	AD AD

Data obtained from Gene-Phenotype relationship data available on OMIM (<http://www.ncbi.nlm.nih.gov/omim>).

ALS13: Ataxin 2 (ATXN2)

A CAG repeat expansion encoding a polyglutamine repeat is found in ataxin 2 (ATXN2), a ubiquitously expressed protein involved in RNA processing, stress granule formation, endocytosis, calcium signaling and controlling metabolism and energy balance. In the normal population, the size varies between 13 and 31 CAG repeats, though 22–23 repeat are the most common (Velazquez-Perez et al., 2017). Repeats of over 35 are associated with fully penetrant spinocerebellar ataxia 2, with those 32–34 showing variable penetrance. Following identification that ATXN2 interacts with TDP-43, intermediate repeats of 27–33 were found to be a risk factor for ALS (Elden et al., 2010), with the intermediate CAG repeat interrupted with a CAA codon (Corrado et al., 2011). Subsequently, a meta-analysis of 9 studies highlighted that whilst there was an increased risk of ALS from 29 CAG/CAA repeats, significance was only reached for 31–33 repeats (Neuenschwander et al., 2014). Interestingly, repeat sizes of 27–28 were found to lower risk of ALS. More recently, a meta-analysis of 16 published studies, along with two large unpublished cohorts of ALS demonstrated an increased risk of ALS with 29–32 CAG/CAA repeats, and this risk increased with the number of repeats (Sproviero et al., 2017). This study also found 27 repeats to have a protective effect.

Following the link with ALS, the role of ATXN2 intermediate repeats in FTD was investigated. Screening of ALS and FTD alongside other neurodegenerative diseases identified 30–33 repeats to be associated with ALS but not FTD (Ross et al., 2011). Subsequently, ATXN2 CAG repeats of ≥ 29 were also found to be associated with ALS and familial ALS-FTD but not sporadic ALS-FTD or FTD (Lattante et al., 2014). A further study of 368 cases also found no significant correlation between FTD and

ATXN2 CAG repeat size though they did find that intermediate repeats (≥ 27) were associated with an earlier age at onset of FTD (Rubino et al., 2019).

Screening of ATXN2 has also identified expansions > 34 in rare cases of both ALS (Corrado et al., 2011; Ross et al., 2011; Van Damme et al., 2011) and FTD (Baumer et al., 2014; Fournier et al., 2018) although no signs of ataxia were reported and neuropathological examination confirmed a diagnosis of ALS. As well as interacting with TDP-43, ATXN2 has also shown to bind to mutant FUS, with intermediate repeats binding both WT and mutant FUS proteins (Farg et al., 2013).

FTD GENES SUBSEQUENTLY ASSOCIATED WITH ALS-FTD AND ALS

Several ALS genes identified through next generation sequencing have previously been identified as being associated with FTD or a syndrome incorporating FTD, such as inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD). These include *VCP* and *CHMP2B*.

ALS14: Valosin Containing Protein (VCP)

Mutations in *VCP* have been described in ALS, FTD and inclusion body myopathy with Paget's disease of bone and FTD (IBMPFD) which is an adult onset disorder characterized by muscle weakness, early onset PDB (see section "Cyclin F (CCNF)") and FTD, though episodic memory is preserved (Kimonis, 1993). Mutations in *VCP* account for 1–2% of fALS cases, are found to be rare in sALS (Koppers et al., 2012) and whilst FTD is recognized in a third of IBMPFD patients,

mutations have been found in FTD cases (Saracino et al., 2018; Wong et al., 2018).

Valosin containing protein (also called as CDC48 or p97) is a hexameric ATPase that is ubiquitously expressed and involved in diverse cellular functions including autophagy, endoplasmic reticulum (ER)- associated degradation (ERAD), chromatin remodeling, DNA repair and other protein quality control pathways (Wang et al., 2016; Shahheydari et al., 2017). ATPase has two domains, D1 and D2 and a regulatory N-domain. A majority of the mutations in VCP have been documented in the N-domain in patients with ALS and/or FTD (Abrahao et al., 2016; Wang et al., 2016; Shahheydari et al., 2017) although additional ALS and FTD mutations have been reported in the D1 domain (Wong et al., 2018). A study reported that mutations in the N-domain, an evolutionarily conserved region in VCP, results in poor hexamer assembly and reduced small ubiquitin-like modifier (SUMO)-ylation of VCP that diminishes its recruitment to stress granules and consequently affects ERAD in a *Drosophila* model of ALS/FTD. In contrast, a recent study that screened 48 patients with FTD reported identified 3 mutations that lie within the D1 domain of VCP and are hypothesized to affect ATPase binding activity (Wong et al., 2018). Interestingly, it has been reported that VCP interacts with FUS (ALS6) (Wang et al., 2015) and Cyclin F (CCNF) (proposed FTDALS5) (Yu et al., 2019) both of which are implicated in ALS. Mutations in *FUS/CCNF* were shown to increase ATPase activity of VCP in the cytoplasm, causing VCP to mislocalize to the cytoplasm (Yu et al., 2019) and trigger accumulation of polyubiquitinated proteins (Wang et al., 2015). VCP is also vital in mitochondrial quality control and IBMFTD patient fibroblasts carrying a mutation in VCP showed uncoupling of mitochondria, reduced mitochondrial membrane potential and ATP production (Bartolome et al., 2013), a feature that is also evident in *SIGMAR1* (ALS16) mutations.

ALS17: Chromatin Modifying Protein 2B (CHMP2B)

Mutation of *CHMP2B* was initially identified in a large Danish family with FTD linked to chromosome 3 (termed FTD-3) (Skibinski et al., 2005). The splice site mutation c.532-1G > C results in the formation of two transcripts encoding two different proteins with a defective carboxyl terminus: CHMP2B^{intron5}, where the intronic sequence between exons 5 and 6 is retained and a single valine is incorporated instead of the final 36 amino acids encoded by exon 6 and CHMP2B^{del10}, where a cryptic splice site is used 10 bp from exon 6, resulting in the insertion of 29 novel amino acids. Subsequently, a Belgian family with FTD-3 was identified, where the c.493C > T mutation lead to truncation of the protein, with the loss of 49 amino acids (van der Zee et al., 2008). In contrast, mutations identified in *CHMP2B* that were associated with ALS were missense mutations (Parkinson et al., 2006).

CHMP2B is a component of the endosomal sorting complex required for transport III (ESCRT-III) complex, which is involved in the maturation of endosomes and autophagosomes. Using cellular and animal models, mutations in *CHMP2B* (both truncated and missense mutations) have been shown to disrupt

endosomal-lysosomal trafficking, through accumulation and enlargement of endosomes (Cox et al., 2010; Zhang Y. et al., 2017; Vandal et al., 2018). The pathology of FTD-3 cases is distinguished by the presence of ubiquitin and p62 (*SQSTM1*; FTDALS3) positive inclusions, which are negative for TDP-43 and Tau (Holm et al., 2007).

OTHER ALS AND FTD GENES

In addition to those genes described above, there are other genes associated solely with fALS and fFTD, including “Pure” ALS genes such as *SOD1* and “Pure” FTLD genes *MAPT* and *Progranulin* (Bennion Callister and Pickering-Brown, 2014). However, it is notable that many of these pure ALS genes also encode proteins that cluster into functional pathways associated with ALS-FTD genes, with mutations in the pure ALS gene affecting similar biological pathways. For example, numerous genes are associated with autophagy/proteasome impairment (*C9orf72*, *SQSTM1*, *TBK1*, *OPTN*, *VCP*, *UBQLN2*, and *CHMP2B*) and/or their proteins are found to be aggregated in cytoplasmic inclusions (*C9orf72*, *SQSTM1*, *OPTN*, *VCP*, *UBQLN2*, *CCNF*, *FUS*, *TDP-43*, and *CHMP2B*) (Figure 2). Many of these genes also encode proteins that have a role in RNA processing (*C9orf72*, *FUS*, *TARDBP*, and *UBQLN2*), whilst others are associated with and dysregulate the mitochondria (*CHCHD10*; *TBK1*; and *VCP*) or the cytoskeleton (*TUBA4A*, *CCNF*, and *SQSTM1*). *SOD1*-ALS, accounting for around 10% of fALS cases, is distinctive in that it is not associated with TDP-43 inclusions, unlike the majority of ALS cases. However, mutations in *SOD1* are associated with similar pathogenic mechanisms, such as disruption to protein quality control, mitochondrial dysfunction, dysregulated axonal transport and RNA processing, in addition to oxidative stress and excitotoxicity. Due to the wide range of biological pathways, gene silencing of *SOD1* is currently in clinical trials as a therapeutic strategy for *SOD1*-ALS patients (van Zundert and Brown, 2017).

Many of the additional ALS genes can also be categorized into these pathways (Table 1 and Figure 2), such as *SETX*, *ANG*, *ATXN2*, *hnRNPA1*, and *MATR3*, which are all involved in RNA processing and *SPG11*, *KIF5A*, and *PFN1* that are associated with the cytoskeleton and mutations in which cause axonal defects (Alsultan et al., 2016). Many of the genes also encode proteins involved in trafficking components within the cell, such as endosomes (*ALS2*, *FIG4*, and *NEK1*) or in the unfolded protein response (*VAPB* and *SIGMAR1*). However, as research has investigated the effect of mutations within these genes, additional secondary pathways have been implicated, such as mutant *SOD1* protein’s effect on protein homeostasis, gene expression and axonal transport, resulting in a complex interactome of direct and indirect effects, which ultimately lead to neurodegeneration.

Whilst there are far fewer genes associated with only FTD, *GRN* (responsible for 5–20% of fFTD) and *MAPT* (responsible for 10–20% of fFTD) are also involved in similar pathways (Figure 3). The progranulin gene (*GRN*) encodes a secreted glycoprotein that is taken up by the cell and cleaved into multiple smaller granulins. The precise function of granulin is

still to be determined, though it has been shown to be involved in multiple pathways including neuronal survival, neurite outgrowth, neuroinflammation, and autophagy (Olszewska et al., 2016). Mutations in *GRN*, leading to haploinsufficiency, are thought to cause FTD through lysosomal defects and reduced clearance of proteins (Ferrari et al., 2019). As with ALS, TDP-43 inclusions are also present, of the FTLD-TDP Type A form (Mackenzie et al., 2011). *MAPT*, encoding the microtubule associated protein tau, stabilizes microtubules through binding to tubulin. Mutations in *MAPT* disrupt this binding and lead to hyperphosphorylated tau aggregates. Recently, mutations in the TATA-box-binding gene (*TBP*), normally associated with spinocerebellar atrophy 17 (SCA17), were identified in a patient with FTD whose MRI showed cerebellar atrophy (Olszewska et al., 2019). The variant was found to co-segregate with disease. Thus, this new FTD gene, which encodes a transcription initiation factor can be categorized as an RNA processing gene.

The identification of a novel gene in a pathway that has not previously been associated with the genetics of ALS or FTD is particularly valuable for highlighting new disease biology and subsequently novel therapeutic targets. In this respect, the identification of mutations in *GLT8D1* and *DNAJC7* in fALS cases are notable discoveries. *GLT8D1* is a glycosyltransferase with an enrichment of familial ALS-associated mutations proximal to the substrate binding site (Cooper-Knock et al., 2019). It was demonstrated that the mutations negatively impact enzyme activity suggesting a loss of function mechanism. Whilst the exact role of *GLT8D1* remains to be discovered, however, glycosyltransferases are known to be involved in the synthesis of gangliosides which are signaling molecules important for motor neuron function (Harschnitz et al., 2014). As such, it is perhaps not surprising that glycosyltransferase dysfunction has already been associated with other neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's disease.

DNAJC7 encodes a heat shock protein (HSP40) which alongside HSP70 facilitates protein homeostasis, through folding new peptides and removing misfolded proteins. Rare protein truncating mutations were identified in *DNAJC7* in ALS cases and were absent from controls and subsequent screening identified further loss of function mutations as well as several rare missense mutations, predicted to be damaging (Farhan et al., 2020). In fibroblasts from a patient with a p.Arg156Ter mutation, protein levels were reduced, suggesting that these mutations may lead to protein aggregation, a characteristic feature of ALS. Further screening of *DNAJC7* and *GLT8D1* in additional cohorts of ALS, ALS-FTD, and FTD cases will establish the contribution of these genes and the roles of their proteins in disease pathogenesis.

CONCLUSION

Amyotrophic lateral sclerosis and FTD have been described as forming a spectrum of disease, with converging mechanisms of neurodegeneration involving RNA processing, stress granules, protein aggregation and autophagy supporting this proposal (Ling et al., 2013; Deng et al., 2017; Nguyen et al., 2019;

Baradaran-Heravi et al., 2020). However, it is also clear that some genes, such as *MAPT*, are quite distinct and therefore it is argued that these should be kept separate (Hardy and Rogeava, 2014). This distinction is also supported by the neuropathology, as whilst the majority of genetic (and sporadic) ALS cases have TDP-43-positive inclusions, along with *C9orf72* and *GRN*-FTD, *MAPT*- FTD does not, similar to *SOD1*-ALS. Thus, these distinctions are important to consider when pursuing diagnostic and prognostic biomarkers or therapeutic strategies.

The application of next generation sequencing, either in the form of targeted, whole exome or whole genome sequencing (WGS) has had a significant impact on the identification of genes associated with these diseases. However, it is also: (i) broadening the range of diseases that we see associated with variants in these genes (Table 2), (ii) broadening the range of genes that you would conventionally associate with ALS and FTD (Blauwendraat et al., 2018; Tripolszki et al., 2019), (iii) increasing the frequency of variants in known ALS and FTD genes within apparently sporadic cases, highlighting the variable penetrance of many of these proposed mutations (Tripolszki et al., 2019), (iv) identifying multiple variants in disease-associated genes within an individual (Cady et al., 2015), which will become increasingly important as personalized medicine based on your genetic mutation enters the clinic and finally (v) illustrating both the variability in frequencies of known genes across populations worldwide (Majounie et al., 2012; Wei et al., 2019), but also the inequality as the majority of these studies are undertaken in the northern hemisphere. It is hoped that WGS of large international cohorts of ALS and FTD such as Project MinE² and GENFI³ will begin to fully understand the genetic contribution to disease and potentially answer why individuals with a particular variant go on to develop ALS, FTD or ALS-FTD.

AUTHOR CONTRIBUTIONS

RR, SH, KC, SS, JC-K, and JK wrote the sections of the manuscript. JK and RR designed and drew the figures. SH drafted Table 1. JK drafted Table 2. JK completed the review of all sections, final edits, and formatting. All authors contributed to the article and approved the submitted version.

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²www.projectmine.com

³<http://genfi.org.uk/>

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Feature Review

Connecting TDP-43 Pathology with Neuropathy

Joseph R. Klim ,^{1,2,4} Greta Pintacuda,^{1,2} Leslie A. Nash,^{1,2} Irune Guerra San Juan,^{1,2,3} and Kevin Eggan^{1,2,*,@}

Transactive response DNA-binding protein 43 kDa (TDP-43), a multifunctional nucleic acid-binding protein, is a primary component of insoluble aggregates associated with several devastating nervous system disorders; mutations in *TARDBP*, its encoding gene, are a cause of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Here, we review established and emerging roles of TDP-43 and consider how its dysfunction impinges on RNA homeostasis in the nervous system, thereby contributing to neural degeneration. Notably, improper splicing of the axonal growth-associated factor *STMN2* has recently been connected to TDP-43 dysfunction, providing a mechanistic link between TDP-43 proteinopathies and neuropathy. This review highlights how a deep understanding of the function of TDP-43 in the brain might be leveraged to develop new targeted therapies for several neurological disorders.

TDP-43 Is Implicated in Neurodegeneration

Efforts to understand the functions of **TDP-43** (see [Glossary](#)) in the brain were spurred on by two major findings: (i) the demonstration that mutations in *TARDBP* can cause familial ALS and FTD [1,2]; and (ii) the discovery that this RNA-binding protein is a major constituent of ubiquitin-positive aggregates within the vulnerable cells of most patients with these diseases [3]. Since these initial findings, TDP-43 pathology has been observed in additional maladies, including primary lateral sclerosis [4], progressive muscular atrophy [5], Guam Parkinson dementia complex [6], Parkinson's disease (PD) with and without dementia [7], a subset of Alzheimer's disease (AD) cases [8], inclusion body myopathy [9], and in as many as half of patients with traumatic brain injury (TBI) [10]. As this list of **TDP-43 proteinopathies** grows, the need to understand the role of TDP-43 in disease pathogenesis increases (see Outstanding Questions).

At present, it remains unresolved to what extent neurodegeneration associated with TDP-43 results from a **loss of function** linked to impaired mRNA processing [11], **gain of functions** (GOFs) related to toxic aggregations [12–14], or a combination of both [15]. In this review, we summarize recent progress toward understanding the consequences of reduced TDP-43 activity within the nervous system as well as new insights into how its accumulation or persistent aggregation in TDP-43 proteinopathies might disrupt neuronal function. We focus particularly on the links between alterations in TDP-43 biology and neuropathy, including a discussion of the most direct evidence connecting TDP-43 with axonal biology through its regulation of the microtubule-binding protein **Stathmin-2** (*STMN2*). We conclude with our views on how molecular interrogation of TDP-43 function is nominating novel potential therapeutic targets that could serve as points of clinical intervention.

Early TDP-43 Studies

TDP-43 is an RNA-binding protein with a broad range of functions linked to RNA metabolism, including transcription, translation, mRNA transport and stabilization, miRNA biogenesis, and long noncoding RNA processing [16]. Yet, it remains to be determined which of these disparate

Highlights

The list of diseases associated with TDP-43 aggregation has grown beyond ALS and FTD.

