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REVIEW

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CURRENT OPINION

Amyotrophic lateral sclerosis: recent genetic highlights

Matthew A. White and Jemeen Sreedharan

Purpose of review
Amyotrophic lateral sclerosis (ALS), like other neurodegenerative diseases, remains incurable, but gene mutations linked to ALS are providing clues as to how to target therapies. It is important for researchers to keep abreast of the rapid influx of new data in ALS, and we aim to summarize the major genetic advances made in the field over the past 2 years.

Recent findings
Significant variation in seven genes has recently been found in ALS: *TBK1*, *CCNF*, *GLE1*, *MATR3*, *TUBA4A*, *CHCHD10* and *NEK1*. These have mostly been identified through large exome screening studies, though traditional linkage approaches and candidate gene screening remain important. We briefly update *C9orf72* research, noting in particular the development of reagents to better understand the normal role of C9orf72 protein.

Summary
Striking advances in our understanding of the genetic heterogeneity of ALS continue to be made, year on year. These implicate proteostasis, RNA export, nuclear transport, the cytoskeleton, mitochondrial function, the cell cycle and DNA repair. Functional studies to integrate these hits are needed. By building a web of knowledge with interlinked genes and mechanisms, it is hoped we can better understand ALS and work toward effective therapies.

Keywords
amyotrophic lateral sclerosis, C9orf72, genetics, RNA processing, TDP-43

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease of adults and is invariably fatal, often within 5 years of disease onset. Importantly, ALS is on a clinical, genetic and pathological spectrum with frontotemporal dementia (FTD). Riluzole, a glutamate modulator and the only disease-modifying agent widely used for ALS, has only a modest effect on survival. Knockdown gene therapies hold promise for those individuals with ALS-causing gene mutations in *SOD1* and *C9orf72*, and clinical trials are underway. However, the majority of ALS is not caused by mutations in these or other identified genes. These apparently sporadic amyotrophic lateral sclerosis (sALS) cases account for around 90% of all ALS, whereas familial amyotrophic lateral sclerosis (fALS), usually dominantly inherited, accounts for the remainder.

The distinction between sALS and fALS, though practical, is increasingly recognized to be artificial, but what is important is that sALS and fALS are often indistinguishable. This suggests that ALS-linked genes in rare fALS cases can inform our understanding of sALS. With this in mind, the dizzying pace of gene discovery in ALS in the past 10 years is to be welcomed, and advances in exome and genome sequencing strategies have been invaluable in this task. Identifying genetic risk factors and modifiers that influence ALS risk in sALS is also adding to our understanding of the mechanisms underlying ALS as a whole.

In this review, we will highlight the latest developments in the genetics of ALS focussing on genes identified in the past 2 years. Refer to Table 1 for a

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KEY POINTS

- The exponential rise in genetic discovery in ALS continues unabated and will continue to do so for some time.
- New genes implicate proteostasis, RNA export, nuclear transport, the cytoskeleton, mitochondrial function, the cell cycle and DNA repair.
- Novel and sophisticated approaches are needed to integrate and model this new knowledge to identify therapeutic targets.

summary of the genes primarily discussed in this article. We will then highlight recent advances in our understanding of *C9orf72*, still the hottest topic in the field. Readers are encouraged to refer to the Amyotrophic Lateral Sclerosis Online Genetics Database website (http://alsod.iop.kcl.ac.uk/) for a comprehensive list of other *ALS* genes or Table 2 for a summary.

NEW *ALS* GENES

TBK1

Perhaps the most significant recent genetic discovery in ALS since *C9orf72* has been the identification of mutations in the *TBK1* gene (encoding TANK binding kinase 1). One study examined 2869 ALS patients and 6405 controls and found nearly 50 variants throughout *TBK1* in ALS patients, including splice-site, loss-of-function (LoF) and missense mutations [1▪]. Another study used whole exome sequencing and linkage analysis to interrogate 252 fALS cases that were negative for *SOD1* and *C9orf72* mutation [2▪▪]. In this study, eight predicted LoF mutations in *TBK1* were found in 13 families (a mixture of truncating and splice-site mutations). The mean age of onset of fALS was 60 years of age, 15% with bulbar onset and with 50% of cases demonstrating cognitive impairment, often frank FTD. LoF mutations were not a contributor to sALS, but nine missense variants were found in both fALS and sALS.

