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REVIEW

Promising animal models for amyotrophic lateral sclerosis drug discovery: a comprehensive update

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ABSTRACT

Introduction: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of motor neurons. Several animal models have been generated to understand ALS pathogenesis. They have provided valuable insight into disease mechanisms and the development of therapeutic strategies.

Areas covered: In this review, the authors provide a concise overview of simple genetic model organisms, including C. elegans, Drosophila, zebrafish, and mouse genetic models that have been generated to study ALS. They emphasize the benefits of each model and their application in translational research for discovering new chemicals, gene therapy approaches, and antibody-based strategies for treating ALS.

Expert opinion: Significant progress is being made in identifying new therapeutic targets for ALS. This progress is being enabled by promising animal models of the disease using increasingly effective genetic and pharmacological strategies. There are still challenges to be overcome in order to achieve improved success rates for translating drugs from animal models to clinics for treating ALS. Several promising future directions include the establishment of novel preclinical protocol standards, as well as the combination of animal models with human induced pluripotent stem cells (iPSCs).

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disorder affecting approximately 4–6 persons per 100 000 worldwide, with only 20% of patients living for five years or more after diagnosis [1,2]. ALS is characterized by a progressive degeneration of motor neurons in the spinal cord and the brain [3]. These clinical manifestations lead to progressive muscle denervation, muscle weakness, muscle atrophy, paralysis and ultimately death. Considerable diversity exists within the disease, with heterogeneity of initial presentation, spreading of disease, progression rates, and survival [4]. Indeed, in addition to motor symptoms, ALS patients can also experience non-motor symptoms including cognitive and/or behavioral impairment, including degeneration in the brain extending to interneurons in frontotemporal lobar degeneration (FTLD) [5,6]. The initial manifestation of ALS varies; some patients present symptoms originating in the spinal region, such as muscular weakness of the limbs (limb onset), while others present symptoms originating in the bulbar region (bulbar onset), characterized by difficulties in speaking and swallowing, known as dysarthria and dysphagia.

The majority of ALS cases (approximately 90%) are sporadic (sALS), and the remaining 10% of cases are familial (fALS). Mutations in more than 40 of them have been associated with ALS and the most common mutated genes are superoxide dismutase-1 (*SOD1*), chromosome 9 open reading frame 72 (*C9ORF72*), fused in sarcoma (*FUS*), and TAR DNA-binding protein (*TARDBP*) (see reviews [7,8]). Several animals have been used to model the major genetic cases of ALS, and they have been instrumental in our mechanistic understanding of ALS and for drug discovery. There are several pathologic mechanisms such as excitotoxicity, oxidative stress, protein misfolding and aggregation, axonal transport dysfunction and degeneration, neuroinflammation, mitochondrial dysfunction, altered RNA processing, endoplasmic reticulum stress and dysregulated nucleocytoplasmic transport that have been associated with ALS onset and progression. Interestingly, at the cellular level, the cytoplasmic mislocalization and aggregation of TAR DNA-binding protein 43 (TDP-43) has been reported in 98% of all ALS cases. Abnormal TDP-43 aggregation results in proteasomal dysfunction, stress granule formation, cryptic splicing, mitochondrial dysfunction and altered RNA metabolism including cryptic splicing-polyadenylation.

Out of over 80 human clinical trials, only riluzole [9], edaravone [10] and more recently Sodium phenylbutyrate and taurursodiol combination [11] modestly slowed disease progression. Moreover, oxidative stress and excitotoxicity are the only two nonspecific pathways targeted by these FDA-approved drugs. Since 2023, the FDA-approved Tofersen (also known as BIIB067, QALSODY), an antisense oligonucleotide (ASO) drug, is indicated for the treatment of ALS in adults

who have the mutation in *SOD1* and proved to reduce the amount of SOD1 protein [12,13]. The success of Tofersen suggests that this therapeutic strategy holds great promise, particularly for correcting other ALS-related mutations ([14] for review). Given the rarity of each genetic variant and the unknown causes of most ALS cases, there is still an urgent need to identify disease-causing pathways to develop better treatment targets for this complex motor neuron (MN) disorder.

Translation of findings from animal models to therapy have largely been focused on the discovery of small molecules targeting pathogenic pathways in ALS, such as excitotoxicity, oxidative stress, mitochondrial function and neuroinflammation. However, over the past decade, novel techniques, such as ASOs, have been developed to target disease-causing mutations and/or restore gene expression (e.g. *STMN2*). In this review, we will focus on the animal models created over the years by the scientific community to study ALS and for drug discovery. We will highlight how the so-called simple models, and more complex models, have been a springboard toward the identification of several drugs with therapeutic potential for this complex disease.

2. Genetics of ALS

Most ALS cases (90%) are of sporadic origin (sALS), appearing spontaneously with no family history, while the remaining 10% are familial (fALS) involving causative genetic mutations. The study of these familial forms has revealed numerous mutations, mainly transmitted in an autosomal dominant mode, and affecting more than 50 genes, involved in the triggering of ALS [2]. In this review, we will focus on the four causative gene responsible for the majority of fALS cases: *SOD1*, *TARDBP*, *FUS* and *C9ORF72*. Apart from those genes that have been clearly implicated in ALS, there are other potentially causative and susceptibility genes that we will not discuss further (see [15] for review).

The GGGGCC hexanucleotide repeat expansion (HRE) mutation in the 5' non-coding region of *C9ORF72* (encoding chromosome 9 open reading frame 72) is responsible for most ALS cases, as it has been identified in ~ 40% and ~ 5% of familial and sporadic cases respectively [16,17]. This HRE comprises 2–20 tandem copies in healthy conditions but is expanded to

hundreds or thousands copies in C9ORF72 patients. Repeat expansion was first associated with reduced *C9ORF72* gene expression [18], suggesting a haploinsufficiency mechanism. Two other disease mechanisms of toxic gain-of-function were then proposed [19,20], associated with 1) toxicity from aggregation-prone dipeptide-repeat proteins (DPRs) translated from the GGGGCC repeat region in by a start codon-independent RAN translation manner and 2) neurotoxic RNA foci nucleated by the GGGGCC repeat. The function of C9ORF72 protein is still unknown, but a role in autophagy and vesicular trafficking has been proposed based on sequence homology with the DENN family of regulators of endocytosis [21,22].