Genetic findings implicate impaired proteostasis in disease pathogenesis, and molecular studies tie decreased protein turnover to TDP-43 aggregation.

Mutations in the glycine-rich domain of TDP-43 incriminate altered phase separation in disease pathogenesis, and new mutations in the RNA recognition motifs potentially affect both RNA binding and solubility.

Altered regulation of critical neuronal transcripts, including *STMN2*, is associated with the loss of TDP-43 function, providing a mechanistic link between TDP-43 and neuropathy.

Aberrant changes in *STMN2* expression in other neurodegenerative diseases spotlight the critical nature of this cytoskeletal regulator.

Key TDP-43-regulated transcripts may serve as biomarkers for detecting, characterizing, and treating associated proteinopathies.

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functions are essential in cell types vulnerable to TDP-43 proteinopathies. TDP-43 was originally identified as a transcription factor responsible for repressing HIV-1 genes [17] and later shown to be a pre-mRNA splicing regulator [18]. Subsequent research has gone beyond single case studies to contribute to a more global characterization of TDP-43, linking its genome-wide activity in splicing regulation to its emerging role in polyadenylation site selection of transcripts found to be altered in neuropathies [19].

Seminal studies have also used animal modeling to explore TDP-43 function in health and disease. Collectively, the various rodent models developed to study TDP-43 proteinopathies (reviewed in detail elsewhere [20]) indicate that abnormal levels of TDP-43 can have lethal consequences. Indeed, TDP-43-null mice are nonviable and selective depletion within the central nervous system (CNS) can lead to neurodegeneration [20]. At the other end of the spectrum, overexpression of either wild-type or mutant TDP-43 also leads to premature death. Given these apparent consequences of perturbing TDP-43 levels, most research groups have been discouraged from pursuing TDP-43 itself as a therapeutic modality and instead have sought to identify novel targets that could suppress TDP-43-related phenotypes resulting from gain or loss of functions.

Mutations in TDP-43 Cause Neurological Disease

Most patients with ALS have no family history of the disease and are regarded as sporadic, whereas ~10% of cases have a first-degree relative with the condition and are considered familial. Shortly after TDP-43 inclusions were first reported in affected neuronal tissues of patients with ALS or FTD, *TARDBP* mutations were also found in patients with either familial or sporadic ALS, with a prevalence of 4% and 1.5%, respectively [1,21]. To date, >50 rare, missense mutations have been characterized [1,2]. Nearly all of them map within the C-terminal low complexity domain, which is intrinsically aggregation prone, suggesting that altering its thermodynamic properties is sufficient to predispose carriers to neurodegeneration (Figure 1). In agreement with this hypothesis, a recent study showed that transient stress triggers cytosolic phase separation of TDP-43, resulting in long-lasting nuclear import defects and cell death [22]. There are also numerous studies demonstrating that mutations in *TARDBP* can lead to elevated levels of TDP-43 [23] through increased stability [24–26] or altered autoregulation [13]. Any of these malfunctions could lead to age-related aggregation, neuroinflammation [27], or nuclear depletion at later stages. Given that increased TDP-43 levels have also been observed in patients with sporadic FTD [28], this pathomechanism should be investigated more broadly.

A few mutations within the RNA recognition motifs (RRMs) of *TARDBP* have also been reported [29–31]. These mutations impair TDP-43 stability and aggregation by affecting its ability to bind (and, therefore, regulate) a subset of essential client RNAs, suggesting that loss of TDP-43 RNA-binding *per se* is sufficient to mediate neurotoxic aggregation [32,33]. However, other studies tend to rule out this possibility, indicating that TDP-43 stability and RNA binding depend upon similar or closely mapping mutations, but are functionally disentangled [34–37]. Resolving these opposing views is particularly relevant for translational studies, because using ectopic RNA substrates to improve TDP-43 solubility has been proposed as a potential therapeutic strategy [38–40].

To model pathogenic mutations similar to those identified in humans, two independent groups introduced point mutations within the C-terminal low-complexity domain of Tdp-43 in mice [13,14]. Although these rodent models differed in their ALS- versus FTD-like phenotypes, they both nominated perturbed TDP-43 autoregulation and, more broadly, alternative splicing, including **cryptic exon** inclusion (nonstandard or poorly conserved exons) and skipping of constitutive

Glossary

Alternative polyadenylation: a well-conserved and tissue-specific RNA-processing mechanism that generates diversity at the 3' end of RNA transcripts.

Cryptic exons: regions of the genome normally skipped by splicing. Inclusion of cryptic exons can destabilize mRNA through nonsense-mediated decay or, if translated, can lead to altered protein structure.

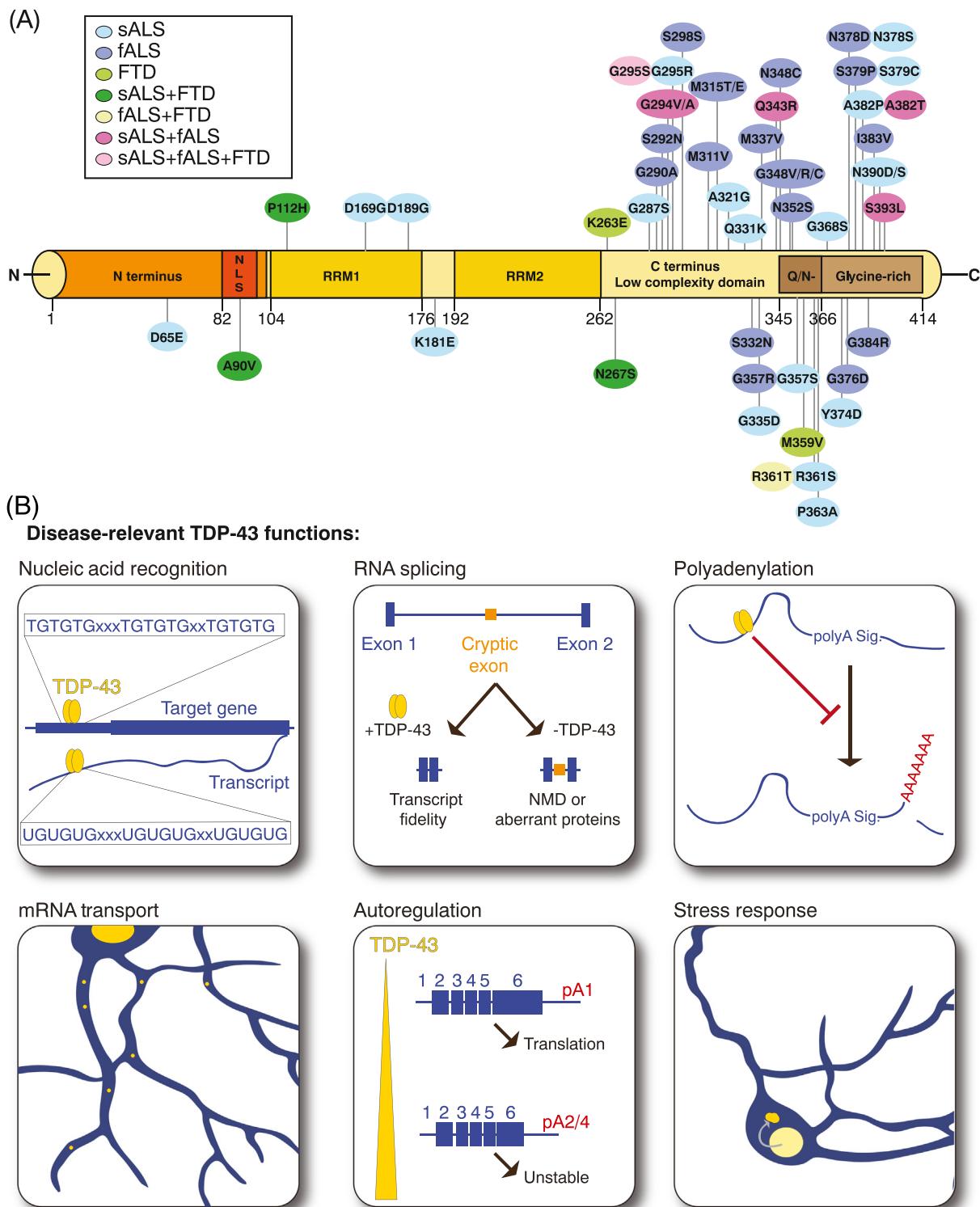
Gain of function (GOF): mutation that results in novel or enhanced activity of a protein. In the context of TDP-43, several GOFs have been proposed, including toxicity mediated by inclusions or increased normal activity of TDP-43.

Loss of function: mutation that results in decreased activity or loss of a protein. In the context of TDP-43, loss of function due to aggregation and nuclear clearance results in altered RNA metabolism.

Stathmin-2 (STMN2): a neural-specific protein that functions in microtubule destabilization and signal transduction pathways important for axonal outgrowth, development, and regeneration.

TDP-43 proteinopathies: a group of neurodegenerative disorders characterized histopathologically by the deposition of TDP-43 protein into insoluble, ubiquitinated, and hyperphosphorylated aggregates. The aberrant accumulation of TDP-43 in vulnerable populations of cells is often associated with the loss of nuclear TDP-43.

Transactive response DNA-binding protein 43 kDa (TDP-43): a protein encoded by the *TARDBP* gene that resembles members of the heterogeneous ribonucleoprotein RNA-binding protein family.



Trends In Neurosciences

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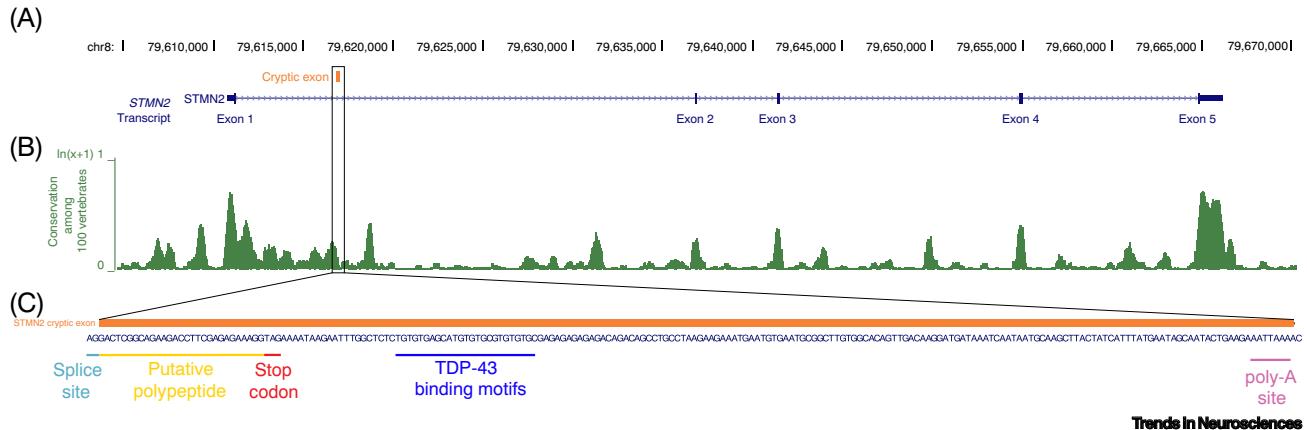


Figure 2. Conservation of the Stathmin 2 (STMN2) Gene Locus. (a) Human STMN2 is located on the long arm of chromosome 8 and is transcribed as several isoforms, generally including five canonical exons. The location of the cryptic exon is highlighted in orange. (B) Conservation among 100 vertebrates along the locus reveals strong conservation at exons as well as some intronic regions. Data extracted from the USC genome browser [<http://genome.ucsc.edu> Dec. 2013 (GrCh38/hg38)] [124,125]. (C) Higher resolution genomic view of the STMN2 cryptic exon (orange) with nucleotide resolution humans. Salient features of the gene are underlined, including the splice acceptor site (teal), the putative coding region (yellow), the stop codon (red), the TDP-43-binding motifs (blue), and the poly-A signal (purple).

exons, as their major phenotypes. Given that the conservation of cryptic exons is low between species (Figure 2) [41], additional work will be necessary to better understand species-specific functions of TDP-43 in regulating RNA metabolism. Such studies will help to resolve the extent to which various animal models are useful for studying the contribution of altered TDP-43 function as it occurs in humans.

Forces Shaping Pathological Aggregation of TDP-43

In addition to pathogenic mutations, nuclear clearance and aggregation of TDP-43 in susceptible cells are the other fundamental observations linking TDP-43 to disease. Given that most patients do not harbor *TARDBP* mutations, it is likely that other upstream pathways converge upon TDP-43 pathology. How this pathology contributes to neural degeneration remains unresolved, with most studies relying on overexpression systems. These studies clearly demonstrate that overexpression of TDP-43 can indeed recapitulate TDP-43 pathology, with toxic consequences in cells as well as organisms [42,43] and further supported by clinical studies of patients with TDP-43 proteinopathies [44]. Given that directly modulating the levels of TDP-43 could be difficult to achieve as a therapeutic strategy, in view of clinic safety, we focus our attention instead on what is known concerning the pathways that regulate the levels, localization, and solubility of TDP-43. Such pathways could offer attractive points of intervention for alleviating the forces in neuronal cell biology pushing TDP-43 into a pathological state.

Disruption of Protein Homeostasis

The disruption of protein homeostasis has been connected to altered TDP-43 localization and solubility. In response to pharmacological inhibition of the proteasome, several groups have

Figure 1. Transactive Response DNA Binding Protein 43 kDa (TDP-43) Protein Structure, Pathogenic Mutations, and Function. (A) TDP-43 comprises several domains: an N-terminal region [amino acid (aa) 1–102] with a nuclear localization signal (NLS, aa 82–98); two RNA recognition motifs: RRM1 (aa 104–176) and RRM2 (aa 192–262); a C-terminal region (aa 274–414), encompassing a prion-like glutamine/asparagine-rich (Q/N) domain (aa 345–366); and a glycine-rich region (aa 366–414). Many dominant mutations have been identified in TDP-43 in patients with sporadic or familial amyotrophic lateral sclerosis (ALS) and in patients with rare frontotemporal dementia (FTD), mostly lying in the C-terminal glycine-rich region. (B) Salient TDP-43 functions implicated in disease pathogenesis. The most common motif identified for TDP-43 is (TG)n, which corresponds to the (UG)n RNA-binding motif. Interaction with RNA allows TDP-43 to regulate pre-mRNA splicing to inhibit the inclusion of cryptic exons as well as influence polyadenylation site selection. Cytosolic roles for TDP-43 include transport of RNA along neuronal processes and response to stresses, including those affecting proteostasis, which can trigger TDP-43 nuclear efflux and localization to stress granules. A multitude of these basic molecular functions contribute to TDP-43 autoregulation, including splicing and polyadenylation. Abbreviations: fALS, familial ALS; NMD, nonsense-mediated decay; sALS, sporadic ALS.

reported aggregation of TDP-43 [45] and in some cases its efflux from the nucleus [46,47]. This striking loss of nuclear TDP-43 associated with altered expression of its transcripts [46] might explain the motor neuropathy observed in humans taking the proteasome inhibitor Bortezomib, a medication used to treat multiple myeloma [48]. It would be informative to evaluate if individuals that succumb to cancer while taking this drug show TDP-43 pathology, which would directly link proteasome inhibition to motor neuropathy and potentially TDP-43 pathology.

Autophagy is another pathway by which cells degrade proteins, particularly those that are long-lived or aggregated. Activation of autophagy has been proposed to enhance turnover of aggregated TDP-43 and mitigate its toxic effects [42,49]. Moreover, the discovery of ALS/FTD-associated mutations in genes encoding proteins involved in autophagy, including *UBQLN2* [50], *TBK1* [51,52], *VCP* [53], *FIG4* [54], *OPTN* [55], *GRN* [56], and *SQSTM1* [57], further implicates alterations in this process in disease pathogenesis [58,59]. Of these proteins, programulin has been demonstrated to specifically reduce insoluble TDP-43 levels to slow axonal degeneration and extend survival in mutant Tdp-43 mice [60]. Interrogating the metabolism of TDP-43 offers a promising venue for future work.

Phase Separation into Biomolecular Condensates

In response to cellular stresses, RNA-binding proteins, including TDP-43, can rapidly localize to aggregate-like structures termed ‘stress granules’. This process has been hypothesized to ensure that translation is limited to essential genes. In a recent screen for small-molecule modulators of TDP-43 recruitment to stress granules, puromycin, which disrupts protein homeostasis by triggering premature polypeptide chain termination, induced TDP-43 recruitment to condensates in cultured neurons [61]. Surprisingly, oxidative stress induced by sodium arsenite, which is commonly used to instigate TDP-43-positive stress granules in cancer cells, did not induce a similar response in neurons [61]. These cell type-specific observations are intriguing given that puromycin poisons cells in part by flooding them with small abortive polypeptides, which may share similar properties with the dipeptides produced by the most common genetic contributor to ALS and FTD: the *C9ORF72* repeat expansion. Once transcribed, the *C9ORF72* repeat expansion can be noncanonically translated into several repetitive polypeptides, which have been demonstrated to alter phase transitions and condensate dynamics [62,63]. Importantly, a recent study showed that polyglycine arginine dipeptide repeat products can seed TDP-43 phase separation [64], providing a potential mechanistic explanation for previous observations that human induced pluripotent stem cell (iPSC)-derived motor neurons treated with puromycin or synthetic dipeptides activated a suite of genes upregulated in the cortex of patients with *C9ORF72* ALS/FTD [65]. These results are consistent with the notion that disruptions in protein homeostasis are indeed upstream of TDP-43 pathology in patients and that investigations of how this leads to cytoplasmic accumulation of TDP-43 in neuronal biomolecular condensates is warranted.