The *TBK1* LoF mutations would be predicted to truncate TBK1, but seven of the eight variants identified by Freischmidt *et al.* produced little or no detectable transcripts or protein products, suggesting that haploinsufficiency of TBK1 is responsible for neurodegeneration. The most 3′ truncation mutation [c.2138+2T>C (p.690–713del)] generated a protein product lacking 22 amino acids from the C-terminal coiled-coil domain 2, which appeared to abolish its ability to bind optineurin (OPTN) *in vitro*. Several of the missense mutations also abrogated the ability of TBK1 to bind OPTN and/or phosphorylate IRF3. It remains unclear how loss of TBK1 function causes neurodegeneration, but aberrant cellular clearance is a possibility given that both OPTN and p62, another ALS-linked protein that TBK1 interacts with, have roles in autophagy [3,4].

TBK1 interacts with OPTN, which binds to ubiquitin chains on mitochondria. This recruits TBK1 to mitochondria and promotes its kinase activation. TBK1 can then phosphorylate the UBAN domain of OPTN, expanding its ability to bind ubiquitin chains. This positive feedback mechanism promotes recruitment and retention of TBK1/OPTN to damaged mitochondria [5]. Further evidence for a role of TBK1/OPTN in the removal of damaged mitochondria involves the PINK1-PARKIN pathway and mitophagy through the polyubiquitination of defective mitochondria. TBK1 physically associates with autophagy adapters OPTN, NDP52 and p62, strengthening the link between ALS and selective autophagy [6], and TBK1 is able to phosphorylate p62 at S403 in the early period of mitochondrial depolarization [7].

Further *TBK1* variants have recently been found in European, Taiwanese and Chinese patients with ALS and/or FTD [8–11]. Strikingly, up to 10.8% of cases of patients presenting with ALS–FTD in a French patient cohort were found to carry LoF mutations in *TBK1*, suggesting that patients

Table 1. Article overview

We aim to summarize the major genetic advances in the field of ALS over the past 2 years:
Significant variation in seven genes have recently been found in ALS:
TBK1 (TANK binding kinase 1) mutations implicate abnormal cellular clearance in ALS and TBK1-ALS could be seen as yet another 'TDP-43 proteinopathy'
CCNF encodes cyclin F and implicates abnormal protein ubiquitination and possible aberrant cell cycle re-entry as mechanisms of neuronal loss in ALS
GLE1 encodes two isoforms involved in multiple cellular functions including nuclear export and stress granule function
MATR3 mutations have been implicated in ALS. Matrin 3 is a 125-kDa nuclear matrix protein that binds DNA and RNA
TUBA4A encoding the Tubulin, Alpha 4A protein further implicates cytoskeletal structural dynamics in ALS pathogenesis
CHCHD10 mutations further implicate mitochondrial dysfunction in ALS
NEK1 mutations are of interest as NEK1 interacts with two known ALS proteins, Alsin and VAPB
Advances in our understanding of *C9orf72* are discussed covering possible mechanisms of disease, development of tools to further study the gene and oligogenicity in ALS

ALS, amyotrophic lateral sclerosis.

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Table 2. Genes implicated in amyotrophic lateral sclerosis