A toxic gain-of-function mutation in the Superoxide dismutase 1 (*SOD1*) gene was found in ~ 20% of fALS and ~ 1–7% of sALS [23]. Most of *SOD1* mutations are missense mutations, but deletions and insertions have also been reported. The main physiological function of *SOD1* is to protect cells against oxidative damage [24,25]. To date, it is still unclear how mutations in *SOD1* lead to ALS. It has been proposed that *SOD1* mutations can result in either a detrimental loss of its enzymatic activity or gain of toxic function due to protein misfolding and aggregation. Mechanistically, excitotoxicity, mitochondrial dysfunction, axonopathy, apoptosis, microglial activation and endosomal trafficking have been reported in ALS SOD1 models [26–29].

Mutations in TAR-DNA Binding Protein (*TARDBP*) are responsible for ~ 4–6% of fALS and ~ 1.5% of sALS cases [30]. This gene encodes for TDP-43, a highly conserved and ubiquitously expressed DNA/RNA-binding protein [31]. TDP-43 is involved in many steps of RNA processing, including transcription, splicing, transport, translation and stress granules formation [32]. Over 50 different dominant heterozygous mutations have been found, mainly affecting the C-terminal domain containing the nuclear localization signal sequence. Mutations in *TARDBP* result in ubiquitin-positive inclusions of TDP-43 protein, predominantly observed in the cytoplasm of neurons and glial cells, and correlate with nuclear depletion of TDP-43 [33,34]. To date, it remains unclear whether loss or gain of function, or a combination of both, is responsible for the pathophysiology of TDP-43.

Finally, mutations in the Fused in Sarcoma (*FUS*) gene account for around 5% of fALS and < 1% of sALS [35,36]. Like TDP-43, FUS is an DNA/RNA binding protein. Mutations in *FUS*, mostly located in the exon 15 which encodes for the nuclear localization signal at the C-terminal region of the protein, are known to cause its redistribution in the cytoplasm and accumulation of protein inclusions [37–40]. ALS-associated FUS mutations have been reported to cause defective DNA damage repair and RNA splicing [41,42].

The identification of these mutations has led to the development of animal models of ALS, with the aim of understanding the physiological and molecular mechanisms underlying the disease.

3. Simple model organisms of ALS

Simple animal models such as worms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), and zebrafish (*Danio rerio*) offer several advantages. They have relatively simple genomes compared to mammals, making them highly amenable to

genetic manipulation. Their short generation times, small size and cost-effectiveness facilitates their maintenance. Simple model organisms are particularly suited for high-throughput screening of chemical libraries, as embryos and larvae can be accommodated in 96-well microplates, and drugs can be easily administered at appropriate developmental stages. Overall, simple animal models offer valuable tools for studying basic biological processes, modeling human diseases, and identifying potential therapeutic.

3.1. C. elegans ALS models

Caenorhabditis elegans (*C. elegans*) is a non-parasitic nematode that is approximately one millimeter long in the adult, with an average lifespan of 2–3 weeks. The nervous system of the worm consists of 302 neurons in the hermaphrodite, and neuronal connectivity has been well mapped and described [43–45]. Indeed, neurons have been found to communicate via approximately 6400 chemical synapses, 900 gap junctions, and 1500 neuromuscular junctions [46] and use most of the known neurotransmitters in the mammalian nervous system. Full genomic sequence comparisons have shown that approximately 35% of the *C. elegans* genome has an orthologous equivalent in humans [47]. *C. elegans* is a valuable genetic model because it is amenable to a wide range of genetic manipulations including forward genetic screens, reverse genetic RNAi screens, rapid mutation mapping, and transgenesis. *C. elegans* assays are fast, low cost, suitable for high-throughput analysis, and genetically tractable with a fully sequenced genome, making the worm an excellent model to study ALS and other neurological diseases [48] (Figure 1).

Human *SOD1* and *C. elegans sod-1* are highly homologous, sharing 71% protein similarity [49]. The toxic gain-of-function have first been observed in transgenic *C. elegans* by introducing human wild-type and ALS-related mutant forms. Overexpression of disease-associated *SOD1* mutations (A4V, G73R and G93A) under the control of a muscle promoter (*unc-54*) in *C. elegans* resulted in low toxicity, including mild cellular dysfunction and protein aggregates with distinct morphological characteristics [50]. Another overexpression *C. elegans* model with pan-neuronal expression of *SOD1*^{G85R} (from the snb-1 promoter) display insoluble SOD1 aggregates in neurons which are associated with locomotor defects, reduced axonal length and impaired neuronal transmission [51,52]. More recently, overexpression of human *SOD1*^{G93A} (from the unc-25 promoter) in the worm's motor neurons also leads to SOD1 aggregates, axon guidance failure, and motor defects [53,54]. Later, another *SOD1*^{G93A} model was described as associated with the same motor deficits, in addition to a reduced lifespan [55].

The only model of *sod-1* deletion in *C. elegans* reports no effect on survival, only on fertility and resistance to oxidative stress [56]. Recently, single-copy ALS SOD1 knock-in models in *C. elegans* have been used as a different genetic approach. Besides modeling the main characteristics of ALS, these models enabled discrimination between the effect of gain and loss of SOD1 function on cholinergic and glutamatergic degeneration [49].

Human TDP-43 and *C. elegans tdp-1* share 57% protein similarity. *C. elegans* with pan-neuronal expression of *TARDP* mutants (G290A, A315T, Q331K, M337V) exhibit neurotoxic features including uncoordinated locomotor phenotypes and abnormal motor neuron synapses including defasciculation of GABAergic motor neurons [57,58]. These phenotypes were particularly correlated with the identification of calcineurin-dependent phosphorylated protein accumulation [59]. Interestingly, loss-of-function mutations in the *C. elegans* TDP-43 homolog *tdp-1* (*tdp-1(ok803)* or *tdp-1(ok781)*) do not induce motor and neurodegenerative phenotypes, but rather increase sensitivity to DNA damage and oxidative stress [60–63]. The expression of the mutant TDP-43^{A315T} in GABAergic motor neurons results in age-dependent motility defects, cytoplasmic insoluble aggregates, neurodegeneration and increased endoplasmic reticulum (ER) stress. These findings recapitulate important features of ALS pathogenesis [64–66].