Exciting discoveries have been made using yeast, fly, and mouse models in relation to genetic modifiers of TDP-43 GOF toxicity. The presence of intermediate-length CAG repeats in *ATXN2*, a gene encoding an RNA-binding protein and biomolecular condensate component, is associated with an increased risk of ALS, while longer lengths are linked to the neurodegenerative disease spinocerebellar ataxia type 2 [35,66]. In a study in mice overexpressing wild-type TDP-43 throughout the CNS, reduction of Atxn-2 was demonstrated to improve motor phenotypes and dramatically extend the severely blunted lifespan of these animals [67]. These findings established reducing *ATXN2* expression as a widely pursued therapeutic target in ALS [67,68]. Although the exact mode of action remains elusive, a model whereby reduced *ATXN2* alters the dynamics of TDP-43 demixing has been proposed [67]. Future studies should consider

the ability of ATXN2 with varying length of polyQ repeats to modulate the dynamics of TDP-43 recruitment into stress granules. It will also be important to discriminate whether ATXN2 regulates TDP-43 levels and whether this might occur through direct modulation of *TARDBP* RNA levels [69]. It could also be that loss of ATXN2 somehow buffers the alterations to TDP-43 client RNAs that occurs when TDP-43 is recruited to biomolecular condensates.

Although stress granules have been proposed to be the crucibles for ALS pathogenesis, the field has not reached a consensus on whether TDP-43 must transition through this phase-separated state on its path to aggregation. Two recent studies demonstrated that TDP-43 aggregates can be found in the absence of stress granule markers [22,40]. These aberrant phase transitions were induced through induced-dimerization of TDP-43 or by addition of pre-fibrillized TDP-43 aggregates to the cultures. Thus, additional investigations should focus on the mechanisms that regulate the transition of TDP-43 into different states. Of note, a recent study implicated post-translational acetylation of TDP-43 as well as chaperone proteins in this context [70].

Altered Nuclear Transport

Given that TDP-43 responds to a variety of cellular stresses by exiting the nucleus and accumulating in the cytoplasm, disruptions of nucleocytoplastic transport through the nuclear pore complex could severely impact normal TDP-43 function. Through mechanistic studies of the *C9ORF72* repeat expansion, several groups connected cytoplasmic TDP-43 accrual with nuclear pore defects [71,72]. Specifically, the arginine-rich dipeptides produced from the C9 hexanucleotide repeat expansion have been shown to interact with karyopherins, a family of nuclear transport receptors, and interfere with cargo loading, thus inhibiting nuclear import of TDP-43 [73]. Disruption of nucleoporins, the main constituents of the nuclear pore complex, has also been confirmed in the motor neurons of patients with sporadic ALS and TDP-43 pathology [74], and nucleoporins were also recently identified within FUS fibrils responsible for TDP-43 seeding [22]. Intriguingly, emerging evidence indicates that mutant and insoluble TDP-43 can sequester nucleoporins, potentially further exacerbating nuclear pore pathology [75]. Given these findings, the pathway relationship between the effects of the *C9ORF72* mutation, TDP-43 nuclear efflux and aggregation, as well as nuclear transport defects, needs to be better resolved.

Neuron-Specific Forces Underlying TDP-43 Pathology

Compared with most other cell types in the body, neurons are unique in their distinctive polarized morphology and their process of communication through electrochemical signals. The fact that electrical activity is perturbed across *in vitro* and *in vivo* models of ALS as well as in patients with ALS, has led to the excitotoxic neurodegeneration hypothesis. According to this hypothesis, excessive firing leads to calcium overload and cell death [76]. While some studies have connected this hyperactivity phenomenon with endoplasmic reticulum (ER) stress triggered by mutant SOD1 [77,78], others recently illuminated an additional point of association between TDP-43 pathology and excessive neuronal firing. Specifically, hyperexcitability triggers TDP-43 mislocalization and, consequently, alternative 3'-untranslated region (UTR) splicing of TDP-43 (a splice form observed in patients [44]), leading to a shortened, toxic form of the protein [79]. Future studies should investigate whether small molecule modulators of neuronal activity can correct this defect.

The integrity of axons might also regulate TDP-43 localization. Following axotomy in mice, two independent groups observed decreased nuclear TDP-43 accompanied by temporary cytosolic redistribution [80,81]. These findings open the possibility that, if nuclear exclusion in response to neuronal injuries fails to resolve, it may lead to motor neuron disease. Currently, the downstream effects of nuclear TDP-43 depletion after neuronal injury are not well understood. For instance, it is unclear whether nuclear efflux of TDP-43 following injury results in changes in the mRNA levels of

known targets in motor neurons. Future work using compartmentalized neuronal cultures might help address these knowledge gaps, given that this approach allows monitoring communication of survival and recovery signals between somatic and axonal compartments in response to axonal injury.

In summary, a multitude of cellular stresses can affect the levels, localization, and solubility of TDP-43. Although there is no general agreement on whether TDP-43 aggregates develop through stress-driven inclusions, understanding these ‘triggers’ of TDP-43 pathology could nominate therapeutic targets that, when modulated, help to eliminate cytoplasmic TDP-43 and restore its nuclear functions. Moreover, these approaches are agnostic as to whether TDP-43 pathology constrains neuronal function through gain- or loss-of-function mechanisms, because both would be resolved by ‘corrective’ compounds to mitigate the pathology.

TDP-43 and RNA Biology

TDP-43 is associated with a multitude of functions connected to RNA metabolism. Rapid development of cross-linking and immunoprecipitation coupled with high-throughput sequencing allowed genome-wide mapping of TDP-43 binding sites [82,83]. In the mouse, Tdp-43 has a genome-wide preference for binding to GU-rich distal intronic sites, and more than half (57%) of the Tdp-43 clusters observed contained at least four GUGU tetramers, with their number correlating with the strength of binding [82]. A similar study mapped TDP-43-binding sites at single nucleotide resolution in human brain tissue, where TDP-43 targets were shown to be enriched for proteins previously implicated in neuronal development and disease [83]. Interestingly, when compared with the mouse, human TDP-43-binding sites appear to cluster within shorter regions of tandem GUGU repeats [83], suggesting another potential mechanism by which TDP-43 has different binding affinities to a given RNA target in disparate species (Figure 2).

Further evidence that TDP-43 affects cell states through fine transcriptional regulation was advanced by artificially modulating TDP-43 levels in different cell types. Notably, in cellular TDP-43 depletion models, several cryptic exons were found to be spliced into mRNAs, often disrupting their translation and promoting nonsense-mediated decay. In accordance with these results, many of the same transcripts were also found to be downregulated in brain tissues from an ALS-FTD cohort, providing direct evidence for loss of TDP-43 function in patient tissues [84]. These results are of additional interest because they are important evidence linking TDP-43-dependent splicing regulation to its emerging role in polyadenylation [85].

Over 70% of mammalian genes undergo **alternative polyadenylation**, where alternative poly(A) sites are utilized, giving rise to several stable isoforms for each transcript [86]. The relative frequencies between isoforms with long or short UTR length in a cell represents a transcriptional fingerprint, which has been shown to change during development and disease [85]. The first evidence of the involvement of TDP-43 in regulating alternative polyadenylation is in the context of its self-regulatory feedback mechanism. At first, it was noticed that TDP-43 has a strong binding affinity for a region close to the 3'UTR of its own mRNA, as an autoregulation mechanism to dose control its concentration and consequent solubility (Figure 1). The same study eventually confirmed that TDP-43 pre-mRNA contains multiple polyadenylation signals in its last intron, and its alternative polyadenylation occurs in response to perturbation of TDP-43 protein levels [87]. In steady-state conditions, most TDP-43 production within cells comes from the transcript that uses the optimal polyadenylation site pA1. However, if TDP-43 concentration increases, excessive binding of TDP-43 proteins interferes with the selection of pA1 and promotes the excision of an alternatively spliced intron (intron 7) containing pA1, with subsequent use of distal suboptimal polyadenylation sites (Figure 1). The resulting isoforms are either retained in the

nucleus or subjected to nonsense-mediated mRNA decay. In a similar fashion, TDP-43 has been shown to promote alternative polyadenylation in many other transcripts. One of many such examples is the mRNA encoding the core pluripotency and reprogramming factor Sox2 [88]. This observation could explain why TDP-43 loss of function leads to preimplantation lethality in mice [89].

The compelling hypothesis that some of the transcripts essential in vulnerable neuronal subtypes could be directly regulated by TDP-43 via alternative polyadenylation was recently advanced in two studies, which linked neuropathies to a subset of transcripts regulated by TDP-43 through differential inclusion of cryptic exons [46,90]. Among these transcripts are *ELAVL3*, encoding an RNA-binding protein similar to TDP-43 and an ALS risk factor [91]; and *AGRN*, encoding a protein vital for the formation of neuromuscular junctions [41]. Importantly, both studies identified *STMN2* (Box 1 and Figure 3), one of the most abundant transcripts in human motor neurons, as a central TDP-43 client RNA.

A Broader Role for TDP-43 in Neural Degeneration

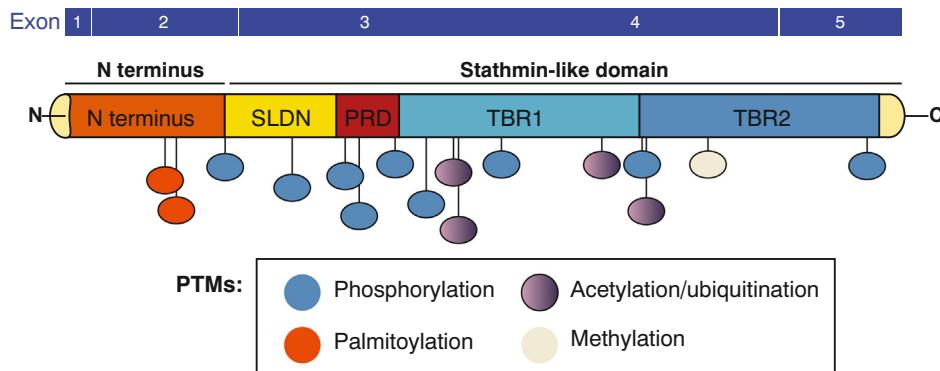
Important studies have nominated a function for TDP-43 in regulating the metabolism of RNAs genetically linked to several additional nervous system disorders, including PD. A significant decrease in *Parkin* mRNA levels was observed upon TDP-43 overexpression in flies, motor cortex of a rat model, and mouse primary neurons. The observed effect was dependent on the TDP-43 RRM1 domain, and it appeared to occur to a lesser extent even when testing the regulation of *Parkin* transgenes lacking introns or 3'UTR regions, suggesting an alternative, intron-independent, mode of regulation [92]. It remains to be addressed whether human TDP-43 regulates human *PARKIN* in a similar way. Despite the demonstrated relevance of this regulation in animal models, its presence in humans should not be presupposed, given the lack of conservation of many TDP-43-binding sites between species, and the substantial variation in alternative splicing and polyadenylation that is also known to exist [41].

A similar species-specificity argument can be raised for the observation that *Arid4b* and *Atxn2* are downregulated, and several additional genes are mis-spliced in a mutant *Tardbp* murine model [13]. Among these is *Mapt*, which encodes the microtubule associated protein tau, and the human ortholog of which is mutated in FTD [13]. Other transcripts

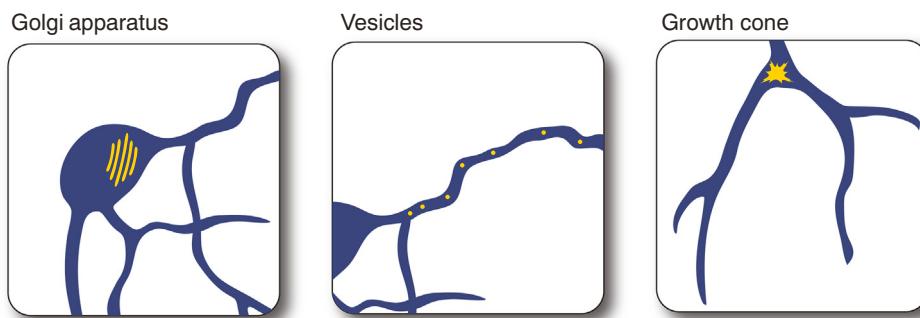
Box 1. What Is Stathmin-2?

The *STMN2* transcript is one of the most abundant in motor neurons and encodes Stathmin-2, also known as SCG-10, one of the four members of the Stathmin family (*STMN1-4*) of microtubule-binding proteins. Unlike *STMN1* and *STMN4*, which are ubiquitously expressed, *STMN2* and *STMN3* are highly enriched in the CNS [126]. In the rat brain, the *Stmn2* transcript is upregulated during development and found at its highest levels shortly after birth (approximately postnatal days 2–6), where it then gradually declines to stable levels through adulthood [127]. This differential temporal expression pattern is well conserved in higher order primates [128]. While downregulated in adulthood, *STMN2* shows a second peak in expression in neurons after injury and can remain increased for days post insult, highlighting the underlying importance of *STMN2* in the maintenance of the adult CNS [95,127,128].

All four Stathmin family members have a similar overall protein structure, each containing a Stathmin-like homology C-terminal domain that is required for tubulin binding. Additionally, the neuronal proteins *STMN3* and *STMN2* contain a specific N-terminal domain, which determines their subcellular localization (see Figure 3 in the main text). While *STMN1* has a cytosolic distribution, *STMN2* is localized to the Golgi apparatus, attached to trafficked vesicle membranes along axons or dendrites and enriched in growth cones during neuronal development or in axonal tips after injury [96,129,130]. To ensure proper neuronal growth and maintenance during development and repair, *STMN2* can antagonize microtubule polymerization either through sequestering tubulin dimers or by directly interacting with the microtubules themselves to promote disassembly at the minus ends, while at the same time having the capacity to promote stability by increasing the rate and extent of polymerization at the plus ends [131].



Subcellular localization:



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Figure 3. Stathmin 2 (STMN2) Protein Structure and Function. STMN2 comprises two domains that can be further subdivided: (i) an N-terminal domain containing a conserved Golgi-specifying sequence with two palmitoylation sites enabling membrane insertion; and (ii) a Stathmin-like domain containing two tubulin-binding repeats (TBR1 and TBR2), which each bind tubulin, a proline-rich domain (PRD) harboring two phosphorylation sites that can be modulated by JNK to potentially modulate the ability of STMN2 to interact with tubulin and promote STMN2 degradation, and a stathmin N-terminal domain (SLDN), which contains a peptide that inhibits tubulin polymerization. Identified post-translational modifications (PTMs) according to PhosphositePlus are marked along the protein structure. The reported subcellular localization of STMN2 protein is also indicated. STMN2 localizes to the Golgi apparatus, is found in vesicles trafficked throughout dendrites and axons, and concentrates within growth cones of developing neurons as well as in regenerating axon tips after injury.

were shown to be stabilized rather than regulated by TDP-43. For instance, levels of the nuclear *NEFL* transcript were not altered in iPSC-derived motor neurons from patients carrying TDP-43 mutations. However, a striking decrease in *NEFL* cytoplasmic protein levels suggests TDP-43-dependent defects in mRNA stability during transport of messenger ribonucleoprotein complexes [93,94].

It will be important to further characterize differential TDP-43 targets in health and disease, and specifically focus on differential alternative polyadenylation events, which might have been under-represented in large-scale studies due to technical limitations of fast-evolving sequencing technologies, currently allowing improved coverage of differential splicing and polyadenylation sites. Finally, combining global transcriptomic and genome-wide RNA-binding assays will enable discrimination between direct targets as opposed to transcripts that are differentially expressed as a secondary consequence of perturbing TDP-43 function. These clarifications will be essential to nominate effective candidates for therapeutic intervention and treatment of TDP-43-related neuropathies.