ALS type	Gene ID	Description	Location
ALS 1	SOD1	Superoxide dismutase 1, soluble	21q22.11
ALS 2	ALS2	ALS2, alsin Rho guanine nucleotide exchange factor	2q33.2
ALS 3	ALS3	Amyotrophic lateral sclerosis 3 (autosomal dominant)	18q21
ALS 4	SETX	Senataxin	9q34.13
ALS 5	SPG11	Spastic paraplegia 11 (autosomal recessive)	15q14
ALS 6	FUS	FUS RNA binding protei	16p11.2
ALS 7	ALS7	Amyotrophic lateral sclerosis 7	20p13
ALS 8	VAPB	VAMP (vesicle-associated membrane protein) associated protein B and C	20q13.33
ALS 9	ANG	Angiogenin	14q11.1
ALS 10	TARDBP	TAR DNA binding protein	1p36.22
ALS 11	FIG4	FIG4 phosphoinositide 5-phosphatase	6q21
ALS 12	OPTN	Optineurin	10p13
ALS 13	ATXN2	Ataxin 2	12q24.1
ALS 14	VCP	Valosin containing protein	9p13.3
ALS 15	UBQLN2	Ubiquilin 2	Xp11.21
ALS 16	SIGMAR1	Sigma nonopioid intracellular receptor 1	9p13.3
ALS 17	CHMP2B	Charged multivesicular body protein 2B	3p11.2
ALS 18	PFN1	Profilin 1	17p13.3
ALS 19	ERBB4	erb-b2 receptor tyrosine kinase 4	2q33.3-q34
ALS 20	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	12q13.1
ALS 21	MATR3	Matrin 3	5q31.2
ALS–FTD1	C9orf72	Chromosome 9 open reading frame 72	9p21.2
ALS–FTD2	CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	22q11.23
ALS	UNC13A	unc-13 homolog A (*C. elegans*)	19p13.11
ALS	DAO	D-amino-acid oxidase	12q24
ALS	DCTN1	Dynactin subunit 1	2p13
ALS	NEFH	Neurofilament, heavy polypeptide	22q12.2
ALS	PRPH	Peripherin	12q13.12
ALS	SQSTM1	Sequestosome 1	5q35
ALS	TAF15	TATA-box binding protein associated factor 15	17q12
ALS	SPAST	Spastin	2p24-p21
ALS	ELP3	Elongator acetyltransferase complex subunit 3	8p21.1
ALS	LMNB1	Lamin B1	5q23.2
	GLE1	GLE1 RNA export mediator	9q34.11
	TBK1	TANK binding kinase 1	12q14.1
	TUBA4A	Tubulin alpha 4a	2q35
	CCNF	Cyclin F	16p13.3
	NEK1	NIMA related kinase 1	4q33

ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia.

presenting with FTD and ALS who do not have *C9orf72* mutations should be screened for *TBK1* mutations [12]. In another study of 104 pathologically confirmed FTLD-TDP patients who were negative for *C9orf72* and *GRN* mutations, 4.8% of patients had mutations in *TBK1*, *OPTN* or both [13]. These mutations were associated with reduced expression of *TBK1* and/or *OPTN* mRNA and protein in cerebellar tissue. This study implicates the OPTN/TBK1 pathway in FTD with TDP-43 inclusions [13]. Pathological analysis of brain and spinal cord from ALS patients with TBK1 mutations also identified TDP-43 positive inclusions [11,14]. Thus, TBK1-ALS is yet another 'TDP-43 proteinopathy'.

CCNF

Linkage analysis followed by whole-exome sequencing of a large ALS–FTD kindred of British ancestry

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identified a missense mutation in the *CCNF* gene [15▪▪]. Further novel *CCNF* variants were identified through screening additional fALS (~600), sALS (~500) and FTD (nearly 200) cases from Europe, North America, Japan and Australia. Five novel missense mutations were found in familial ALS and/or FTD, and 19 mutations (missense, nonsense and frameshift) were seen in sporadic ALS and/or FTD. Most mutations affected highly conserved residues, and none were seen in controls. No clear genotype–phenotype pattern was evident. An additional replication study of 611 sALS cases and 1424 controls demonstrated an enrichment of mutations in ALS. In summary, mutations in *CCNF* occur throughout the world and account for 0.6–3.3% of fALS cohorts.

CCNF encodes cyclin F, a ubiquitously expressed protein, which is known to function in two major biological processes. First, cyclin F helps to form an E3 ubiquitin-protein ligase complex and transfer activated ubiquitin to target proteins for degradation. Overexpression of ALS mutant cyclin F isoforms appeared to result in an increase in ubiquitinated proteins and, notably, an increase in TDP-43 ubiquitination [15▪▪]. Whether the underlying mechanism is due to a toxic gain of function and whether cyclin F-linked ALS is a 'TDP-43 proteinopathy' *in vivo* are important questions requiring further investigation.

Cyclin F also has a role in coordinating the cell cycle. Cyclin F levels rise through interphase, peaking in G2 and then dropping at mitosis [16]. Cyclin F is not a highly abundant protein and appears unstable. Unlike other cyclins, its degradation is independent of the ubiquitin proteasome system but requires the C-terminal PEST region of the protein [17]. Cyclin F is a substrate binding subunit of SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complexes and interacts with the ribonucleotide reductase family member 2. Ribonucleotide reductase plays a critical role in the conversion of ribonucleotides to deoxyribonucleotides necessary for replicative and repair DNA synthesis.