C. elegans models have also been used to study ALS-associated mutations. *fust-1* shares 38% identity and 56% similarity with human *FUS*. Pan-neuronal overexpression of human *FUS* mutations (S57Δ, R514G, R521G, R522G, R524S and P525L) in *C. elegans* results in cytosolic FUS aggregates, locomotor impairment, synaptic dysfunction, reduced lifespan [65,67]. FUS C-terminal pathological form (FUS501) overexpression in worm also leads to impaired synaptic ultrastructure of GABAergic motor neurons and neuromuscular junction (NMJ) vesicles, reduced postsynaptic currents [68]. This elegant study done in *C. elegans* implicates a role of FUS in the organization of synaptic vesicles and synaptic transmission at the NMJ. The first single-copy transgenic human FUS nematode (S57Δ) results in age-dependent paralysis and hypersensitivity to acetylcholinesterase inhibitor, treatment suggesting dysfunction in the GABAergic system [69]. A more recent study reported the creation of a novel knock-in model in worm (R524S and P525L mutations). These mutations are associated with stress-induced locomotor defects, impaired neuronal and muscle autophagy and neuromuscular dysfunction [70]. A deletion model *fust-1 (tm4439)* has also been described. It is characterized by motor deficits and GABAergic motor neuron degeneration [71].

Finally, *alfa-1*, the conserved homolog of C9ORF72 in *C. elegans*, allowed the research community to model all three pathways caused by the repeat expansion in the gene. Deletion mutations in *alfa-1* result in motility defects leading to an age-dependent paralysis, impaired nuclear transport, increased stress sensitivity, degeneration of motor neurons [72] and dysregulation in endolysosomal homeostasis [73]. From a translational perspective, this result is important because it confirms the functional similarity of *alfa-1* to its human counterpart C9ORF72 [74] and thus the model's relevance. In addition to modeling C9ORF72 loss-of-function, the introduction of C9ORF72 hexanucleotide repeats or DPRs also cause neurotoxicity and motor impairments. For instance, the transgenic *C. elegans* expressing 75 G_4C_2 repeats developed a shortened lifespan, locomotor defects and distinct dipeptide repeat protein aggregates [75]. Muscle expression of G_4C_2 repeats (from 5 to 120) under the *myo-3* promoter [76] in worms leads to the formation of RNA foci. Similarly, pan-muscular expression of certain DPRs accelerates paralysis [77].

Overall, the *C. elegans* model offers a wide variety of genetic tools that can be adapted to the genetic diversity of ALS pathology. However, it has several limitations, especially in phenotype analysis related to ALS pathophysiology. The

short life cycle of *C. elegans* allows for large-scale, high-throughput *in vivo* drug screening studies – as discussed in the next section. However, it does not permit to reproduce the progression of neurodegenerative symptoms that characterize ALS disease, in particular the degeneration of motor neurons leading to progressive motor phenotypes, and the slow onset of abnormal protein conformations and inclusions. Neurotransmission is simplified, and the worms lack a conserved neuroinflammatory system. The model cannot be used to study neuroinflammation, which is a major contributor to ALS disease.

3.2. Drosophila ALS models

The fruit fly *Drosophila* was introduced as an animal model in the early 20th century and has since become one of the most widely used models in biology ever since [78]. Its small size, ease of breeding, short life cycle (10 days from fertilized embryo to adult fly), and the power of available genetic tools [79] make it an ideal model for studies in biology. Complete genome sequence comparisons have shown that around 75% of the *Drosophila* genome has an orthologous equivalent in humans [80–82]. Its nervous system contains approximately 100,000 neurons [83], whose synaptic transmission uses mechanisms similar to those found in humans [84]. Among the genetic tools most commonly used in *Drosophila*, the most important are: chemical and insertional mutagenesis, the inducible UAS/GAL4 system [85], the temporal and regional gene expression targeting (TARGET) method, allowing precise control of Gal4 system [86]. A genomic-comprehensive transgenic RNA interference (RNAi) library has been successfully used to identify genetic modifiers associated with human disease [87]. More recently, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats with associated Cas 9 protein (CRISPR/Cas9) have been successfully employed in *Drosophila*. Several fly models have provided new insights into the cellular and molecular pathways underlying ALS progression (see [88,89] for review) (Figure 1).

The first *Drosophila* models of ALS were created shortly after the identification of SOD1 as the main causative gene. The first study reported shortened lifespans and infertility in *Drosophila* SOD1 (*dSod1*)-null animals [90]. This phenotype is notably rescued by overexpression of human SOD1 in adult motor neurons [91]. Later, flies expressing the human *SOD1* mutation (A4V or G85R) [92] exhibit motor impairment, synaptic dysfunction and accumulation of human SOD1 protein in motor neurons, as well as increased heat shock proteins (HSP) in glial cells. Interestingly, expression of the human *SOD1*^{G85R} in specific cell types, either motor neurons or glia, shortened *Drosophila* longevity when exposed to ALS-linked environmental insults [93,94]. Interestingly, the expression of *SOD1*^{G93A} in *Drosophila* thoracic muscles causes ALS phenotype comprising motor behavior defects, decreased lifespan, and causes mitochondrial pathology [95].

The knock-in *Drosophila* model for *SOD1* (G37R, H48R, H71Y, and G85R) results in a strong lethality [96]. Escaper adult flies show severe locomotion defect, muscle atrophy and denervation [96]. At the NMJ level, *dSod1*^{G85R} adult mutants are characterized by BMP ligand-dependent reduction in bouton number, mEPSP frequency, and muscle capacitance and locomotion defect [97].

Deletion mutations in the fly *TARDBP* ortholog *TBPH* results in larval lethality and reduced lifespan in adults escapers, as well as locomotion impairment [98–102]. At the level of the NMJs, some groups observed a reduction in the number of axonal branching and synaptic boutons [98,103], or an increase in synaptic boutons [100], and impaired synaptic transmission [101]. Overexpression of both *TBPH* and *TDP-43* in *Drosophila* results in toxic phenotypes with a reduced lifespan and age-progressive locomotor defects and NMJ degeneration [99,101,104–110].