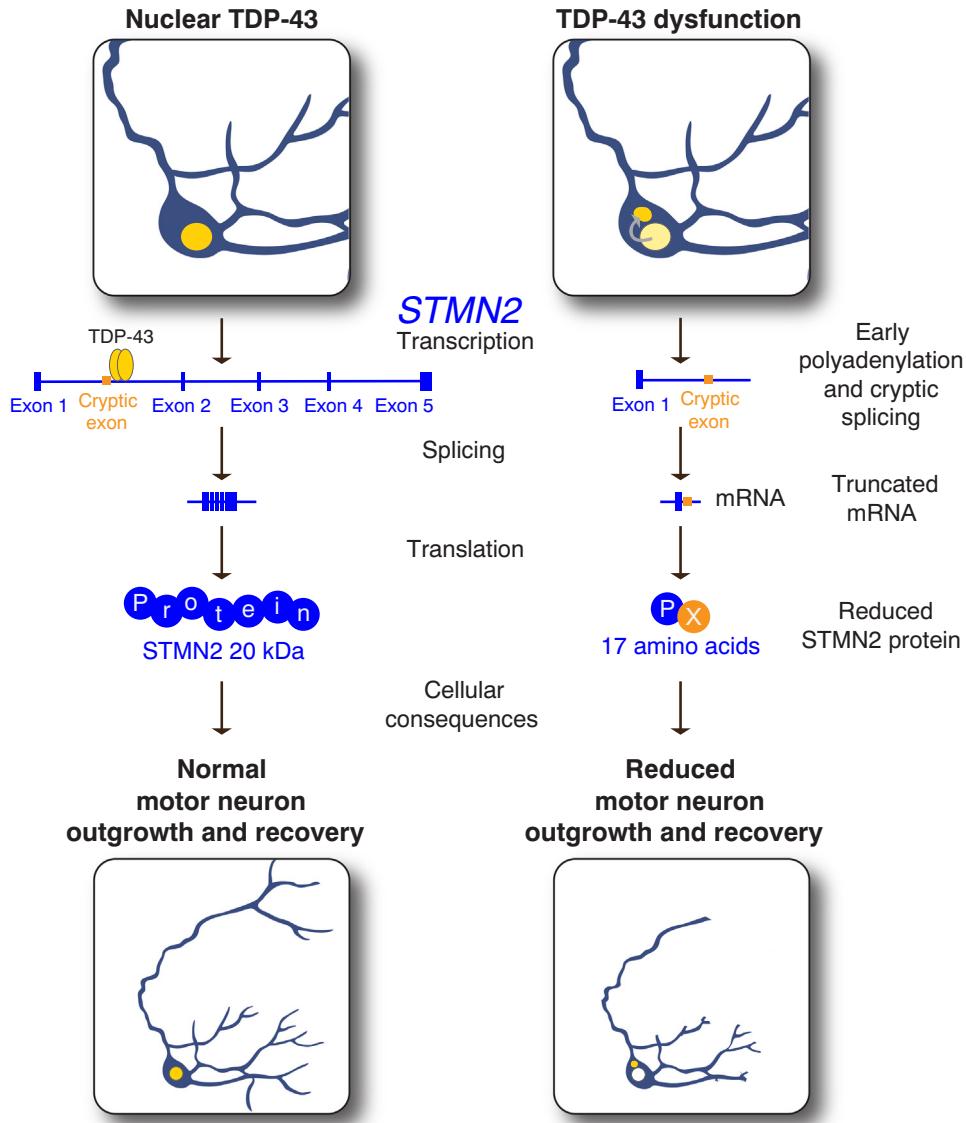


Figure 4. Proposed Model for Transactive Response DNA-Binding Protein 43 kDa (TDP-43) Regulation of Stathmin 2 (STMN2). A pathological hallmark of amyotrophic lateral sclerosis (ALS) is the nuclear loss of TDP-43 and its aggregation. We propose a model of TDP-43 regulation of STMN2 where it binds to STMN2 pre-mRNA upon the intron between exons 1 and 2. Either a reduction in TDP-43 levels or nuclear egress leads to early polyadenylation and splicing of a cryptic exon, resulting in a truncated STMN2 mRNA transcript. The blunted transcript encodes a putative 17-amino acid polypeptide, thus leading to reduced levels of STMN2 protein. Loss of STMN2 leads to reduced neurite outgrowth and axonal repair after injury.

TDP-43 as a Regulator of STMN2 and Axonal Biology

The aforementioned regulation of STMN2 by TDP-43 is a prime example of how this RNA-binding protein can define cell state integrity through its binding of principal transcripts (see Figure 4). Stabilization of STMN2 levels by TDP-43 enables neuronal outgrowth and suggests a direct link connecting TDP-43 pathology with progressive neuropathy [46,90]. STMN2 expression is all but eliminated in stem cell-derived human neurons after TDP-43 depletion and is significantly

reduced in postmortem spinal cords from patients with ALS [46,90]. Similarly, neuronal cells harboring *TARDBP* mutations express modestly lower levels of *STMN2*, but it is unclear whether this results from the inability of mutant TDP-43 to regulate proper *STMN2* polyadenylation, splicing, a combination of the two, or some other aspects of RNA metabolism [46,90]. An additional question left unresolved is whether *STMN2* downregulation is a consequence of mutant TDP-43 being unable to regulate the transcript or a consequence of lower levels of TDP-43 in these cells. To resolve this matter, the relative ability of mutant and wild-type TDP-43 to bind to the *STMN2* pre-mRNA should be examined.

The implication of *STMN2* loss and its impact on motor neuron function *in vivo* needs to be addressed. *In vitro* studies of rodent neurons demonstrated that knock down of *Stmn2* can negatively impact neuronal outgrowth and regrowth [95–98]. Thus far, however, complete loss of *STMN2* *in vivo* has only been assessed in *Drosophila*, a system that contains a single stathmin-like gene (*sta1*). Loss of *sta1* results in impaired synaptic stability and, according to an independent report, posterior paralysis, potentially through presynaptic disassembly of innervated neuromuscular junction in mutant flies [99,100]. In mice, knock down of *Stmn2* in the midbrain was recently demonstrated to cause dopaminergic neuron loss and deficits in coordinated motor skills [101].

Establishment of complete loss-of-function mammalian models will be critical to understand whether *STMN2* is essential for maintenance of large projection neurons and neuromuscular junctions *in vivo*. Although loss-of-function mouse models could address the distinct functions of *STMN2* in the motor system, they would still be limited in their potential to resolve its connection to TDP-43 proteinopathies. Specifically, TDP-43-binding motifs within intron 1 of *STMN2* are only found in higher order primates (Figure 2). This observation posits a roadblock for *in vivo* modeling of Tdp-43-mediated *Stmn2* regulation in rodents that can be overcome by humanizing the locus through bacterial artificial chromosome (BAC) transgenics or genome editing.

Altered *STMN2* expression levels have been observed in several neurodegenerative diseases. In the context of motor neuron diseases, *STMN2* expression has been found to be downregulated in human motor neurons derived from patients with spinal muscular atrophy (SMA), but the precise mechanism of this decrease is currently unclear [102]. The shared multifunctional role in RNA metabolism of the disease-causing gene in SMA, survival of motor neuron 1 (*SMN1*) and *TARDBP*, along with the splicing abnormalities observed in both diseases, point toward a potential convergence on a dysfunctional spliceosome in the degenerative processes underlying these two motor neuropathies [103]. In AD, abnormal *STMN2* expression patterns were detected in the temporal cortex and hippocampus of patients [104]. The discrepancy between changes in transcript and protein levels found in the AD-affected brain regions in these studies suggests a potential alteration in the post-transcriptional regulation of *STMN2*. A more recent study demonstrated that *Stmn2* directly binds to Amyloid Precursor Protein (APP), which promotes APP distribution to the cell surface, potentiating increased APP processing in rodent hippocampal neurons [105]. Remarkably, overexpression of *Stmn2* in the hippocampus of an AD mouse model led to a significant reduction in β-Amyloid peptide (Aβ) deposits, a proteolytic product of APP, positioning *Stmn2* as a prospective modulator of Aβ plaque formation. Beyond AD, a network analysis nominated decreased *STMN2* expression in patients with PD as a point of intersection between potential disease pathways and known PD risk genes [101].

While it is clear that restoring or increasing *STMN2* expression may be beneficial in some contexts, there is also evidence that this restoration must be finely tuned: in a transgenic SOD1 mouse model of ALS, increased *Stmn2* expression was associated with Golgi fragmentation

[106]. These findings highlight the dynamic aspect of microtubule regulation and the importance of sustaining stable levels of this cytoskeletal regulator in motor neurons.

Beyond disruption of *STMN2* regulation, what other cellular pathways connect TDP-43 function with axonal biology? In neurons, a portion of cytoplasmic TDP-43 is actively transported along axons in a bidirectional and microtubule-dependent manner. Axonal TDP-43 colocalizes with other RNA-binding proteins in the presynaptic terminals and in the neuromuscular junctions of both *Drosophila* and mouse models [94,107–109]. Interestingly, some disease-causing mutations in TDP-43 impair axonal transport of *Nefl* mRNA-containing granules in *Drosophila*, rodent cortical neurons, and in motor neurons derived from patients with ALS [94]. The previously unappreciated role of TDP-43 in mRNA transport sheds light on additional levels of regulation for some of its well-characterized neuronal mRNA targets [82]. Understanding how altered TDP-43 levels or mutations affect the spatial distribution of another subset of these targets might illuminate the pleiotropic effects of TDP-43 pathology in neuronal function.

Collectively, the evidence suggests an interplay between TDP-43 function and proper axonal maintenance. In the soma, TDP-43 is essential to sustain physiological and stable mRNA levels of key players of the neuronal cytoskeleton (i.e., *STMN2*). Separately, the role of TDP-43 role in axonal mRNA transport is crucial for soma–periphery communication of survival and recovery signals. Thus, TDP-43 dysfunction and, hence, impairment of pathways relevant for axonal biology, might disrupt neuronal maintenance and survival mechanisms, leading to disease initiation or progression.

Biomarkers for TDP-43 Proteinopathies

Although it is clear that TDP-43 pathology occurs in most patients with ALS/FTD and even a subset of patients with other neurological conditions, there are currently no approaches to identify the extent of TDP-43 accumulation or aggregation in living individuals (Table 1). Many efforts have been made to develop biomarkers for motor neuron degeneration in general, including biofluid measures of motor neuron-specific miRNAs, p75, and neurofilament [110–114]. Although neuronal degeneration has been hypothesized to precede the accumulation of these factors in biofluids, this has not been formally resolved. However, there is the idea that an imaging-based or fluid biomarker that beacons TDP-43 disruption would greatly enhance the ability to study the dynamics of TDP-43 pathology in patients and the interplay of TDP-43 with motor neuropathy. Such a biomarker would also be invaluable for evaluating therapeutics designed to resolve TDP-43 pathology.

Direct *in vivo* measurement of TDP-43 remains elusive, and its ubiquitous expression creates complications in identifying and isolating the pathological protein specific to the CNS.

Table 1. ALS Biomarkers

Biomarker	Biofluid	Change relative to controls	ALS	Disease mimics	Non-neurological diseases?	TDP-43 specific?
<i>miR-143</i>	Serum	↓	✓	✓	✓	✗
<i>miR-218</i>	Serum, CSF	↓	✓	✓	✓	✗
<i>NfH</i> or <i>NfL</i>	CSF, serum, plasma	↑	✓	✓	?	✗
<i>P75</i>	CSF, urine	↑	✓	✓	?	✗
<i>Cryptic STMN2</i>	Postmortem spinal cord	↑	✓	?	?	✓

Interestingly, forms of TDP-43 associated with TDP-43 pathology have been identified in exosomes, small vesicles secreted from cells, some of which easily pass the blood–brain barrier, suggesting that exosomal isolation and subsequent detection of pathological TDP-43 would be advantageous [115]. As explored broadly in this review, TDP-43 has a crucial role in RNA metabolism. Therefore, biofluid measures of RNAs that are regulated by TDP-43, or proteins they encode, should display expression changes in close concert with alterations in TDP-43 to serve as proxy measures for TDP-43 pathology. *STMN2* is a compelling example of such an RNA because it is one of the most abundant transcripts in human motor neurons, yet its abundance declines rapidly if TDP-43 becomes perturbed [46]. Furthermore, TDP-43-dependent alterations in *STMN2* transcript structure have already been confirmed in post-mortem samples from patients with either ALS or FTD [116], whereas neither aged-matched controls nor patients with mutant SOD1, who are not known to produce TDP-43 protein inclusions, showed the presence of this cryptic exon [90]. For this molecular finding to be developed into a biomarker, sensitive measures for the *STMN2* RNA or its protein in cerebrospinal fluid (CSF) or potentially serum will need to be developed. Recent progress in purifying RNAs and proteins from CNS or serum-derived exosomes may facilitate this effort [117,118].

While measuring total *STMN2* levels in biofluids will certainly be informative, measuring the truncated *STMN2* RNA and protein species produced when TDP-43 becomes perturbed may be even more valuable. Given that full-length *STMN2* is an abundant motor neuron RNA, its levels may decline with motor neuron loss regardless of TDP-43 status. Also, if other disease states converge on *STMN2*, as has been suggested for PD [101], they may or may not occur through a TDP-43-dependent splicing mechanism. By contrast, monitoring alternatively spliced transcripts, such as cryptic variants of *STMN2* that only occur when TDP-43 is perturbed, might provide a more specific signal that tracks closely with the extent of TDP-43 pathology. While it is currently unknown whether the modified open reading frame encoded by this cryptic *STMN2* RNA is translated, the predicted peptide could also serve as another specific signal for the occurrence of TDP-43 pathology.

In this same vein, it appears logical to pursue additional nervous system RNA interactors of TDP-43 to form a panel of potential biomarkers. Attractive additional targets include *MAPT*, *ELAVL3*, *AGRN*, and *PARK2*. These genes, as mentioned earlier, show altered expression as a result of TDP-43 perturbation. Similar to *STMN2*, *AGRN* shows cryptic splicing upon TDP-43 knockdown, and significantly decreased levels of protein in the CSF of patients with sporadic ALS, thus providing two potential measures of disease progression [41,46,119]. Similarly, *ELAVL3*, also involved in neuronal differentiation and regulation [120], shows significant downregulation during TDP-43 depletion in human motor neurons as well as the creation of an alternatively spliced form [46]. Decreased *ELAVL3* expression was also identified as part of an early and predictive sporadic ALS gene signature that persists to end-stage disease [121]. *MAPT*, which encodes the neuronal protein Tau, is often found aggregated in forms of FTD and AD [122]. Recently, *Mapt* was identified to be alternatively spliced as a result of Tdp-43 dysfunction [13], thus making both its transcript and protein potentially useful for assessing TDP-43 pathology. Importantly, the changes in RNA metabolism that occur upon relocalization are stereotyped within a given cell type and are also, in some cases, highly specific to cell types in which TDP-43 is ultimately perturbed. As a result, measures of multiple TDP-43 client RNAs might eventually be combined to infer not only the total number of cells with TDP-43 pathology, but also whether this pathology is occurring in astrocytes, oligodendrocytes, microglia, or even specific neuronal subtypes. While we currently do not have a sufficiently detailed catalog of TDP-43 client RNAs in disparate cell types and how they are modified by loss of TDP-43 activity, further studies of the kind summarized here should eventually enable this goal.

Concluding Remarks

Development of new RNA-seq technologies has improved our understanding of how TDP-43 regulates RNAs and how alterations in this process may disturb nervous system function. Studies of TDP-43 client RNAs, including *STMN2*, provide clear evidence that TDP-43 function is reduced or lost in at least a subset of ALS and FTD cases [46,90,116]. Overall, studies thus far suggest a model in which certain disruptions to protein homeostasis resulting from aging, environmental exposure, injury, or ALS/FTD-causing mutations lead to TDP-43 mislocalization and altered RNA metabolism. While the abundance of many transcripts changes due to loss of TDP-43 function, the precipitous loss of *STMN2* provides compelling evidence that key roles in the pathology are played by cellular processes linking TDP-43 pathology and the disruption of mechanisms protecting the axon and preventing neuropathy. It will be crucial to determine whether truncated *STMN2* is specific to ALS and FTD or is found more broadly in other TDP-43 proteinopathies.

Decades of ALS/FTD genetic studies have fueled substantial efforts toward the development of therapies targeting specific subsets of patients. For example, antisense oligonucleotide (ASO) clinical trials are underway for therapies designed to reduce the expression of mutant *SOD1* and *C9ORF72* [68]. These depletion strategies are ideally positioned as a potential treatment for forms of disease caused by mutations with clear toxic GOFs. By contrast, gene replacement strategies are a potential therapeutic avenue for restoring normal levels of gene expression decreased due to mutations, such as the inflammation-suppressing *C9ORF72* product [123]. For TDP-43 proteinopathies, however, the pathway to the clinic is less straightforward. We hope that the molecular findings related to TDP-43 proteinopathies highlighted in this review might help spark innovation. Perhaps among the leading candidate targets in this context is *STMN2*. TDP-43 regulation of *STMN2* has the potential to serve as a disease biomarker or even a therapeutic target for splice-switching antisense oligonucleotides, given the success of nusinersen, an ASO that promotes productive splicing of *SMN2*, for the motor neuron disease spinal muscular atrophy [68]. Nevertheless, further investigations are needed into the regulation of *STMN2* by TDP-43, including whether its reduction is beneficial or pathogenic. It is also possible that other ‘missing links’ connecting TDP-43 pathology with neuropathy, beyond *STMN2*, might be identified and validated in future studies.

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Declaration of Interests

J.R.K. is an employee of Faze Medicines and a shareholder in Faze Medicines and QurAlis. K.E. and J.R.K. are authors on a pending patent that describes methods and compositions for restoring *STMN2* levels surfaces (WO/2020/150290). K.E. is a founder of Q-State Biosciences, QurAlis, and EnClear Therapies, and currently employed at BioMarin Pharmaceutical. K.E. is an author on a pending patent that describes compounds and methods for treating neurodegenerative diseases (WO2020107037).

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Outstanding Questions

TDP-43 regulates hundreds of transcripts. Do a small number of these target genes account for disease pathogenesis and progression, or are TDP-43 proteinopathies the result of many modest molecular ‘paper cuts’ collectively summing to major dysfunctions?

Can restoration of a small number of TDP-43 target RNAs (e.g., *STMN2*) serve as a clinical strategy for TDP-43 proteinopathies, or do therapeutic approaches need to focus on pathways upstream of TDP-43 to restore a broader set of transcripts?

More than 50 mutations associated with disease have been identified in *TARDBP*. What are the consequences of these mutations on TDP-43 function, and do they lead to distinct or common defects?

The ability of TDP-43 to phase separate into biomolecular condensates is well established, but how is this process regulated in response to cellular stresses? Are there signal transduction cascades that regulate the ability of TDP-43 to specifically respond to these insults? If so, what are they?

TDP-43 pathology has also been observed in astrocytes. Does TDP-43 pathology in non-neuronal cell types also lead to significant alterations in RNA metabolism? Does pathology in non-neuronal cells contribute to disease onset or progression?

Reduced *STMN2* expression has been observed in other TDP-43 proteinopathies. Is the mechanism behind reduced *STMN2* expression also the consequence of premature polyadenylation and inclusion of a cryptic exon, or is it the result of neuronal loss?