Attempts by postmitotic neurons to re-enter the cell cycle can lead to apoptosis and neuronal loss characterized by increased expression of cyclins. In a neurotrophin withdrawal model of primary motor neuron apoptotic cell death, nuclear condensation and DNA fragmentation can be observed along with activation of caspase-3 and caspase-9. The cyclin-dependent kinase inhibitors olomoucine, roscovitine and flavopiridol have been demonstrated to suppress the death of motoneurons. Taken together with the observation that cyclin D1 and cyclin E expression rises following removal of neurotrophic support, it can be envisaged that postmitotic neuronal loss occurs as neurons attempt to re-enter the cell cycle inappropriately [18]. Treatment with nerve growth factor decreases cyclin F levels in PC12EY cells suggesting that cyclin F is involved in NGF-mediated cell cycle events during the differentiation of these cells [19].

GLE1

Recessive mutations in *GLE1*, encoding the RNA-processing protein Gle1, cause the lethal congenital contracture syndrome 1 and lethal arthrogryposis with anterior horn cell disease [20,21]. Candidate screening of *GLE1* in 173 fALS and 760 sALS patients recently identified novel nonsense and missense mutations in two sALS cases and a splice-site mutation in a fALS case [22▪▪]. The nonsense transcript produces no functional protein, whereas the splice-site mutation alters the C-terminus, preventing the mutant isoform from localizing at the nuclear pore where it is critical for mRNA export. These *GLE1* mutants also failed to rescue motor neuron pathology in zebrafish lacking *GLE1*. Thus, the mutations seem to cause a critical loss of function.

GLE1 encodes two isoforms, Gle1A and Gle1B. Gle1B localizes to the nuclear pore complex and shuttles between the nucleus and cytoplasm [23] and self-associates via its coiled-coil domain forming oligomers necessary for its function in mRNA export [24]. The unique C-terminal 43 amino acid region of Gle1B mediates binding to the C-terminal non-FG region of the nucleoporin CG1/NPL1 [23], whereas nuclear rim localization of Gle1B is dependent on 29 N-terminal residues that interact with Nup155 [25]. These observations are interesting given that nuclear transport has recently been implicated in motor neuron degeneration [26,27].

Gle1 also has important roles in translation termination [28,29] and initiation [30]. Interestingly, the Gle1A isoform specifically has been found to associate in stress granules, which is an area of intense research in ALS [31]. Furthermore, overexpression of Gle1A or a disease-associated isoform led to the formation of cytoplasmic protein aggregates [32]. This suggests that, rather than loss of function, cellular stress may occur through increased cytoplasmic Gle1 activity.

MATR3

Exome sequencing of a family of European ancestry with dominantly inherited ALS and dementia identified a mutation in the *MATR3* gene causing a F115C missense change [33▪▪]. Analysis of a further 108 fALS cases identified a T622A mutation in a 66-year-old Sardinian and a first cousin who presented with

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rapidly progressive ALS at 64 years of age. Analysis of 96 British ALS cases identified a P154S variant in a sALS case. Further large screens of European, Canadian and Australian ALS cohorts identified three variants in *MATR3* including a V394M missense mutation and two splice-site mutations [34–36], whereas a Taiwanese study of ~200 cases found just one mutation (A72T) in a patient with bulbar-onset ALS [37]. Overall, MATR3 mutations may account for less than 1% of ALS. Interestingly, there is clinical heterogeneity in the phenotype associated with *MATR3* mutations, with some cases having very slow progression longer than 15 years, or clinical and electromyographic findings more in keeping with distal myopathy with vocal cord paresis [38–40].

Matrin 3 is a 125-kDa nuclear matrix protein that binds DNA and RNA. It remains unclear how ALS-linked mutations might cause disease, with initial pathological and cellular studies showing little or no mislocalization of matrin 3 [33▪▪,41]. There is some evidence that TDP-43 and matrin 3 may interact [42] but more work is needed to determine if *MATR3*-ALS is indeed a TDP-43 proteinopathy.