Similarly to TDP-43, expression of wild-type and mutant human *FUS* (R524S or P525L) in eyes results in progressive retinal degeneration, associated with axonal loss [111–113]. Gain-of-function mutants have been shown to be either lethal when *FUS* is ubiquitously expressed [114,115], or associated with reduced lifespan when pan-neuronal [112,113]. At the NMJ synaptic level, few phenotypes have been described in flies : a reduction in the number of synaptic boutons, associated with functional deficits, increased apoptosis in motor neurons, synaptic transmission impairment and disturbed locomotion [111,114,116,117]. Several studies have hypothesized a role for FUS in degeneration, notably through reduced mitochondrial transport [118,119]. Characterization of *cabeza* (*caz*) mutant, the fly homolog of FUS, reveals a key function for the gene in neuronal development, which is not responsible for maintaining adult neuronal function [120–122].

The study of the *C9orf72* gene in Drosophila model has focused on gain-of-function models, due to the absence of an ortholog. The toxicity of RNA foci nucleated by the GGGGCC repeat has first been modeled in *Drosophila*. These studies showed that the expression of expanded repeat RNA is sufficient to cause neurodegeneration in the eye and motor neurons [123]. For instance, the expression of a $(G_4C_2)_{48}$ RNA repeat in *Drosophila* recapitulates ALS-associated phenotypes such as locomotion defect and branching defects [124]. Interestingly, a Drosophila model expressing 160 G_4C_2 repeats flanked by human intronic and exonic sequences formed nuclear RNA foci in glia and neurons [125].

Since then, the *Drosophila* model has been used extensively to assess the pathogenicity of C9ORF72-associated ALS (see [126] for review). C9ORF72 DPRs toxicity has also been studied in *Drosophila*. Flies expressing arginine-rich dipeptide-repeats [127], specifically in glutamatergic neurons [128], shows toxicity-related phenotypes and severe neurodegeneration, whose underlying mechanisms have been investigated in several studies [129–134]. Pan-neuronal expression of DPRs leads to neurodegeneration and cell death in the *Drosophila* central brain, and causes age-related motor impairment and neurodegeneration [135]. At the synaptic level, the expression of arginine-rich dipeptide-repeats leads to a decrease in the number and function of neuromuscular synapses, associated with reduced muscle size, and locomotion defects [136,137]. Even more recently, the specific expression of polyGR in *Drosophila* muscle [138] resulted in morphological and functional mitochondrial defect.

Species / homology to human genome	C9ORF72	SOD1	TARDBP	FUS
D. melanogaster >40%	GOF, expression of expansion repeats : 30 (G4C2), 48 (G4C2), 160 (G4C2) UAS- 8,28,58 (G4C2) DPRs 50 repeats, GR 80 repeats	LOF : Knockout GAL4/UAS-induced overexpression of WT and mutants: 1A4V, G85R, G37R, H48R, H71Y, G93C G41D, I113T	LOF : Knockdown, RNAi 38377,38379 GAL4/UAS-induced overexpression of WT and mutants: D169G, G298S, A315T, M337V, N345K, G287S, G348C, A382T, N390D,Q367X	LOF : Knockout GAL4/UAS-induced overexpression of WT and mutants: R518K, R521C, R521G, R521H,R524S, P525L
C. elegans >60%	LOF : Deletion (ok3062) GOF, expression of expansion repeats : 75 (G4C2), 5-120 (G4C2), 29 (G4C2) DPRs 50 repeats	Stable transgenic mutants: G85R,G93A, G37R,G127X, 1A4V, H71Y, L84V	LOF: Deletion (ok803,ok781) Stable transgenic mutants: G290A, A315T, A382T, M337V, Q331K, G348C	LOF : Deletion (tm4439) Stable transgenic mutants : R495X, R514G, H517Q, R521G, R522G, R524S, P525L, FUS513, FUS501, S57Δ
D. rerio >70%	LOF : morpholino knockdown, miRNA knockdown GOF, expression of expansion repeats : 35,38,70,72,89,90 (G4C2) DPRs 40,80, 200,1000 repeats	Overexpression of mutants (mRNA): G93A, G37R, A4V Stable transgenic mutants: G93R, T70I	LOF : morpholino knockdown, knockout Overexpression of mutants (mRNA): A315T, G348C, A382T Stable transgenic mutants: A382T	LOF : morpholino knockdown, deletion Overexpression of mutants (mRNA): R521H, R521C, S57Δ, H517Q, R521G Stable transgenic mutants: R521H, R521C
M. musculus R. norvegicus 99%	LOF : Knockout (rat, mouse) GOF, expression of expansion repeats : 66, 80, 450, 500, 100-1000 (G4C2) 80 (G4C2) (rat)	Stable transgenic mutants: 1A4V, G93A, G37R, G46R, G48Q, G86R, G85R, D90A, G127X G93A, H46R (rat)	Stable transgenic mutants: A315T, M337V, G298S, Q331K, G348C, F210I M337V (rat)	LOF : Knockout Stable transgenic mutants: R521G R521C (rat)

Figure 1. Overview of simple genetic models (zebrafish, *C. elegans* and *Drosophila*) and rodent models that recapitulate ALS pathology. Abbreviations: LOF, loss of function; GOF, gain of function.

Drosophila has played a key role in the modeling of ALS. However, as with *C. elegans*, the progressive onset of different motor phenotypes or pathophysiological markers of the disease cannot be accurately described due to the relatively short life cycle. In the absence of a C9ORF72 ortholog, current fly models are limited to the analysis of HRE-induced toxicity gain. The limited selection of motor and behavioral tests is another limitation. As a result, eye degeneration has been and is still commonly utilized as a phenotypic marker in the model. We want to stress the importance of thoroughly confirming the specificity of this phenotype in genetic analysis to avoid misleadingly suggestive induced toxicity.

3.3. Zebrafish ALS models

Zebrafish have become increasingly popular as a simple verte-brate model organism for studying vertebrate biology, phy-siology and human disease due to several advantages it offers [139–142]. Zebrafish shares approximately 70% of human genes, with a high degree of identity conservation [143,144]. Zebrafish embryos develop ex utero, so rapidly that at 3 days post fertilization (dpf), embryogenesis is complete, reaching adulthood at 3 months post fertilization (mpf). The transpar-ency of zebrafish embryos facilitates imaging and makes observation of organ development, cell migration, and ALS-relevant pathology possible. Zebrafish stereotypes movement patterns enable behavioral tests to examine changes in motor activity. In particular, it offers many advantages for the study of neuromuscular diseases [141], with the increasing develop-ment of new methods for pharmacological screening and quantitative analysis of the neuromuscular junction [145,146].