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The Hitchhiker's Guide to Nucleocytoplasmic Trafficking in Neurodegeneration

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Abstract

The widespread nature of nucleocytoplasmic trafficking defects and protein accumulation suggests distinct yet overlapping mechanisms in a variety of neurodegenerative diseases. Detailed understanding of the cellular pathways involved in nucleocytoplasmic transport and its dysregulation are essential for elucidating neurodegenerative pathogenesis and pinpointing potential areas for therapeutic intervention. The transport of cargos from the nucleus to the cytoplasm is generally regulated by the structure and function of the nuclear pore as well as the karyopherin α/β, importin, exportin, and mRNA export mechanisms. The disruption of these crucial transport mechanisms has been extensively described in the context of neurodegenerative diseases. One common theme in neurodegeneration is the cytoplasmic aggregation of proteins, including nuclear RNA binding proteins, repeat expansion associated gene products, and tau. These cytoplasmic aggregations are partly a consequence of failed nucleocytoplasmic transport machinery, but can also further disrupt transport, creating cyclical feed-forward mechanisms that exacerbate neurodegeneration. Here we describe the canonical mechanisms that regulate nucleocytoplasmic trafficking as well as how these mechanisms falter in neurodegenerative diseases.

Keywords ALS/FTD · Huntington's disease · Alzheimer's disease · Proteinopathy · TDP-43 · FUS

Introduction

Neurodegenerative diseases describe a highly heterogeneous set of disorders where progressive and irreversible neuronal death leads to detrimental symptoms dependent upon the subset of neurons that are impacted. While patients will exhibit various degrees of heterogeneity in regard to the specific brain regions involved in their disease as well as the severity of symptom onset and progression, for most patient populations, the progression of these diseases will slowly cost patients their independence and ultimately their lives. While promising clinical trials appear to be on the

horizon, very few existing therapeutics have disease modifying effects. A substantial hurdle to developing effective therapeutics is the lack of causative and targetable cellular pathways that directly lead to neurodegenerative phenotypes. Therefore, comprehensive understanding of the pathogenesis of neurodegenerative diseases is critical for finding successful therapies. Here we review the possible mechanisms leading to neurodegeneration in the context of altered nucleocytoplasmic transport.

The movement of proteins, RNA and other cargos between the nucleus and the cytoplasm broadly encompass the cellular mechanisms controlled by nucleocytoplasmic transport machinery. There is substantial evidence to suggest an association of nucleocytoplasmic trafficking deficits with neurodegeneration [1–5]. Furthermore, an overarching theme that has emerged in recent years is that the failure of cargo transport across the nuclear envelope is not unique to a single neurodegenerative disease, nor one genetic insult [1–5]. However, more work remains to be completed to determine, precisely, how nucleocytoplasmic trafficking deficits may cause neurodegeneration. To properly survey the disrupted landscape of nucleocytoplasmic trafficking in neurodegeneration, it is essential to understand how a

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healthy cell manages nucleocytoplasmic transport. Thus, we first review the principles and components of canonical nucleocytoplasmic transport, and then follow with how these highly regulated transport mechanisms are proposed to be disrupted in several neurodegenerative diseases.

Canonical Nucleocytoplasmic Transport

Structure and Architecture of the Nuclear Pore

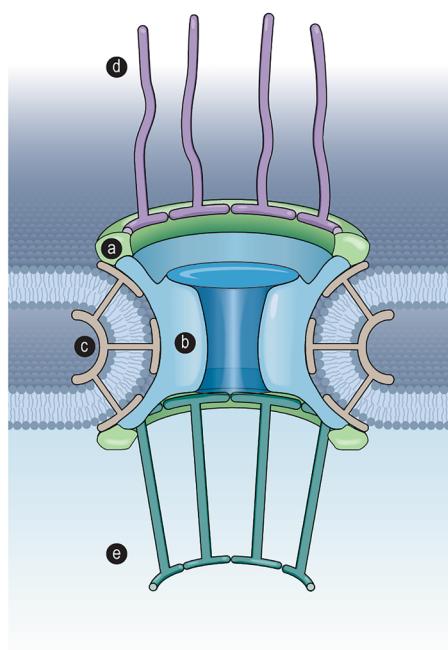
The nuclear membrane acts as a critical divide between the nuclear and cytoplasmic compartments of all eukaryotic cells [6, 7]. Its structural architecture provides a semi-permeable separation between the nucleus and the cytoplasm, which allows cytoplasmic access to genetic material, creating opportunities for complex gene regulation responses to stimuli outside of the nucleus. Unknown structures identified on the nuclear envelope using electron microscopy in the 1950s were first hypothesized to be regularly interspaced fusions of the inner and outer nuclear membrane and were later termed “nuclear pores” [8, 9]. These highly conserved structures are present in all eukaryotes, and are likely the result of early evolutionary events [10]. At the center of each nuclear pore is the massive, ~ 125,000 kDa nuclear pore complex (NPC) [8, 10–12]. The NPC displays eight-fold symmetry around a central channel, and additionally exhibits

symmetry on an axis perpendicular to the central channel; where there exists a symmetrical nuclear and cytoplasmic ring on either side of the inner ring complex [12–14]. Additionally, asymmetric components of the NPC, the nuclear basket and cytoplasmic filaments, extend processes out from the NPC [14].

Each NPC consists of ~ 500–1000 proteins, which—due to the symmetry of the NPC—are comprised of multiple copies of only about 30 unique proteins known as nucleoporins (Nups) [14–16]. These ~ 30 unique Nups can be categorized into 5 groups, based on their structural role in the NPC: the coat nucleoporins, inner ring nucleoporins, transmembrane nucleoporins, cytoplasmic filament nucleoporins, and the nuclear basket nucleoporins (Fig. 1). Although the structure of nucleoporins and NPCs are highly conserved, they display poor genetic conservation, suggesting that broad structures not conserved by genetic sequence still perform similar functions across species [17, 18]. Figure 1 provides the human nomenclature, structural component, and the potential role in disease for each Nup [14, 19–24].

Coat Nucleoporins

This subset of Nups dictates the structure of the nuclear and cytoplasmic rings (Fig. 1a). In humans this “Y” shaped structure is composed of: Sec13, Seh1, Nup96, Nup75, Nup107, Nup160, Nup133, Nup37, Nup43, and ELYS [25],



Nucleoporin	Component of the NPC	FG-Repeats	Disease Associations				References
			ALS	HD	AD	Others	
Sec13 Seh1 Nup96 Nup75 Nup107 Nup160 Nup133 Nup37 Nup43 ELYS	Coat Nups	↑	R	M T	P	Δ	[12,19,22,31]
	Coat Nups						[12,19,31,114]
	Coat Nups						[12,19,31]
	Coat Nups						[12,19,31]
	Coat Nups						[12,19,22,31,89,92,113,114]
	Coat Nups						[12,19,22,31]
	Coat Nups						[12,19,22,32]
	Coat Nups						[12,19,22,32]
	Coat Nups						[12,19]
	Coat Nups						[12,32,20]
NDC1 POM210 POM121	Transmembrane Nups	↑ M M	M	Δ	Δ R	PP	[12,19,22,31,87]
	Transmembrane Nups						[12,19,22]
	Transmembrane Nups						[12,20,22,92]
Nup205 Nup188 Nup93 Nup155 Nup53 Nup54 Nup58	Inner Ring Nups	M T ↑ M ↓ M M ✓ ✓	Δ	R	P	PP	[12,19,22,31,89,92]
	Inner Ring Nups						[12,20,22,31]
	Inner Ring Nups						[12,21,22,31,92,114]
	Inner Ring Nups						[12,21,22,31,92,114]
	Inner Ring Nups						[12,19,92]
	Inner Ring Nups						[12,22,19,31]
	Inner Ring Nups						[12,19,22,31,92]
	Inner Ring Nups						[12,21,22,31,92]
Nup62 Nup58	Inner Ring Nups/Cytoplasmic Filaments	✓ ↓ M	M T	T	T	Δ R	[12,21,22,31,90,92,114,196,197]
	Inner Ring Nups/Cytoplasmic Filaments						[12,21,22,23,31,92,113]
Rae1 Nup42 Nup88 Nup214 DDX19 Gle1 RanBP2	Cytoplasmic Filaments	✓ M T M ↑ M M	Δ	R	PP	PP	[12,21,22,31]
	Cytoplasmic Filaments						[12,23,31]
	Cytoplasmic Filaments						[12,20,22,31,90,92,196]
	Cytoplasmic Filaments						[12,20,22,31,92]
	Cytoplasmic Filaments						[12]
	Cytoplasmic Filaments						[12,22,24,31,113]
	Cytoplasmic Filaments						[12,19,22,92]
Nup153 Nup50 Tpr	Nuclear Basket	✓ ✓ ↓ M	M T	P	R	PP	[12,19,22,92,113,196]
	Nuclear Basket						[12,20,31,113,114]
	Nuclear Basket						[12,20,22,31,114]

Genetic Modifiers: ↑ = enhancers, ↓ = suppressors; Mislocalization: T = Tissue, M = Disease Model; Expression: PP = Protein, RR = RNA; Mutation = Δ

Fig. 1 The structure and architecture of the nuclear pore complex. The structure of the human nuclear pore complex is comprised of 5 major groups. Coat nucleoporins (a), inner ring nucleoporins (b), transmembrane nucleoporins (c), cytoplasmic filaments (d), and the

nuclear basket (e). Right panel: structural grouping of the human nucleoporins and the associations with ALS, AD, HD, and other non-neurological diseases

[26](#). 16 coat nucleoporin complexes make up each of the nuclear and cytoplasmic rings by intricate interlocking of the upper arm with the center stalk of the “Y” region [\[12, 27\]](#). The topographical structure of these complexes has been somewhat conserved, but species divergence does exist [\[25, 27\]](#).

Inner Ring Nucleoporins

Forming the central channel of the NPC, the inner ring structure contains Nup205, Nup188, Nup93, Nup155, Nup53, Nup54, Nup58, Nup62, Nup98 (Fig. 1b) [\[15, 28\]](#). These Nups crystal structures have revealed that flexible linkers mediate the interaction of this complex, allowing for the flexibility of the inner ring [\[29\]](#). The flexibility of these Nups and their ability to interact with cargos play crucial roles in proper trafficking through the nuclear pore [\[12\]](#).

Transmembrane Nucleoporins

The pore membrane proteins (POMs) act as anchors to secure the NPC in position by utilizing a transmembrane domain to interact with the nuclear envelope (Fig. 1c). The human nuclear pore contains NDC1, POM210, and POM121 [\[12, 15, 30\]](#). These components are some of the most poorly characterized features of the NPC with regard to their structure and function [\[12, 30\]](#). While the sequence conservation of POMs is particularly poor, it is likely that the function of transmembrane nucleoporins is conserved [\[12, 30\]](#). POMs contain large often unstructured regions that have been hypothesized to interact either with the nuclear envelope or with the soluble components of the NPC [\[30\]](#). While little is known about the function and regulation of human transmembrane nucleoporins, deletion of POMs in fungi produces no overt survival phenotype and they have been shown to be nonessential for cellular function [\[31, 32\]](#). However, some studies indicate that these proteins play an important role in NPC assembly and nuclear membrane structure. Indeed, evidence suggests POM121 and NDC1 are important during NPC biogenesis and nuclear membrane homeostasis [\[33, 34\]](#).

Cytoplasmic Filaments and the Nuclear Basket

These two components of the nuclear pore are considered asymmetric because they have rotational symmetry like the components above, but not symmetry across the nuclear envelope. The cytoplasmic filaments are long projections that reach into the cytoplasm from the NPC (Fig. 1d) [\[35\]](#). They consist of the following nucleoporins: Rae1, Nu42, Nup88, Nup214, DDX19, Gle1, and RanBP2 (also commonly referred to as Nup358) [\[12\]](#). A principal role of these Nups is the spatial restriction of DDX19 and RanBP2, which

are utilized for bulk mRNA and protein export, respectively, across the NPC to the cytoplasm [\[12, 36–38\]](#). On the opposite side of the nuclear membrane, the human nuclear basket nucleoporins (Nup153, Nup50, and Tpr) also play roles in the organization of transport machinery across the nuclear pore by facilitating the recognition and binding of nuclear import and export factors in the nucleus (Fig. 1e) [\[12, 36\]](#).

Passive Diffusion Through the Nuclear Pore

The NPC regulates trafficking through many mechanisms. The first degree of regulation of transport through the nuclear pore is the diffusion barrier created by the intrinsically disordered phenylalanine—glycine (FG)-rich repeats [\[10, 12\]](#). FG-repeats are common structural components of different nucleoporins spanning different structural regions and play multiple roles in regulating trafficking through the NPC. These FG-repeats form an intrinsically disordered domain in the central channel of the NPC that allows for the passive diffusion of small molecules (<40 kDa) but creates an energetically inefficient method of transport for larger molecules [\[39\]](#). It is interesting to note that the majority of the FG-repeat Nups are not essential, except for Nup98 which constitutes the largest contribution to the diffusion barrier [\[40, 41\]](#).

Facilitated Transport Through the Nuclear Pore

The Ran Gradient

The direction of—and energy for—cargo transport through the NPC is regulated by the gradient of Ras-related nuclear protein (Ran) [\[42\]](#). This small GTPase adopts different conformations in its GTP or GDP bound state, modulating its affinity for transport factors [\[42\]](#). The concentration of RanGTP, with higher levels in the nucleus, and RanGDP, with higher levels in the cytoplasm, is a tightly regulated, cyclical process resulting in an actively maintained Ran gradient in which the concentration of RanGTP to RanGDP mediates nucleocytoplasmic transport [\[42\]](#). Cytoplasmic levels of RanGDP are maintained by the strictly cytoplasmic localization of Ran GTPase-activating protein (RanGAP) and Ran binding proteins (RanBPs) [\[43–45\]](#). RanGAP together with RanBPs contribute to higher cytoplasmic levels of RanGDP by facilitating the hydrolysis of RanGTP that enters the cytoplasm (Fig. 2e, l, and q) [\[43–45\]](#). Cytoplasmic RanGDP is shuttled into the nucleus by a dedicated transporter, nuclear transport factor 2 (NTF2) (Fig. 2f, m) [\[46\]](#). Once in the nucleus, Ran’s chromatin-associated guanine nucleotide exchange factor (RCC1) promotes the exchange of GDP for GTP on Ran, increasing the concentration of nuclear RanGTP and completing the cyclical mechanism for

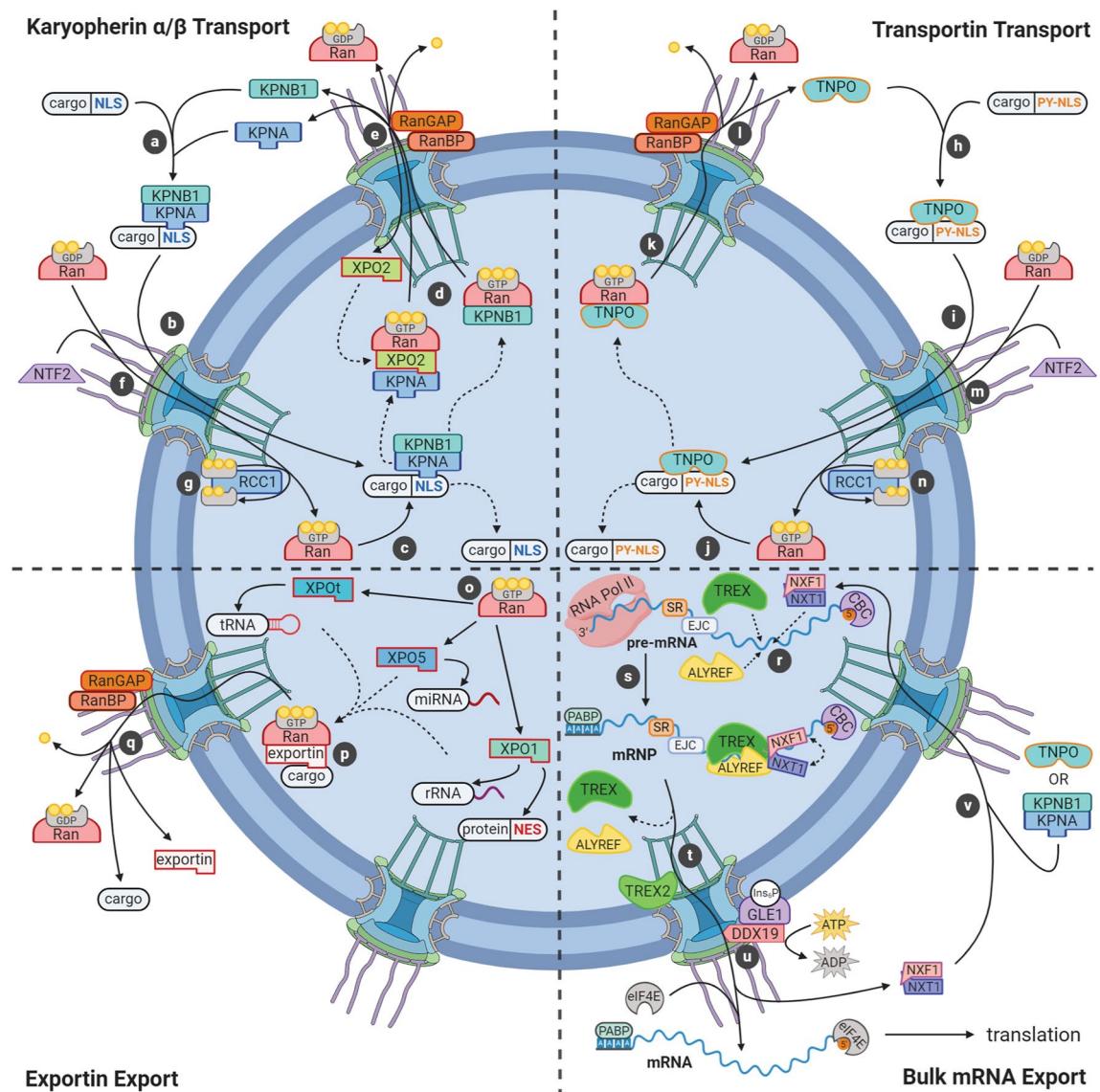


Fig. 2 Canonical nucleocytoplasmic trafficking mechanisms. Top left: Karyopherin α/β mediated import. KPNA binding NLS containing cargo with KPNB binding (a). The tripartite complex is translocated through the NPC (b) and dissociated by RanGTP binding to KPNB1 (c). KPNA recycling to the cytoplasm by XPO2 bound to RanGTP, while KPNB1 recycling is facilitated by its direct binding to RanGTP (d). On the cytoplasmic side of the NPC, the hydrolysis of RanGTP via RanGAP and RanBP dissociates KPNB1 and KPNA export complexes, producing RanGDP and releasing the karyopherins in the cytoplasm (e). RanGDP is shuttled to the nucleus by NTF2 (f), where RCC1 exchanges Ran's GDP for GTP (g). Top right: Transportin. Cargo with a PY-NLS requires TNPO binding (h) and is transported through the NPC (i). Cargo is released after RanGTP binds TNPO (j). TNPO is recycled to the nucleus bound to RanGTP (k). TNPO and RanGTP dissociation is facilitated by RanGAP and RanBP (l), and the Ran gradient is maintained with NFT2 and RCC1 (m, n). Bottom left: Exportin. In the presence of RanGTP, exportins

bind tRNA, miRNA, rRNA or NES containing protein as cargo (o). The resulting RanGTP and cargo bound exportin (p) is translocated through the NPC and dissociates after hydrolysis of RanGTP (q). Bottom right: Bulk mRNA transport. RNA export to the cytoplasm begins during transcription as the nascent strand emerges from RNA Pol II where the TREX complex and ALYREF bind the RNA between the EJC and the 5' end of the pre-mRNA (r). This promotes the recruitment and conformational change of NXF1-NXT1, essential to the hand-off of RNA (r). After the dissociation of RNA Pol II and the binding of PABP to the 3' polyA tail, the mRNP proceeds to the NPC (s). Releasing TREX and ALYREF, the mRNP is drawn through the NPC where NXF1 may interact with TREX2 as it enters the channel (t). On the cytoplasmic side of the NPC, DDX19, powered by ATP hydrolysis and stimulated by GLE1 bound to Ins₆P, promotes the dissociation of the mRNP into its mRNA and export factor components (u). NXF1-NXT1 is recycled to the nucleus by either the TNPO or the KPNA/KPNB1 import pathways (v)

the maintenance of the Ran gradient, essential for nuclear import and export (Fig. 2g, n) [47, 48].