TUBA4A

Identification of *TUBA4A* (encoding the Tubulin, Alpha 4A protein) as a candidate gene associated with fALS is intriguing as it further implicates cytoskeletal structural dynamics in ALS pathogenesis [43▪▪]. Exome sequencing and rare variant analysis of a discovery cohort identified five nonsynonymous *TUBA4A* changes within exon 4. These changes included four missense variants – R320C/H, R215C, A383T and a nonsense mutation W407X. Four of these mutations were predicted to be deleterious, whereas three nonsynonymous changes found in 4300 controls were predicted to be benign. A replication study revealed an excess of *TUBA4A* mutations among patients and identified a T145P variant that segregated with disease. With the exception of A383T and G43V, no ALS-associated *TUBA4A* mutations were seen in 13 023 controls. Clinically, patients with *TUBA4A* mutations display spinal-onset ALS with both upper and lower motor neuron features with a low prevalence of FTD. Time will tell how common a cause of ALS *TUBA4A* mutations are.

In-vitro studies suggested that *TUBA4A* mutations confer a dominant-negative property on the protein. Mutant TUBA4A is inefficient at forming α-tubulin/β-tubulin dimers and poorly incorporates into microtubules *in vitro*. Variants also inhibit the general assembly of the cellular microtubule network and thus reduce structural stability. TUBA4A^{W407X} can form aggregates and therefore may sequester other tubulin binding proteins or impair cellular proteostasis.

CHCHD10

Bannwarth *et al.* [44▪▪] described a large kindred with slowly progressive ALS, FTD, cerebellar ataxia, mitochondrial myopathy and mitochondrial DNA deletions. Failing to identify mutations in genes normally associated with mitochondrial disease, they undertook whole exome sequencing and identified a missense mutation in *CHCHD10* (c176C>T; p.S59L), which encodes the coiled-coil helix domain-containing protein 10. This same mutation was also identified in another kindred with classical ALS–FTD, with or without Parkinsonism. CHCHD10 is a mitochondrial protein located in the intermembrane space and enriched at cristae junctions. Both endogenous and overexpressed CHCHD10^{S59L} altered mitochondrial cristae ultrastructure and caused fragmentation of the mitochondrial network. Respiratory chain deficiency was also observed, suggesting that CHCHD10 is critical for maintaining ATP production and oxygen consumption [44▪▪]. Mitochondria from *CHCHD10* mutant fibroblasts also showed poor genome repair after oxidative stress [45].

Subsequent studies have identified *CHCHD10* mutations in European populations in association with a variety of phenotypes, mostly ALS and FTD, but also Charcot–Marie–Tooth type 2 and spinal muscular atrophy [46–55]. In these populations, *CHCHD10* mutation accounts for less than 1% of ALS and FTD. Interestingly, studies from China suggest that although *CHCHD10* mutations are also rare in ALS, they may be a more significant cause of FTD, perhaps accounting for nearly 10% of cases in this population [56–58].

NEK1

Another minor hit from the exome sequencing study of Cirulli *et al.* [1▪] was dominant LoF mutations in *NEK1*. More recently, this gene was implicated in a separate whole exome sequencing study of 265 fALS index cases and 827 control individuals [59]. More screens will help determine how common a cause of ALS and/or FTD *NEK1* mutations are, but it is notable that initial studies suggest that NEK1 interacts with two known ALS proteins, Alsin and VAPB [1▪].

Other studies have shown that the C21orf2 protein can interact with NEK1. C21orf2 affects cell proliferation after DNA damage. DNA repair was

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shown to be less efficient in C21ORF2-depleted cells and homologous recombination was impaired. This deficit could be rescued by the overexpression of NEK1 [60]. NEK1 is a member of the NIMA (never in mitosis A) related kinase family of serine/threonine kinases involved in the early cellular response to genotoxic stress and plays an important role in preventing cell death induced by DNA damage [61,62]. Foci of damaged DNA in NEK1 null cells remain after removal of a toxic insult, and NEK1 null cells develop unstable chromosomes at a rate much higher than identically cultured wild-type cells [63]. NEK1 also plays a role in mitochondrial function regulating a pathway of mitochondrial cell death through phosphorylation of voltage-dependent anion channel 1 (VDAC1) on serine 193 [64].