Finally, one of the main advantages of the zebrafish model is the ease of genetic modification, fostered by the sequencing of the entire zebrafish genome in 2013. The genetic loss-of-function includes the expression of dominant negative con-structs by (i) antisense morpholino oligonucleotides (AMO) transient knockdown of gene expression [147] or (ii) knockout using several genome editing methods such as ZFNs, TALENs and CRISPR-Cas9 (see [140,148] for review). The genetic gain-of-function includes (i) injecting blastocysts with mRNA for global expression or (ii) by driving expression in small sets of neurons from DNA constructs using neural-specific or induci-ble genomic regulatory elements (e.g. the UAS/Gal4 system) [149]or (iii) by CRISPR/Cas9 knock-in of point mutations [150–152] and more recently by using CRISPR-based cytosine base editors [153]. Several ALS-causing genes have been studied in zebrafish using loss- or gain-of-function approaches (Figure 1).

Transgenic *sod1* zebrafish larvae expressing mutants (G93R, G93A) exhibit abnormal NMJ and mitochondrial dysfunction, while adults show a progressive locomotor decline, altered mus-cle respond and motor neuron loss [154,155]. Co-injection of three SOD1 mutants (G93A,G37R,A4V) induces a motor axono-pathy specific for motor neurons in zebrafish larvae [156]. Another group reported comparable results, including increased sensitivity to oxidative stress, in a transgenic zebrafish line expressing the missense T70I *sod1* mutation [157]. At the func-tional level, a study conducted on the transgenic SOD1[G93R] zebrafish model has allowed elucidating the role of interneuron dysfunction in the early stages of ALS [158]. In addition to an NMJ loss, the group described a reduced glycinergic transmission (spontaneous miniature glycinergic excitatory postsynaptic cur-rents (mEPSCs)) onto motor neurons of *sod1* zebrafish larvae.In zebrafish embryos, overexpression of either wild-type or mutant (A315T, G348C, A382T) human *TARDBP* with mRNA injection led

to motor axonopathy and locomotor defects [159,160]. At NMJ level, expression of *TARDBP*[G348C] mRNA resulted in impaired neuromuscular synaptic transmission, reduced frequency of mEPCs, reduced quantal transmission and orphaned presynaptic and postsynaptic structures. Interestingly, similar phenotypes were observed upon zebrafish *tardbp* knockdown with an antisense morpholino oligonucleotide, suggesting that both loss- and toxic gain-of-function might be involved in the molecular mechanism of pathogenesis [160]. As for *C. elegans* model, motor impairments in zebrafish expressing mutant TDP-43 (G348C) were partially ameliorated by compounds that mitigate endoplasmic reticulum stress, such as methylene blue and salubrinal [66]. Zebrafish knockout models were then created for the two orthologous *TARDBP* genes (*tardbp* and *tardbpl*) [161,162]. Interestingly, reduced survival, motor and axonal defects described in double knockouts can be compensated with a novel *tardbpl* splice form (Tardbpl-FL). To date, only one CRISPR/Cas9-based knock-in of *FUS*- and *TARDBP*-related missense mutation has been created in zebrafish [151].

The overexpression of human *FUS* mutants (R495X or G515X) leads to disrupted transportin-mediated nuclear import and accumulation in cytosolic stress granules [37,163]. Cytosolic mislocalization and accumulation in stress granules has been also described in cultured zebrafish cells from larvae expressing mutant FUS (R521C) [164].

Transient knockdown of zebrafish *fus* with an AMO were shown to have impairments in locomotor activity with abnormal motor axon projections [165]. Functionally, this model shows increased motoneuron excitability, reduced fidelity of synaptic transmission and quantal content and abnormal NMJ structure [166]. However, the first CRISPR-Cas9-based *fus* knockout zebrafish lack motoneuronal and locomotor defects [167]. Another mutagenesis-generated *fus* knock-out model, summarizes ALS-associated phenotypes such as behavioral deficits, NMJ defects including reduced motor neurons axonal length, and altered tau transcription [168]. Overall, despite these conflicting results, mechanisms involving loss of function or haploinsufficiency are likely relevant to FUS- associated pathology in zebrafish model.

Different zebrafish models that have been developed to study the pathological mechanisms of ALS related to the C9orf72 gene (see [169] for review). Expression of G_4C_2 repeats in *c9orf72* with DNA constructs injections results in toxic RNA foci formation and apoptotic cell death [170]. Sense and antisense RNA repeats induce motor axonopathy in zebrafish larvae [171]. This C9orf72 zebrafish model permitted to decipher a new potential pathogenic mechanism by demonstrating that RNA toxicity and RNA cytoplasmic formation can contribute to the pathogenesis of C9orf72-associated ALS/FTD.

The role of DPR protein toxicity in C9ORF72 ALS has also been demonstrated in the zebrafish using transgenic UAS responder lines forcing the translation of poly-GA or poly-GR protein [172,173]. The sustained expression of G_4C_2-linked DPR proteins increases mortality, impairs locomotion, leads to motor axonopathy, and promotes DPR protein aggregation in zebrafish larvae. Similarly, a stable transgenic zebrafish line expressing C9ORF72-related hexanucleotide repeats recapitulate the motor behavioral deficits, muscle atrophy, motor neuron loss and early mortality [174].

Models of C9ORF72 loss of function or haploinsufficiency have also been established in zebrafish. The transient knockdown with AMO injection [175] or overexpression of a nonfunctional mutant [176] results in motor axonopathy, increased apoptotic death and abnormalities of spontaneous and evoked swimming. Our group recently developed a new loss-of-function zebrafish line using the stable expression of a specific miRNA targeting the 3'-UTR region of *C9orf72* gene [177]. C9-miR fish replicates the hallmarks of ALS as it causes locomotor deficits, motor neuron axonopathy and degeneration, muscle atrophy and TDP-43 mislocalization. At NMJ level, this model showed reduced mEPCs frequency and impaired release of quantal synaptic vesicles. Lastly, a new loss of function zebrafish line was generated by targeting exon 2 using CRISPR/Cas9 [178]. The model lacks a neurogenerative phenotype in the spinal cord but is characterized by neuronal loss in the retina.