Karyopherin α/β Transport

Large protein complexes destined for the nucleus that struggle to passively diffuse across the FG-repeat domain of the NPC typically contain a nuclear localization signal (NLS) or a nuclear export signal (NES) [49–51]. These specific nuclear transport peptide sequences facilitate the binding of nuclear transport factors which promote transport through the NPC. In what is considered the “classical” transport pathway, karyopherin α (KPNA) binds directly to cargo containing a classical NLS [52]. Seven KPNA isoforms are thought to exist in humans and while there is some interesting specificity of the different isoforms, KPNA2 is considered to be the primary import protein for cargo with a classic NLS [53]. KPNA interacts directly with karyopherin β (KPNB1) through KPNA’s potent NLS domain, creating a tripartite cargo-KPNA-KPNB1 nuclear import complex (Fig. 2a) [16, 53]. Additionally, KPNB1 can also bind directly to some NLS sequences without the KPNA adaptor protein [16]. Once a cargo is bound to a nuclear import complex, the entire complex is guided through the NPC by KPNB1 due to its interactions with the intrinsically disordered FG-repeat domain of the central channel Nups (Fig. 2b) [54, 55]. After successful passage across the nuclear pore and into the nucleus, the import complex dissociates when RanGTP binds KPNB1 (Fig. 2c). KPNB1 recycling and return to the cytoplasm requires binding of RanGTP, while KPNA recycling requires RanGTP dependent nuclear export factor, exportin-2 (XPO2) (Fig. 2d) [56]. Immediately following translocation through the intrinsically disordered FG-repeats, the coordination of RanGAP with RanBPs promotes the innate GTPase activity of Ran, leading to hydrolysis of RanGTP, and disassembly of the karyopherin export complex, thereby releasing the export cargo (Fig. 2e) [57, 58].

Transportin Transport

A different nuclear transport mechanism was discovered by the identification of a new type of NLS, the PY-NLS, which is not recognized by the classical karyopherin α/β machinery, but instead by another group of nuclear transport receptors called transportins (TNPO) [59–62]. Consisting of Transportin1, 2A, and 2B, this transport family functions in a similar manner to the classical transport machinery described above, in which transportin binds to its cargo (Fig. 2h), mediates the interaction with FG-repeat Nups to allow for translocation through the NPC (Fig. 2i), dissociates from its cargo in a RanGTP dependent manner (Fig. 2j), is exported back to the cytoplasm in complex with

RanGTP (Fig. 2k) and freed to bind new cargo following hydrolysis of RanGTP by RanGAP (Fig. 2l) [63, 64]. The difference in cargo recognition appears to be largely mediated by transportins’ recognition of cargo with the PY-NLS, unique from the classical NLS [65]. However, experimental evidence suggests that there are transportin dependent cargos that do not contain a PY-NLS, suggesting multiple recognition motifs for transportin mediated nuclear import [63, 66]. Additionally, post-translational modifications near the PY-NLS can alter transportin mediated transport [63].

Exportin Transport

Export of proteins and certain types of RNA from the nucleus, is mediated by seven exportin proteins in humans [67]. Exportins load their cargo in a RanGTP dependent manner, whereby the RanGTP binding increases exportins affinity for its cargo (Fig. 2o) [42]. Translocation of the exportin-cargo-RanGTP complex (Fig. 2p) through the NPC is mediated by exportin’s interactions with the FG-repeat Nups of the central channel. Once in the cytoplasm dissolution of the export complex and unloading of the cargo occurs through the hydrolysis of RanGTP via RanGAP, thus decreasing exportin’s affinity for its cargo (Fig. 2q) [57, 58]. Exportin 1 (XPO1) is the most prevalent and heavily utilized export receptor, which recognizes protein cargo with an NES [42]. Interestingly, XPO1 can also mediate rRNA, snRNA, and some mRNA export by binding to adaptor RNA binding proteins [42]. Different exportin isoforms have different targets. For example, XPOt is responsible for nuclear export of tRNA, and XPO5 is responsible for miRNA and rRNA export (Fig. 2o) [68, 69].

mRNA Transport

mRNA transport through the nuclear pore occurs through a different mechanism from the Ran mediated nucleocytoplasmic transport mechanism described above [69, 70]. Nuclear export of mRNA is a dynamic process where messenger ribonucleoprotein (mRNP) undergo various remodeling events from biogenesis to maturation and export [69]. The transcription and export (TREX) complex is a key player that initially associates with nascent RNA from transcribing RNA Pol II (Fig. 2r) [71–73]. During mRNA processing, recruitment of a TREX subunit, the RNA export factor ALYREF, is essential for nuclear export after RNA maturation (Fig. 2r) [74]. TREX and ALYREF contain binding sites that mediate the binding of the nuclear RNA export factor 1 (NXF1)—nuclear transport factor 2 like export factor 1 (NXT1) heterodimer [75, 76]. The NXF1-NXT1 interaction with ALYREF triggers the transfer of RNA from ALYREF to NXF1 (Fig. 2r) [77]. This hand-off of RNA is essential because NXF1 has the ability to drive the translocation

through the FG-repeats of the nuclear pore [77]. These binding events signal the maturation of pre-mRNA into an NXF1-NXT1-mRNP complex (Fig. 2s), which then translocates to the nuclear basket, a process that may be conducted by TREX2 whose scaffolding component has been shown to directly interact with NXF1 (Fig. 2t) [78–80]. Once at the nuclear basket, recognition, docking, and subsequent translocation of the mRNP proceeds through interactions between FG-repeat Nups and NXF1 (Fig. 2u) [81]. As an mRNP passes through the NPC and enters the cytoplasm, its cargo mRNA and receptor complexes are dissociated via the ATP-dependent RNA helicase activity of DDX19 (human ortholog of Dbp5), in association with Nup214 stimulated by GLE1, which is bound to its co-factor inositol hexaphosphate (IP6) (Fig. 2u) [82]. The remodeling concludes when the cap-binding protein, eukaryotic translation initiation factor 4E (eIF4E), replaces the cap binding complex (CBC) added during the pre-mRNA stage, preparing the transcript for translation [83]. NXF1-NXT1 can be recycled to the nucleus by either the TNPO or the KPNA/KPNB1 pathway (Fig. 2v). Nuclear export of mRNA appears to be a relatively inefficient process. Studies have indicated that only about 30% of mRNPs that interact with the NPC are successfully passed through the diffusion barrier [84, 85]. While still unclear, it is postulated that the export receptor's capacity for hydrophobic FG-repeat interactions is tempered by the size and composition of the mRNP, a balance that determines the solubility of the cargo-receptor complex in the hydrogel meshwork and, thus, its propensity to translocate through the nuclear pore [39, 86].

A Delicate Relationship Between Neurodegenerative Diseases and Nucleocytoplasmic Transport

The cytoplasmic aggregation of nuclear RNA binding proteins (RBPs), repeat expansion associated gene products, tau, and other proteins is often a common hallmark of neurodegenerative diseases. Emerging evidence indicates that the dysfunction of the dynamic and structural components of nucleocytoplasmic transport may be intricately tied to pathological protein aggregation [1, 87–90]. However, the exact mechanisms underlying this relationship and its implications on neurodegeneration are not fully understood. Disrupted RNA metabolism, as a downstream effect of aberrant RBP localization and aggregation, has become a widely accepted and commonly observed disease phenotype of ALS/FTD, although there has been limited evidence to support the idea that it directly leads to neuronal degeneration [1, 87, 91–93]. Similarly, although evidence suggests that aggregates of tau and repeat expanded gene products in Alzheimer's Disease (AD) and Huntington's Disease (HD), respectively, may

directly impede nucleocytoplasmic transport and damage NPC machinery, it is not clear whether their presence is a driving force or a downstream by-product of disease progression. Here we will review proposed nucleocytoplasmic trafficking abnormalities across different neurodegenerative diseases, their relationship to aberrant protein aggregation, and how this complex dynamic may contribute to neuronal cell death.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that leads to the progressive loss of motor function [94]. Due to overlapping symptoms and genetics ALS is now commonly accepted as a spectrum disorder with frontotemporal dementia (FTD), where patients experience cognitive impairment due to the degeneration of the frontal and temporal lobes leading to social and behavioral changes [95–97]. The exact mechanisms leading to ALS/FTD remain unclear but nucleocytoplasmic trafficking abnormalities appear to play a central role in disease pathogenesis.

Chromosome 9 Open Reading Frame 72 (C9orf72)

The etiology of ALS/FTD is diverse and growing with over 40 genetic abnormalities currently associated with ALS [98, 99]. One of the most well characterized genetic abnormalities associated with the ALS/FTD spectrum to date is the chromosome 9 open reading frame 72 (*C9orf72*) repeat expansion [95–97]. First identified in 2011 the GGGGCC (G_4C_2) hexanucleotide repeat expansion in the first intron of the *C9orf72* gene is responsible for ~50% of all familial ALS and ~20% of sporadic ALS cases [95–97]. Healthy individuals have less than 23 repeats of the intronic G_4C_2 sequence of *C9orf72*, while patients with *C9orf72*-mediated ALS/FTD can exhibit anywhere from 30 to several hundred repeats [95–97]. Three commonly accepted but not mutually exclusive hypotheses exist to explain the neurodegenerative phenotype introduced by *C9orf72* repeat expansion: 1) *C9orf72* haploinsufficiency, 2) accumulation of G_4C_2 sense and C_4G_2 antisense RNA-foci, and 3) the production of dipeptide repeats (DPRs) via repeat-associated non-AUG (RAN) translation (Fig. 3b) [100–112]. The direct mechanism for how the *C9orf72* repeat expansion causes ALS/FTD pathogenesis remains elusive but is currently being extensively investigated.

Early genetic screens in *S. cerevisiae* and *D. melanogaster* identified crucial components of the nucleocytoplasmic transport machinery as modifiers of *C9orf72* disease models [113–115]. Specifically, in regards to the NPC, the coat nucleoporins, Nup107 and Nup160, the nuclear basket component, Nup50, as well as inner ring components, Nup155 and Nup98, were found to be suppressors of *C9orf72*

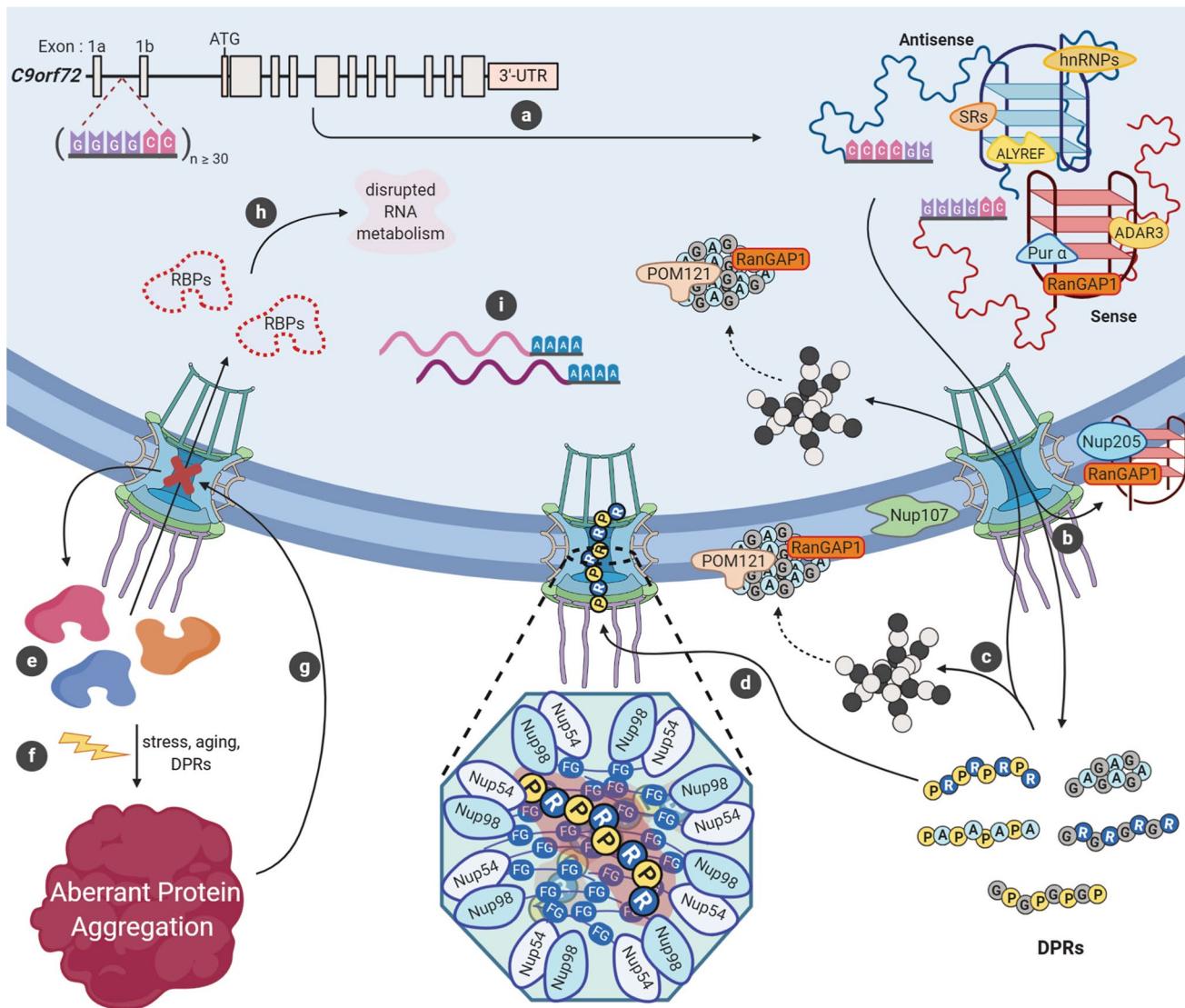


Fig. 3 Nucleocytoplasmic trafficking deficits caused by the *C9orf72* repeat expansion. The sense and antisense transcription of the (G₄C₂) expansion of the *C9orf72* gene between exon 1a and 1b leads to the formation of sense and antisense RNA foci found to sequester Pur α, RanGAP1, ADAR3, SRs, hnRNPs, and other proteins not shown (a). Expanded *C9orf72* RNA results in cytoplasmic/perinuclear RNA foci, which colocalize with RanGAP1 and Nup205, and produce DPRs via RAN translation (b). DPRs aggregate in the cytoplasm and nucleus, where polyGA aggregates may cause the mislocalization of POM121 and RanGAP1 (c). PolyPR aggregates in the cytoplasm and nucleus may cause the mislocalization of POM121 and RanGAP1 (c). PolyPR clogs the NPC due to strong interactions with the FG-repeats of the central channel nucleoporins,

Nup54 and Nup98, whereby it may promote the conversion of the FG hydrogel sieve to a more solid, fibrillar state (d). Impaired import and subsequent accumulation of soluble RBPs (e) coupled with factors like stress, aging, and DPR interaction (f) leads to the formation of insoluble protein aggregates that further disrupt nucleocytoplasmic transport (g), resulting in a feed forward mechanism of toxicity. In addition, the depletion of RBPs from the nucleus has a detrimental effect on RNA metabolism (h). PolyA RNA retention in the nucleus (i) is another common feature of *C9orf72* ALS/FTD models and may be a result of disrupted RNA metabolism and NPC defects

phenotype (Fig. 1) [113, 114]. In contrast, coat nucleoporin, Seh1, inner ring components, Nup62 and Nup93, nuclear basket components, Nup50, Nup153 and Tpr, the cytoplasmic filament component, GLE1, and the transmembrane nucleoporin, NDC1, were found to be enhancers of the observed *C9orf72* phenotype (Fig. 1) [113–115]. Additionally, components central to nucleocytoplasmic transport,

such as protein transport factors, Ran, RanGAP, RCC1, transportins, exportins, and karyopherin α and β, and RNA export factors, NXF1, GLE1, and ALYREF, were shown to be modifiers of *C9orf72*-phenotypes [113, 115].