LATEST DEVELOPMENTS IN C9orf72

The *C9orf72* gene remains the most lucrative area of research in ALS right now. The astounding progress in our understanding of *C9orf72* biology is due to the novelty of the gene mutation, an intronic hexanucleotide repeat expansion (HRE) and myriad questions regarding its mode of toxicity; the frequency of the mutation, which accounts for 22–61% of fALS, 6–19% of sALS and around 25% of familial FTD in European populations; the fact that *C9orf72* mutation is associated with TDP-43 pathology, the hallmark protein of ALS and half of FTD cases and the fact that *C9orf72* links both ALS and FTD, which are on a clinicopathological spectrum. The history and progress of 'C9-ALS–FTD' have recently been well reviewed elsewhere [65–67], but a few points are worthy of note.

(1) Although the debate over protein (dipeptide repeats, DPRs, generated by RAN translation) and RNA toxicity (RNA foci, G-quadruplexes, sequestration of RNA-binding proteins) in C9-ALS–FTD continues, mouse knockout studies have shown that loss of *C9orf72*, while not obviously toxic to the nervous system, can cause severe immune dysregulation and lymphoma [68–70]. One study suggested a role for C9orf72 in autophagy [71]. It has therefore been suggested that human *C9orf72* knockdown therapies should specifically target the HRE-containing allele to avoid the potential effects of complete loss of C9orf72.
(2) The recent development of novel antibodies directed against the short and long isoforms of the C9orf72 protein is welcome [72▪▪]. These tools demonstrated that the long isoform is present in the cytoplasm *in vivo*. The short isoform localizes to the nuclear membrane, but in ALS patients it seems to relocate to the plasma membrane, and in these cells TDP-43 mislocalizes as well [72▪▪]. These results are intriguing given recent interest in nucleocytoplasmic transport disruption in models of C9orf72 mutation (reviewed in [27]). More experimental tools are needed to elucidate the normal functions of C9orf72 protein, whose biology remains poorly understood.
(3) Novel transgenic mouse models have recently been developed, which variably recapitulate features of ALS [73–76,77▪▪]. One BAC-transgenic model in particular develops physical and pathological phenotypes relevant to human disease, including, importantly, TDP-43 proteinopathy [77▪▪]. Intriguingly, this model found that antisense transcripts were better correlated with disease than sense transcripts. These models may prove valuable in developing biomarkers and therapies for ALS–FTD.
(4) Oligogenicity in ALS is a topic of growing interest and appears to be more common in fALS than sALS [78]. One particularly interesting example is the coexistence of intermediate expansions in *ATXN2* with the *C9orf72* HRE [78–80]. It has been suggested that ATXN2 expansion may push *C9orf72* HRE carriers toward an ALS or ALS–FTD phenotype rather than one of the many other phenotypes associated with the mutation. Further genotype–phenotype studies are warranted, as determining how oligogenicity in ALS modifies phenotypes will no doubt lead to important insights into disease pathogenesis and yield credible therapeutic targets.

CONCLUDING REMARKS

Significant genetic advances continue to be made in ALS as we have summarized above. These clues implicate almost every area of cell biology, in particular proteostasis, RNA processing and the neuronal cytoskeleton. Having focussed our attention on these new genes, and advances in *C9orf72*, we have not had an opportunity to describe a number of other intriguing advances made in our understanding of established ALS genes. These include a potential role for TDP-43 in cryptic exon splicing [81], hydrogel studies of hnRNPs, DPRs and FUS, which show that ALS mutations can impair proteostasis and axonal local translation [82–85], the characterization of the first SOD1 ENU mutant knock-in mouse that develops a motor phenotype [86] and a large Turkish study identifying a number of intriguing new rare variants in ALS cases [87]. Thanks to project MinE (www.projectmine.com)

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and other gene-hunting projects, we are set to identify yet more ALS-causing genes. These genetic discoveries clearly indicate the complexity of the mammalian motor system and how it can be made to suffer in so many different ways. However daunting this complexity may seem, our growing knowledge will empower us to accelerate progress toward finding ways to slow and potentially halt the neurodegeneration that occurs in ALS.

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Conflicts of interest

There are no conflicts of interest.

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