Among the three simple animal models selected for this review, the zebrafish stands out as the sole vertebrate and therefore the most complex organism. However, it is important to acknowledge that there are some limitations associated with this model. First, the zebrafish lineage experienced a complete duplication of the genome, making it challenging to model gene silencing due to the presence of multiple orthologs for some human genes. It is necessary to consider the genetic interactions between duplicated genes, as has been shown for the *tardbp* genes [161,162].

4. Rodent models of ALS

Prior to the development of simple models, mouse and rat models have been important tools to better understand the early and late physiological mechanisms of ALS-associated and to evaluate potential therapeutic strategies ([179] for review) (Figure 1).

The first ALS mouse model was created shortly after the discovery of the first *SOD1* causative gene [23]. The dominant gain-of-function mutation SOD1-G93A causes motor neuron disease in transgenic mice [24], which has since been widely used, notably to identify ALS-related cellular changes [180–184]. Symptoms include loss of spinal motor neuron, paralysis, and early death. The different models created express a varying number of copies of the transgene, and the time of onset of symptoms is variable [185–187]. Other mouse models were then developed with human *SOD1* mutation overexpression: G85R [188], G37R [189], G86R [190], D90A [191], H46R [192] and D83G [193]. These mice develop progressive degeneration of lower and upper motor neurons, distal axonopathy, intraneuronal and glial inclusions and muscle wasting and atrophy, sometimes leading to paralysis.

Early descriptions of the first SOD1 KO mouse indicated an absence of motor phenotype. Further studies showed this to be a model of chronic oxidative stress [194]. SOD1 null mouse models are much rarer, but those described later also show progressive and oxidative stress-mediated distal motor axonopathy [195], reduction in muscle mass and oxidative damage in skeletal muscle leading to reduced motor performance [196]. Importantly, rat models overexpressing human *SOD1* mutant (h*SOD1*[G93A]) also display pathological features such

as motor neuron axonal loss, muscle wasting and paralysis of both hindlimbs and one forelimb [197,198].

Many murine models of TDP-43 mutations have been generated. The first models were created by ubiquitous overexpression, under the control of the *Prnp* promoter, of wild-type and mutant (A315T, M337V, Q331K) human cDNA [199–202]. This method has proven to be very severe, with early onset of the motor phenotype, aggregates of ubiquitinylated proteins and premature death, but no or very few TDP-43 inclusions. Conversely, expression of TDP-43 mutants (A315T and G298S) under the control of an endogenous TDP-43 promoter [203] results in ubiquitinylated TDP-43 inclusions, but no paralysis-inducing motor phenotype or reduced life span. The same BAC transgenic approach was used in rats, which rapidly developed paralysis and died after a month [204]. To limit the extremely severe effect, probably due to peripheral toxic effects of the ubiquitous expression, other models of neuronal TDP-43 overexpression driven by the *Thy1.2* promoter have been established [205–207]. The results show a toxic effect of wild-type human protein in phenotypic mice, which is exacerbated by mutant overexpression. Different groups have used an inducible overexpression model at different developmental stages, using the *CaMK2* promoter [208–210]. The mice are characterized by moderate motor neuron loss, progressive motor and cognitive impairment, and slightly earlier death. Finally, conditional expression of the TDP-43 mutant in the brain and spinal cord, through the NEFH (neurofilament heavy chain) promoter, results in an ALS-specific neurodegenerative phenotype with loss of motor neurons, denervation in the NMJ and TDP-43 mislocalization in mice [211] and motor neuron loss, loss of hindlimb grip strength and paralysis in rats [212]. Although rarer, TDP-43 knockout models also exist in murine models. Because complete loss of function is lethal, two groups created a model knocking out TDP-43 specifically in the postmitotic motor neurons [213,214]. This model results in modest loss of motor neurons, astrogliosis and accumulation of ubiquitinated material in motor neurons. The knock-in method has also been used for the *Tardbp* gene, in particular for the M323K mutation, which causes motor neuron death [215], the Q331K mutation which causes cognitive deficits [216], the M337V and G298S mutations, which specifically affect neuromuscular junctions without overt neurodegeneration [217], and the N390D mutation which covers several important phenotypes including TDP-43 aggregation and motor neuron degeneration [218].

TDP-43 plays a crucial role in mRNA splicing of many transcripts and mislocalization of TDP-43 in ALS leads to aberrant splicing of transcripts, foremost *Stathmin2* (STMN2). Cryptic exon inclusion in *STMN2* mRNAs results in a decreased STMN2 protein level in many patients with ALS. Interestingly, *Stmn2±* mice display NMJ denervation and motor deficits [219]. Recently, Baughn and colleagues [220] engineered mice to carry a *Stmn2* gene partially humanized by insertion of the human *STMN2* cryptic splice and polyadenylation sequences but without TDP-43 binding. Using this mouse model, they showed the efficacy of ASOs to correct Stmn2 pre-mRNA misprocessing and restored its protein levels. However, the effects of these ASOs have yet to be tested on functional/behavioral outcomes in animal models. Nevertheless, restoration of STMN2 expression using an ASO (QRL-201) is currently a strategy being tested in a Phase 1 clinical trial (NCT05633459).

As in the case of TDP-43, many FUS mouse models have been designed [221]. Interestingly, only a gain-of-function of the mutated protein (conditional knock-in mutation) mimics ALS phenotypes, with progressive motor neuron degeneration, neuromuscular junction defects, and FUS mislocalization without aggregation [222–225]. Mice with ubiquitous overexpression of human wild-type FUS [226], truncated variant FUS 1–359 under control of the prion *Thy1* promotor [227], and FUS R521C mutation [41] develop such a severe motor and neuroinflammatory phenotypes that premature death generally occurs between 3 and 4 months of age. Overexpression of the FUS R521C mutation also leads to ALS-like phenotypes in rats with motor neuron degeneration, denervation atrophy of skeletal muscle and progressive paralysis [228]. Loss of FUS function, conversely, leads to a pathological and behavioral phenotype that is not characteristic of ALS [229].