In agreement with the idea that nucleocytoplasmic transport is altered in *C9orf72*-mediated ALS/FTD, RanGAP1, was shown to directly bind to the *C9orf72* repeat

expansion (Fig. 3a), and was later shown to form intranuclear inclusions [89, 116]. These findings suggest that RanGAP1's ability to regulate the Ran gradient is altered in *C9orf72*-mediated ALS/FTD [89, 116]. Not surprisingly, disruptions of the Ran gradient have been observed in the presence of the *C9orf72* repeat expansion, a phenotype that is rescued with overexpression of RanGAP1 [89]. Further evidence of disrupted nucleocytoplasmic transport components has been shown in mice overexpressing the GA dipeptide repeat, which form cytoplasmic and intranuclear aggregates that colocalize with RanGAP1 and POM121 (Fig. 3c) [117].

In addition to RNA-foci interaction with RanGAP1, evidence also suggests that *C9orf72* RNA-foci sequester RNA-binding proteins, including ALYREF, Adenosine Deaminase Acting on RNA (ADAR3), heterogeneous nuclear ribonucleoproteins (hnRNPs), Pur-alpha, and serine arginine (SR) proteins (Fig. 3a) [116, 118, 119]. Additionally, the disruption of proper RBP function may be exacerbated by DPRs. GR and PR overexpression models in HEK293T cells identified over a hundred interactors of the arginine containing DPRs, including disease causing RBPs with low-complexity domains (LCD) [120, 121]. DPR interaction with LCD containing proteins reduces the capacity of membrane-less organelles for liquid–liquid phase separation (LLPS), a characteristic essential for the dynamic nature of RBPs, stress granules, nucleoli, and the diffusion barrier of the nuclear pore complex [120, 121]. The LLPS of proteins, a normally reversible process whereby proteins shift from a soluble or “de-mixed” state to liquid droplet state, can be a seeding point for the deposition of protein aggregates—commonly seen in ALS/FTD pathology—when aging, stress, and DPR interaction are in play (Fig. 3f) [122, 123]. Through similar mechanisms, PR repeat proteins have been shown to promote the fibrillation of the FG-repeats on Nup98 and Nup54, thereby clogging the NPC and reducing nuclear transport of RNA and proteins (Fig. 3d, i) [120, 124]. However, a recent study from Vanneste and colleagues provides evidence that these arginine containing DPRs do not directly interfere with nucleocytoplasmic transport and highlights a need for additional studies clarifying the role DPRs may play in the pathogenesis of *C9orf72*-mediated ALS/FTD [125].

Taken altogether, it is clear that nucleocytoplasmic transport can be disrupted in *C9orf72* mediated ALS/FTD, through mechanisms leading to the sequestration of transport machinery and aberrant cytoplasmic RBP accumulation and aggregation (Fig. 3e–g). Aside from direct impairment of nuclear import and export, the nuclear depletion of RBPs causes a widespread disruption of RNA metabolism (Fig. 3h) that will be described in the context of TDP-43 and FUS below.

Transactive Response DNA Binding Protein (TARDBP)

Considering the diversity in the etiology and patient presentation within the ALS/FTD disease spectrum, it is surprising that there may be shared mechanisms leading to neurodegeneration in many patient subgroups [126–128]. Indeed, cytoplasmic accumulation and nuclear depletion of the RNA binding protein, encoded by the TARDBP gene, TAR-DNA binding protein-43 (TDP-43) is found in ~95% of all ALS cases and ~45% of FTD cases (FTD-TDP), irrespective of etiology [126, 127, 129, 130]. Notably, TDP-43 proteinopathies are not limited to ALS and FTD with TDP-43 pathology. In fact, TDP-43 positive inclusions are also a characteristic of certain cases of HD, AD, Parkinson’s disease, chronic traumatic encephalopathy, and certain inclusion body myopathies [126, 130–133]. Despite extensive research, it is unclear whether cytoplasmic TDP-43 accumulation leads to a nuclear loss of function or a cytoplasmic toxic gain of function, or a combination of both [93, 129, 134, 135]. Whether wild-type TDP-43 inclusions are drivers of disease progression, or simply a by-product of a degenerating neuron, remains to be seen. Considering that aberrant TDP-43 inclusions are a shared occurrence in multiple diseases we will discuss TDP-43’s functional role in cellular homeostasis and its potential contribution to neurodegenerative disease pathogenesis in more detail below.

TDP-43 is an essential RNA binding protein involved in many steps of RNA metabolism, including: transcription, splicing, maturation, stability, transport, translation, and micro and long non-coding RNA processing [134]. It has further been proposed to be involved in the formation and maintenance of stress granules, and the regulation of stress granule nucleating protein expression [136]. TDP-43 binds to RNA with high specificity via its two RNA recognition motifs (RRMs). Containing both an NLS and an NES, TDP-43 plays roles in both the nucleus and the cytoplasm and, therefore, its trafficking across the nuclear membrane is essential for its proper function. Nuclear import of TDP-43 is facilitated by the classical KPNA/KPNB1 pathway (Fig. 2a–e) [137]. Interestingly, expression of the nuclear export factor XPO2, which is required for KPNA recycling (Fig. 2d), is reduced in the brains of FTD-TDP patients, potentially contributing to the cytoplasmic accumulation of TDP-43 in these patients [137]. The nuclear export of TDP-43 has been predicted to be recognized as an NES-containing cargo by XPO1. Indeed TDP-43 contains two NES sequences that have been predicted to interact with XPO1 [138]. However, recent studies have suggested these two NES sequences are neither necessary nor sufficient for nuclear export, indicating they may be nonfunctional and that TDP-43 may not be actively transported out of the nucleus [138]. In support of this hypothesis, pharmacological and genetic disruption of facilitated nuclear export

pathways (Fig. 2o–q), suggest that TDP-43 is not exported by any of the major export pathways (XPO1, XPO5 or with mRNA through bulk RNA export) [138, 139]. Instead, export of TDP-43 is thought to be mediated through passive diffusion [138].

Considering the prevalence of TDP-43 mislocalization it is essential to understand how the aberrant cytoplasmic accumulations (Fig. 4l) form and how these accumulations are involved in nucleocytoplasmic trafficking defects. The aggregation of TDP-43 is most commonly attributed to its LCD, housed within the C-terminus of the protein. The LCD is home to the majority of ALS associated *TARDBP* mutations [140]. In addition to being unusually disordered and aggregation prone, the C-terminus of TDP-43, in fragmented form, is a highly toxic and prevalent component of the cytoplasmic inclusions seen in ALS pathology [141, 142]. It seems possible that the ability of TDP-43 to undergo LLPS, may create opportunities for TDP-43 to form insoluble aggregates (Fig. 4n) [123, 143, 144]. Indeed, under stress, with age, and in mutated forms, cytoplasmic TDP-43 droplets become more solid and gel like, facilitating aggregation (Fig. 4p) [123, 144–146]. The specific mechanism detailing how endogenous TDP-43 aggregations form in neurodegenerative diseases remains unknown. Artificial introduction of TDP-43 inclusions recruit pathogenic species that mimic the TDP-43 inclusions observed in patients [147]. The low complexity domain on TDP-43 drives the formation of these neurotoxic TDP-43 inclusions and promotes further recruitment of nuclear TDP-43 to such inclusions [147]. Interestingly, aberrant TDP-43 liquid–liquid phase separation can be rescued by enhancing TDP-43 interaction with RNA [147], suggesting that the aggregation of TDP-43 in the cytoplasm may be the result of impaired mRNA export from the nucleus [147]. Mann *et. al* suggest the possibility that increased nuclear retention of RNA renders cytoplasmic TDP-43 more aggregation prone due to reduced cytoplasmic RNA [147].

TDP-43 aggregation may contribute to neurodegeneration by disrupting the nucleocytoplasmic transport machinery [92]. Overexpression of TDP-43 C-terminal fragments either co-aggregate with, or cause the mislocalization of coat Nups (Nup107 and Nup160), inner ring Nups (Nup35, Nup58, Nup62, Nup93, Nup98, Nup205), transmembrane Nups (POM121 and POM210), cytoplasmic filament Nups (Gle1, Nup88, Nup214, RanBP2) nuclear basket Nups (Nup153 and Nup155), and nuclear transport components (XPO5, NXF1) (Fig. 4r). This indicates that cytoplasmic aggregation of TDP-43 is highly disruptive to the NPC (Fig. 4t) [92]. While, the overexpressed system employed in these experiments may exaggerate the degree of NPC interaction, these studies strongly suggest that TDP-43 cytoplasmic inclusions have the capacity to disrupt the NPC and its components (Fig. 4t) [92]. Notably, despite the substantial dysregulation

of the NPC and nucleocytoplasmic transport, the Ran gradient appears normal in these experiments [92].

In addition to the gain-of-toxic function due to TDP-43 aggregates, it is also possible that the nuclear depletion and loss of function of TDP-43 is a toxic contributor resulting from TDP-43 mislocalization to the cytoplasm [127]. These two mechanisms describing cellular stress from TDP-43 proteinopathy are likely not mutually exclusive. As stated above, TDP-43 is a master regulator of splicing and it has been shown to act as a splicing repressor for cryptic exons [148–150]. Proper TDP-43 function has been shown to prevent the inclusion of specific exons, termed cryptic exons, by negatively regulating splicing at these sites [150]. Loss of function of TDP-43 due to its depletion leads to the inclusion of these cryptic exons which could allow for improper handling throughout an RNAs translation and maturation often leading to the decay of the host transcript (Fig. 4u) [148–150]. Indeed, downregulated genes from RNA sequencing datasets of samples with depleted TDP-43 are enriched for cryptic exons [148]. The inclusion of cryptic exons has been observed in C9orf72-mediated ALS/FTD [150]. Interestingly, cryptic exons are highly variable in different cell types, a finding that may provide clues to explain selective vulnerability in neurodegeneration [149]. Considering that TDP-43 interacts with thousands of transcripts, substantial work remains to be completed to understand how the inclusion of cryptic exons is the result of TDP-43 mislocalization and what role they play in the pathogenesis of neurodegenerative diseases. One such possibility is TDP-43's altered regulation of stathmin-2 in ALS [151, 152]. Depletion of TDP-43, mimicking loss of nuclear TDP-43, leads to the pre-polyadenylation of stathmin-2 and decreased protein expression [151, 152]. Interestingly, stathmin-2 is thought to play a role in neurite outgrowth and microtubule dynamics [151, 152]. The loss of stathmin-2 following depletion of TDP-43, causes failure of neuronal regeneration after axotomy, a phenotype that is rescued by stathmin-2 transduction and pharmacological inhibition of c-Jun N-terminal kinase [151, 152].

Fused in Sarcoma (FUS)

Another widely studied, dysregulated RBP implicated in neurodegenerative disease is the FUS protein [135, 153–157]. Historically, FUS pathology was considered to be a rare observation, only attributed to the abnormal aggregation of mutant FUS in ~ 5% of ALS cases (ALS-FUS), and the abnormal aggregation of wild-type FUS in ~ 10% of FTD patients (FTD-FUS) [153, 154, 158, 159]. While initial studies were focused primarily on pathological FUS inclusions, more recent work provides evidence that the spinal motor neurons of sporadic ALS patients display a diffuse cytoplasmic mislocalization of FUS, with no coinciding

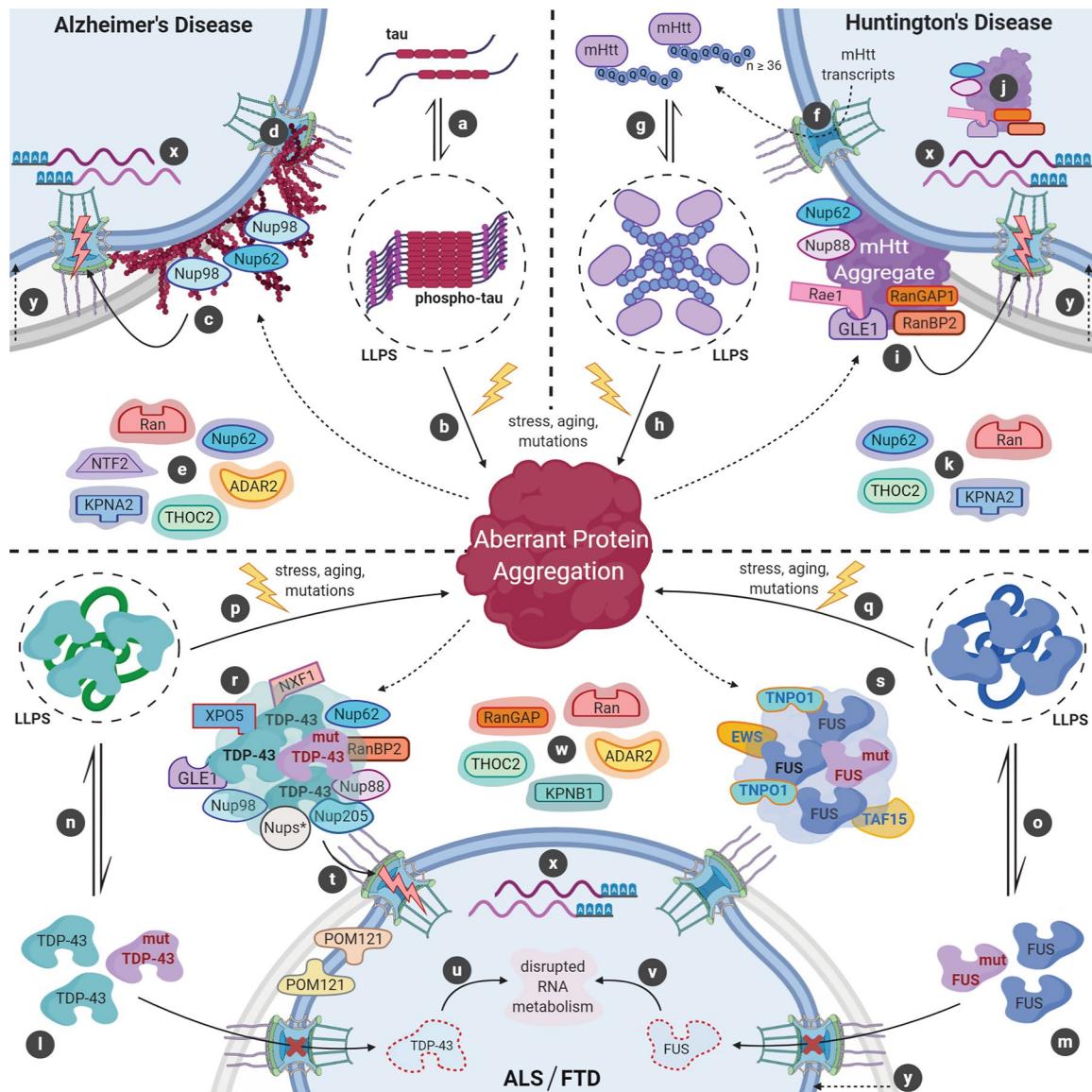


Fig. 4 Nucleocytoplasmic trafficking deficits in neurodegenerative diseases. Top Left: Alzheimer's disease. The phosphorylation state of tau protein determines its capacity for LLPS, where hyperphosphorylation promotes a more inseparable phase (*a*). Dysregulation of tau phosphorylation from stress, aging, and/or mutations cause it to aggregate into NFTs (*b*). Phospho-tau positive NFTs interact with the nuclear envelope, where they disrupt the NPC, and co-aggregate with Nup98 and Nup62 (*c*). NFT's physical occlusion of the NPC (*d*). Impaired Ran gradient, mislocalized or aggregated Nups, RBPs and transport factors act as additional evidence of nucleocytoplasmic trafficking disruptions (*e*). Top Right: Huntington's disease. mHtt harbors over 36 glutamine repeats (*f*). The polyQ tract within mHtt promotes its LLPS (*g*) which can lead to its pathological aggregation through aging and cellular stress (*h*). Toxic perinuclear aggregates of mHtt disrupt the NPC, evidenced by their sequestration of Nups, Ran-related proteins, and other transport factors (*i*). Nuclear mHtt aggregates are also common in Huntington's patients although their contribution to disease progression may be less severe (*j*). Similar to

tau in AD, mHtt can lead to an impaired Ran gradient and the mislocalization and/or aggregation of proteins (*k*). Bottom: ALS/FTD. A common feature of ALS/FTD is the impaired import cytoplasmic accumulation of TDP-43 (*l*) and FUS (*m*). Both of these proteins can undergo LLPS distinct from stress granules, mediated by their LCDs (*n*, *o*). With aging, stress, and/or mutations in these LCDs, TDP-43 or FUS can shift to an aggregated state (*p*, *q*). TDP-43 aggregates can include at least 14 Nups as well as several nuclear transport factors (*r*). TDP-43 aggregates lead to further disruption of the NPC and induce mislocalization of POM121 and POM210 to perinuclear puncta (*t*). The binding interactions of FUS aggregates are less elucidated, although FTD-FUS co-aggregates with other FET proteins and TNP01 (*s*, blue font). AD, HD, and ALS/FTD display a disrupted Ran gradient, and mislocalized RBPs and transport components (*w*). Nuclear loss of TDP-43 or FUS disrupts RNA metabolism (*u*, *v*). Nuclear accumulation of polyA-RNA (*x*) and distortions in the nuclear envelope (*y*)

aggregation [157]. In agreement with these results, mice with mutation-induced cytoplasmic mislocalization of FUS without detectable cytoplasmic aggregation, exhibit age dependent progressive motor and cognitive deficits [160]. These recent studies suggest that the cytoplasmic inclusions of FUS observed in postmortem tissue may not be the only toxic contribution provided by FUS dysregulation [160]. Considering FUS may have a wider contribution to ALS/FTD pathogenesis than once predicted, it is essential to understand the mechanisms by which FUS dysregulation occur, how these differ between ALS-FUS and FTD-FUS patients, and how they are distinct from the mechanisms driving TDP-43 pathology.