Most C9ORF72 knockdown [230] and knockout [231–237] in mice mainly develop an inflammatory phenotype, sometimes associated with a reduced lifespan, but no locomotor deficits or motor neuron degeneration. A more recent study has shown that knockdown of C9ORF72 in mice, using a miR-RNAi sequence targeting a region located in exon 8 of the gene, causes mild strength loss and deficits in the neuromuscular junction and impaired social interaction, but still no motor neuron disease [238]. Interestingly, several studies in mice [239,240] and rats [241] support the hypothesis of a synergistic effect of c9orf72 loss- and gain-of-function mechanisms in promoting ALS pathology. In this sense [239], have shown that loss of C9orf72 and haploinsufficiency exacerbate motor behavior deficits in a dose-dependent manner. Viral G_4C_2 repeat expansion mouse models recapitulate hallmark features including motor and cognitive deficits, TDP-43 inclusions, cortical neuron loss [242,243] and NMJ abnormalities [244]. DPR formation has been described in an inducible gain-of-function model that exhibits locomotor phenotype and muscular dystrophy [245].

Here, we provide a brief overview of the large number of mouse models that have been developed for ALS. This heterogeneity in the phenotypes described, and their onset complicates the selection of the most appropriate model for subsequent translational studies. For instance, the use of the SOD1 model predominates in mouse preclinical studies. It has led to significant progress in describing some of the cellular mechanisms leading to selective MN degeneration. However, these animals do not reproduce an essential marker of pathology, namely TDP-43 inclusions, and show a rapid progression of the disease. These characteristics question their relevance in a preclinical study. Moreover, the development of new knock-in models including humanized mouse, in which the mouse locus is replaced by the human orthologous sequence, is increasingly being used to study ALS. This strategy can generate clinically relevant animal models. However, a humanized mouse gene can have undesirable effect on splicing and phenotypic data must be analyzed with caution.

5. Translational value of ALS animal models for therapy

5.1. Small molecules

In addition to provide a better comprehension of pathogenic mechanisms, the simple ALS models have been used to screen therapeutic compounds [246] (Figure 2). An *in vivo* chemical screening platform for ALS genes, conducted in worms and fish, revealed that methylene blue efficiently attenuated the TDP-43 and FUS phenotypes, notably motor impairments, by promoting a protective ER stress response [64,66]. The efficacy of this compound has since been demonstrated in a mouse model of ALS [247]. Interestingly, methylene blue has been shown to be an effective inhibitor of TDP-43 aggregation in a human cell model. In addition, Vaccaro and colleagues showed that compounds structurally related to methylene blue, such as salubrinal, guanabenz and phenazine, also exhibited potent suppression of mutant TDP-43 protein proteotoxicity [66].

A drug screening in both *C. elegans* and *zebrafish* models ($TARDBP^{A315T}$ and $TARDBP^{G348C}$, respectively) identified a potential therapeutic compound, pimozide, which, after validation in $SOD1^{G37R}$ mice, is currently in clinical trial (NCT03272503) [248]. The pimozide, a derivative-TRVA242 T-type calcium channel antagonist, was shown to increase synaptic transmission at the NMJ, reduce motor defect and rescue pathological phenotypes at the NMJ [248]. Later, another high-throughput screen reported a novel molecule, TRVA242 as effective in restoring motor phenotypes in worms and zebrafish [249]. Additionally, the compound TRVA242 was found to ameliorate morphological and synaptic abnormalities of NMJ in zebrafish and mouse ALS models [249].

The ease of pharmacological phenotype analyses in the $TARDBP^{A315T}$ worm model allowed the selection of the compound α-methyl-α-phenylsuccinimide (MPS) as an effective neuroprotector to ameliorate locomotor defects and reduce GABAergic motor neurons loss [250]. The same worm model $TARDBP^{A315T}$ [65], together with the model $FUS^{S57Δ}$ [69] also allowed the repositioning of the probiotic *Lacticaseibacillus rhamnosus* HA-114 as a neuroprotector restoring motor defects by promoting mitochondrial β-oxidation [251]. The same group proposes an interesting regenerative approach to rescue axonal abnormalities using the poly (ADP-ribose) (PAR) polymerases (PARP) PARP-1 and PARP-2 in the $mFUS^{S57Δ}$ worm model [252]. A screening strategy using the $SOD1^{G93A}$ *C. elegans* model identified novel genetic modifiers that prevent SOD1 protein aggregation and suppress toxicity [253]. The $SOD1^{G93A}$ worm model was pharmacologically studied with the diabetes drug metformin. It proved effective in reducing neurodegeneration and extending life expectancy by upregulating autophagy [55]. Finally, the *C. elegans* model has also been used to validate hits from cell-based screens, such as the compounds LDN-0130436 [254] and PROTAC2 [255], which decreases GABAergic motor neuron degeneration and decrease TDP-43 aggregation, respectively.

A behavior-based drug screening system indiscriminately identified 12 compounds in another *C. elegans* TDP-43 toxicity model not described in the review, dnc-1/dynactin 1 knockdown [256]. This study provides strong support for the use of simple organisms, as among the compounds that improved the motor defects and axonopathy were two FDA-approved drugs for ALS : riluzole and nifedipine [257].

Finally, McGown and colleagues created a neuronal stress reporter line, lying on the increased expression of the fluorescent heat shock stress protein hsp70 following neuronal stress (hsp70-DsRed) [158]. This model was used as an additional *in vivo* model to validate the efficacy of the FDA-approved ALS drug riluzole in stabilizing sodium channels and reducing excitotoxicity, hence providing new insight into a novel mechanism of this treatment [258]. After reporting a new zebrafish model expressing C9orf72 HRE that recapitulates ALS phenotypes, the same group described for the first time and in several other C9orf72 expansion models an abnormal activation of the heat shock response (HSR) that correlates with disease progression. Importantly, ivermectin, like riluzole, reduced HSR activation in both C9orf72 and SOD1 zebrafish models, offering new therapeutic perspectives [174].