The relationship between TDP-43 and FUS is interesting considering their structural and functional similarities: both have RNA recognition motifs, an LCD, and an NES and NLS; both are predominantly nuclear, yet play roles in mRNA stability and translation in the cytoplasm; and both are involved in transcription and splicing [161–163]. Indeed, the LCD of FUS is thought to mediate its capacity for LLPS, disruptions of which may seed FUS aggregation similar to the manner by which TDP-43 is thought to aggregate (Fig. 4o, q) [164]. Also, the nuclear export of FUS appears to be similar to TDP-43, given FUS has 2 predicted XPO1-mediated NES sequences that do not mediate its nuclear export [138]. Instead FUS export is regulated by passive diffusion through the nuclear pore and not by facilitated transport pathway [138, 165]. However, they have key differences that might explain why TDP-43 and FUS inclusions are mutually exclusive [153, 154, 158, 159]. For example, they utilize different transport pathways. The nuclear import of TDP-43 is facilitated by the KPNA/KPNB1 pathway (Fig. 2a–e), while FUS nuclear import is conducted by Transportin-1 (TNPO1) via the TNPO pathway (Fig. 2h–l) [63, 65, 162, 166]. FUS mutations in ALS-FUS patients, have been shown to disrupt the binding of the TNPO1 receptor, which would lead to FUS's cytoplasmic accumulation in a manner independent from drivers of impaired TDP-43 nuclear import (Fig. 4m) [162, 166–170]. Interestingly, neuronal FUS has been shown to be dysregulated in conditions of stress due to failures of TNPO1 [165]. Intriguingly, astrocytes do not display FUS mislocalization under the same conditions, suggesting FUS may have cell-type specific contributions to disease [165]. Additionally, different manifestations of post-translational modifications of FUS, may play a role in its different presentations in ALS and FTD [171–179]. Specifically, the methylation of FUS residues adjacent to the PY-NLS can alter FUS's interaction with TNPO1 [171–179]. Dimethylation, characteristic of ALS-FUS, reduces this interaction, and hypomethylation, characteristic of FTD-FUS, increases it [171–179]. In support of these findings, FTD-FUS inclusions are positive for TNPO1 and other members of the FET protein family, EWS and

TAF15, while ALS-FUS inclusions are not (Fig. 4s) [180]. These differences are intricately tied to the unique presentations of each disease and parsing out why these differences occur is important to characterize disease progression.

Similar to TDP-43, one consequence of FUS aggregation and mislocalization may also be a loss of function due to a nuclear depletion. While TDP-43 and FUS have similar roles in RNA processing and regulation of alternative splicing, FUS is thought to regulate a largely distinct set of transcripts in comparison to TDP-43 [181]. Depletion of FUS leads to broad misregulation of RNAs, including the dysregulation of expression for over 600 mRNAs as well as the dysregulation of over 350 splicing patterns (Fig. 4v) [181]. Interestingly, while both TDP-43 and FUS are important regulators of RNA metabolism, only 86 RNAs are regulated by both proteins, suggesting that the disruption to RNA metabolism due to FUS dysfunction impacts a different group of RNA compared to TDP-43 dysfunction [181]. These studies suggest that neurodegeneration resulting from FUS dysfunction may be distinct from neurodegeneration caused by TDP-43 dysfunction [181].

hnRNP A1 and hnRNP A2B1

Both TDP-43 and FUS are members of a large subclass of RNA-binding proteins known as hnRNPs, which have been shown to have diverse transcriptional control through their roles in RNA maturation and neuronal RNA transport granules. Additionally, these proteins have been associated with stress granule formation, which is critical to the cellular stress response [182–184]. Like TDP-43 and FUS, rare dominant mutations in the LCD of hnRNP A1 and hnRNP A2B1 have been associated with ALS [182–184]. Interestingly, these mutations are most frequently associated with multisystem proteinopathy, a rare inherited disease in which patients experience the degeneration of muscle, bone and/or the central nervous system [182–184]. The mutations in hnRNP A1 and hnRNP A2B1 have been shown to cause their mislocalization from the nucleus to the cytoplasm and to pathologically alter stress granule dynamics by enhancing their tendency to fibrillize and thereby disrupting their capacity to undergo LLPS [182–184]. These cytoplasmic inclusions have also been shown to colocalize with cytoplasmic TDP-43 aggregates and have been associated with TDP-43 nuclear depletion [182–184].

ADAR2

Our lab has shown that the RNA editing enzyme Adenosine Deaminase Acting on double-stranded RNA 2 (ADAR2) forms cytoplasmic inclusions in C9orf72-mediated ALS/FTD and in Alzheimer's Disease (Fig. 4e, w) [185]. ADAR2 utilizes KPNA1 and KPNA3 to traffic into the nucleus [186,

[187]. While it is unclear how ADAR2 accumulations form in ALS and AD it is possible that dysregulation of KPNA1 or 3 would lead to abnormal cytoplasmic inclusions of ADAR2. In addition to its cytoplasmic accumulation we provided evidence to suggest aberrant function of A to I editing in C9orf72-mediated ALS/FTD with enrichment of A to I editing aberrations in the EIF2 pathway, which may lead to global translational inhibition [185]. ADAR2 is a nuclear enzyme and its presence in the cytoplasm alters the function of the enzyme, suggesting that the altered cellular localization in disease represents a mechanism for its dysregulation [185]. There has been a revolution in the understanding of the function of A to I editing in both normal cellular function and in disease [188]. Dysregulation of ADAR2 and its normal cellular function would lead to altered regulation of RNA transcripts that would have yet unknown cellular consequences. While many RNA editing abnormalities have been associated with neurodegeneration [188], due to the massive number of uncharacterized A to I editing sites further experiments are needed to establish the specific role of altered RNA A to I editing events in the pathogenesis of neurodegenerative diseases.

Superoxide Dismutase 1 (SOD1)

Mutations in the SOD1 gene were the first genetic association with ALS [189]. Patients with SOD1 mutations do not have TDP-43 or FUS pathology [190–193]. However, large SOD1 accumulations exist in patients with these SOD1 mutations [190–193]. The dichotomy that appears to exist between neurons with SOD1 accumulations compared to TDP-43 and FUS accumulations is interesting and suggests different mechanisms leading to neurodegeneration [190–193]. Further evidence to support the unique nature of SOD1 suggests that SOD1 exhibits a distinct transcriptome compared to that of C9orf72 patients [194]. It is possible that some components of the nuclear pore and nucleocytoplasmonic transport are disrupted as a result of the SOD1 G93A mutation [195]. However, conflicting reports suggest that more experiments are required to understand nucleocytoplasmonic transport deficits in SOD1 mediated ALS [92].

Sporadic ALS

In contrast to familial ALS, there have been few studies which describe nucleocytoplasmonic transport deficits in sporadic ALS. Limited number of studies report impaired nucleoporins (Nup62) and KPNB1 in cells with TDP-43 aggregates [196, 197]. Considering the challenges associated with studying and modeling sporadic forms of ALS it is not surprising that few studies have begun to describe nucleocytoplasmonic trafficking deficits. However, as stated above, TDP-43 aggregations have the capacity to disrupt the

nucleocytoplasmonic trafficking machinery and it is possible there will be future disruptions identified in sporadic ALS.

Huntington's Disease

The degeneration of striatal medium spiny projection neurons as well as cortical pyramidal neurons due to the presence of an expanded CAG repeat in the Huntington (*HTT*) gene is known as Huntington's disease [198]. Individuals with more than 39 CAG repeats in the *HTT* gene develop progressive motor, cognitive and psychiatric symptoms leading to a steady decline in physical health and quality of life culminating in death 10–30 years after disease onset [199]. Translation of *HTT* with an abnormal number of CAG repeats, leads to huntingtin protein with an expanded poly-glutamine (polyQ) tract (mHtt) (Fig. 4f). Due to this polyQ tract and proline-rich region, mHtt undergoes LLPS (Fig. 4g), potentially leading to cytoplasmic, perinuclear, and nuclear aggregates through factors such as aging and other stressors (Fig. 4h–j) [200]. There is substantial evidence that suggests these aggregates disrupt the NPC and nucleocytoplasmonic trafficking in Huntington's disease.

Immune-electron microscopy performed on cells over-expressing mHtt show distortion of the nuclear membrane (Fig. 4y) and cytoplasmic inclusions of NPC proteins in cells with perinuclear inclusions of mHtt (Fig. 4k) [201]. Additionally, early analysis of protein interactions suggested that polyQ accumulations interact with nuclear pore components, specifically FG-repeat Nups found in the cytoplasmic filaments (DDX19, RanBP2, and Nup214), the nuclear basket (Nup153), and the inner ring nucleoporin Nup62 [202].

More recent studies elaborated on these previous publications, firmly associating nucleocytoplasmonic trafficking abnormalities with HD [90, 203]. First Gasset-Rosa et al. confirmed that mutant huntingtin accumulations lead to abnormal nuclear envelope morphology [203]. Using a mHtt mouse model, an increasingly severe nuclear envelope pathology in the homozygous compared to heterozygous animals suggested a dose-dependent phenotype [203]. These findings were confirmed in human induced pluripotent neural progenitor cells and in human postmortem tissue further associating mutant huntingtin and nuclear membrane abnormalities with disease state [203]. In addition to nuclear membrane pathology, HD models and patients displays overt nucleocytoplasmonic trafficking deficits [203]. mRNA export is dysregulated, as evidenced by increased mRNA levels in the nuclei of cortical regions in a mouse models and Huntington's disease human postmortem tissue (Fig. 4x) [203]. Concurrently, work done by Grima et al. showed a disruption of the Ran gradient in motor neurons differentiated from hiPSCs from HD patients [90]. Evidence for disruption of the NPC comes from the discovery in human post mortem tissue showing that patients with Huntington's disease exhibit a

mislocalization or aggregation of the Ran GTPase activating protein, RanGAP1, in the frontal cortex, striatum, cerebellum, and hiPSC neurons (Fig. 4k) [90]. Additionally, they found that Nup62, a component of the central channel of the inner ring of the NPC, was mislocalized only in the striatum and displayed a diffuse cytoplasmic pathology in human tissue and hiPSC neuronal models (Fig. 4k) [90]. The Ran gradient deficit can be rescued both by inhibition of nuclear export via Exportin-1 inhibition with KPT-350 treatment; and interestingly, inhibition of O-GlcNAcylation, a common post translational modification for nucleoporins [90].

Alzheimer's Disease

AD is the leading cause of dementia worldwide [204]. The age related progressive cognitive decline of episodic memory is the most common clinical feature of Alzheimer's disease [204]. Early studies utilizing electron microscopy identified a close association between neurofibrillary tangles (NFTs) and the NPC in neurons of post mortem tissue from patients with Alzheimer's disease (Fig. 4d) [205]. Furthermore, recent studies using iPSC-differentiated neurons have shown that tau accumulation disrupts nuclear lamina and nucleocytoplasmic transport [206]. Mechanisms for tau aggregation have parallels to RBP and mHtt aggregation—mutation and the phosphorylation states of tau have been implicated in its aberrant LLPS (Fig. 4a, b) [207, 208]. Given the prevalence of tau-pathology in AD and the emerging link between nucleocytoplasmic trafficking deficits and neurodegenerative diseases, elucidating the impact of tau inclusions on the NPC is critical to understanding disease pathogenesis.

Conflicting reports exist on the nature of Nup62s localization in Alzheimer's disease postmortem tissue [88, 209]. Early studies found that Nup62 staining on abnormal nuclei in neurons with NFTs was localized primarily to the nuclear membrane and identified no aberrant localization [209]. More recent studies have suggested that similar to Huntington's disease, Nup62 is mislocalized in neurons from AD patients (Fig. 4e) [88]. Due to difficulties in Nup62 labeling the authors suggest it is difficult to determine if cytoplasmic aggregation exists due to lipofuscin autofluorescence, but instead suggest diffuse cytosolic mislocalization and depletion at the nuclear membrane [88]. Nup414 labeling, which includes Nup62, further suggests the dysregulation of FG-repeat Nups [88]. Indeed, Nup98, the largest contributor to the FG-repeat diffusion barrier, interacts directly with phospho-tau and is mislocalized in AD hippocampal neurons [88]. Not surprisingly, the direct interaction of tau with the NPC leads to disruption of the NPC diffusion barrier and the Ran gradient (Fig. 4c) [88]. However, Nup54, POM121, Nup88, Nup153, Nup133, and Nup214 were shown to not have apparent differences in expression or distribution in

neurons positive for tau tangles, which suggests normal function of the transmembrane, cytoplasmic filaments, nuclear basket, and coat nucleoporins, as well as some of the inner ring nucleoporins, indicating there is not a complete breakdown of the NPC [88, 209]. Nonetheless, similar to nuclear envelope aberrations found in other neurodegenerative diseases, neurons from patients with Alzheimer's disease exhibit aberrantly shaped nuclei (Fig. 4y) [209].

Neurons with and without NFTs also contain cytoplasmic accumulations of the RanGDP transporter, NTF2 (Fig. 4e) [209]. Failure of the nuclear import of RanGDP would lead to the disruption of the Ran gradient in AD and disruption of Ran mediated nucleocytoplasmic transport [210]. This supports the disruption of the Ran gradient in AD CA1 hippocampal tissue, with phospho-tau inclusions [88]. Considering that the interactions of the karyopherin family with NTF2 are thought to expedite transport kinetics, it would not be surprising that NTF2 accumulations accompany additional nuclear import deficits [211]. Interestingly, induced cytoplasmic artificial beta sheets that form amyloid-like aggregates, inhibit nuclear export of mRNA, likely due to their forced mislocalization and co-aggregation of KPNA2 and KPNA4, RanBP1, and THOC2 suggesting that the presence of beta-sheets in NFTs found in AD may further disrupt nuclear trafficking adding additional sources of cellular stress (Fig. 4e) [212, 213].

Therapeutically Targeting Nucleocytoplasmic Transport Defects

The prevalence of nucleocytoplasmic trafficking throughout neurodegenerative diseases creates an enticing opportunity for therapeutic intervention. To this point the nuclear export inhibitors KPT-350, KPT-335, and KPT-276 have been shown to have neuroprotective effects [89, 90, 92, 139]. However, the mechanism of this neuroprotection remains unclear [139]. Considering the recent evidence suggesting that TDP-43 and FUS are not targets of exportin mediated transport, it is unknown whether KPT compounds are directly modulating TDP-43 or FUS nucleocytoplasmic transport [138, 139, 165]. Biogen has recently initiated a Phase1 safety trial with an XPO1 inhibitor in ALS patients to be completed by the end of the fiscal year 2020 [214]. Interestingly though, low non-toxic concentrations of KPT compounds are not able to inhibit nuclear export, emphasizing even more that the detailed mechanisms for how KPT compounds might confer neuroprotection in neurodegenerative disease have yet to be identified [139].

Considering the widespread presence of protein aggregation in these diseases, removing these aberrant protein aggregates may alleviate the associated neurotoxic phenotypes [146, 215]. Interestingly, nuclear import receptors can

disassemble aberrant insoluble protein aggregates that have been associated with neurodegenerative diseases [216]. Specifically, KNPA and KNPB have been shown to be able to disaggregate TDP-43 aggregates and TNPO1 was able to disaggregate FUS aggregates as well as other aggregates of proteins with a PY-NLS [216]. These findings suggest that failure of nuclear import can be rescued by supporting the possibly overwhelmed endogenous nuclear import receptors (Ex. KNPA, KNPB, and TNPO1) [216]. If disaggregation of aberrant fibrils using nuclear import receptors can also restore normal transport of RNA binding proteins to the nucleus, it may be possible to rescue toxic cytoplasmic aggregates, alleviate nuclear depletion of RBPs and thereby protect against neuronal loss in neurodegenerative diseases [215, 216].

Conclusion

The widespread prevalence of impaired elements of nucleocytoplasmic trafficking machinery throughout instances of neurodegeneration suggest the existence of a common mechanism of dysfunction. The potential of overlapping causative disease mechanisms creates exciting opportunities for multifaceted therapeutic interventions. While an enormous body of work remains to fully understand the mechanisms behind possible therapeutic interventions in nucleocytoplasmic transport, early evidence suggests multiple exciting opportunities. These trafficking aberrations have been well established and their role in neurodegenerative diseases has been increasingly strengthened. However, the initiating factor that leads directly to detrimental nucleocytoplasmic trafficking deficits remains unclear. Nevertheless, it seems likely that regardless of how trafficking deficits begin, the consequences act in a feed-forward mechanism that will further disrupt nucleocytoplasmic trafficking resulting in more severe consequences. Once nucleocytoplasmic trafficking defects have taken place, how this cellular disruption causes neurodegeneration is still largely unknown. It is likely that nuclear depletion and increased cytoplasmic concentration leading to aberrant aggregations will contribute to cellular stress through feed-forward disruptions of RNA metabolism and nucleocytoplasmic transport. The identification of toxic species that result from trafficking deficits may result in additional opportunities for therapeutic interventions.

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