The *Drosophila* ALS model was also subjected to pharmacological screening, leading to the identification of the PPARγ agonist pioglitazone as a neuroprotective compound improving locomotor function but not the lifespan of TDP-43-expressing flies [259]. This new compound was then tested in a phase II clinical trial [260]. The results showed that pioglitazone did not produce any significant improvements in terms of slowing the progression of the disease. More recently, α-Lipoic acid was shown to attenuate oxidative stress, neurotoxicity and improve motor activity in the $hSOD1^{G85R}$ fly model [261]. In a recent study using LDS-$(G_4C_2)_{44}$ and $(G_4C_2)_{30}$ transgene constructs in flies, the authors evaluated the ability of three compounds (TMPyP4, PJ34 and KPT-276) to reduce G_4C_2 toxicity which is manifested by reduced lifespan, eye degeneration and motor defects [262]. TMPyP4 and PJ34 are the most potent compounds and ameliorate the disease phenotype by extending lifespan and improving climbing ability. It has previously been reported that TMPyP4 attenuates G_4C_2 repeat-dependent neurotoxicity in Drosophila by suppressing hexanucleotide repeat-mediated nuclear import deficits by modifying the structure of the repetitive RNA. These results represent a major breakthrough in the therapeutic potential of this compound [263].

Mouse model remains one of the most widely used ALS animal models, especially for the preclinical studies. There have been as many preclinical studies on mouse models as there are potential pathophysiological mechanisms for this complex pathology ([264] for review). Here, we review the most significant translational studies resulting from work done on mouse models of ALS (Figure 2).

Shortly after its creation, the mSOD1 model was the subject of a study aimed at reducing oxidative stress using vitamin E (α-tocopherol) [265], which failed to show clinical efficacy [266]. The same conclusion has been reached for other compounds with antioxidant and anti-inflammatory properties [267,268]. More recently, vitamin B12 (methylcobalamin) has been reevaluated for its antiglutamatergic and neuroprotective properties in mice [269]. Its clinical study is still underway and shows promising results in early stage ALS ([270]. The motor performance of SOD1-G93A mouse model was evaluated to assess the effect of

	Organism	Compound	Reference	Clinical trial
Small molecules	*D. melanogaster*	TMPyP4	Zhang et al. 2015	-
		α-Lipoic acid	Wang et al. 2018	NCT04518540 No phase
		TMPyP4,PJ34 and KPT-276	Lee et al. 2024	-
	C. elegans	LDN-0130436	Boyd et al. 2014	-
		MPS	Wong et al. 2018	-
		HA-114	Labarre et al. 2022	NCT06051123 Phase 2
		PARP-1 / PARP-2	Tossing et al. 2022	-
		Metformin	Xu al. 2022	NCT04220021 Phase 2
		PROTAC 2	Tseng et al. 2023	-
	D. rerio	Pimozide	Patten et al. 2017	NCT03272503 Phase 2
		Ivermectin	Shaw et al. 2018	-
		TRVA242	Bose et al. 2019	-
	R. norvegicus	AB1010 (masitinib)	Trias et al., 2016	NCT03127267 Phase 3
	M. musculus	vitamin E (α-tocopherol)	Gurney et al. 1996	NCT04140136 Phase 2
		Lithium carbonate	Shin et al. 2007	2020-000579-19 Phase 2
		Pioglitazone	Kiaei et al. 2005 Schutz et al. 2005	NCT00690118 Failure
		Tamoxifen	Wang et al. 2012	NCT00214110 Failure
		Trehalose	Castillo et al. 2013	NCT05136885 Phase 3
		vitamin B12 (methylcobalamin)	Ikeda et al. 2015	NCT03548311 Phase 3
		IMS-088	Patel et al. 2015 Pelaez et al. 2023	-
		SAR443820	Yuan et al. 2019	NCT05237284 Failure
		Pridopidine	Ionescu et al. 2019	NCT06069934 No phase
		MAP4Ki (PF6260933)	Liu et al. 2024	-
		FP802	Yan et al. 2024	-
ASOs / gene therapy		ATXN2 ASO BIIB105	Becker al. 2017	NCT04494256 Phase 1
		SOD1 ASO BIIB067	McCampbell et al. 2018	Tofersen Approved
		FUS ASO ION363	Korobeynikov et al. 2022	NCT04768972 Phase 3
		C9orf72 ASO WVE-004	Liu et al. 2022	NCT04931862 Failure
		STMN2 ASO QRL-201	Baughn al. 2023	NCT05633459 Phase 1
		AAV9/NF242	Droppelmann et al. 2024	-
Antibody therapy		Tegoprubart (AT-1501)	Lincecum et al. 2010	NCT04322149 Phase 2
		AP-101 (NI005)	-	NCT05039099 Phase 2
		ANX005	Andrews-Zwilling et al. 2022	NCT04569435
		D3-1	Minamiyama et al. 2023	-

Figure 2. Translational value of ALS animal models for therapy. Summary of small compounds and other therapeutic strategies that have been identified in various animal models of ALS and are currently in clinical trials, with the corresponding phase of development.

the main FDA-approved drugs: riluzole [271] and edavarone [272], together with the rat SOD1- H46R model [273]. In the light of riluzole, other compounds aimed at modulating the glutamatergic pathway have been tested in the clinic without much success. A very recent study, partially conducted in the SOD1[G93A] mouse model, used a recent pharmacological principle that, in contrast to classical NMDA receptor (NMDAR) pharmacology, allows selective elimination of extra-synaptic NMDAR-mediated sensing without disrupting synaptic function [274]. The compound, named FP802, which thus selectively inhibits the TwinF interface, is a very promising new therapeutic avenue as it improves motor performance, extends lifespan, and prevents motor neuron loss in SOD1[G93A] mouse model.

Some autophagic enhancers are currently being developed in the clinic, after being validated in mouse models of ALS. We can notably cite the example of Tamoxifen, which has been shown to reduce TDP43 protein aggregation in motor neurons of the FTLD-U mouse model [275].

A very important part of current translational research is focused on the inflammatory component of the pathology [276]. Much of this development involves mouse models of ALS, particularly to test the efficacy of anti-inflammatory treatments such as pioglitazone [277–279]. For example, Pelaez and colleagues show that inhibiting the NF-κB pathway with IMS-088, derived from withaferin A (ImStar Therapeutics Inc.) improves cognitive and motor function, increases motor neuron dendritic branching and restores synapses in a newly generated mouse model hFUS[R521G/Syn1] [280]. Another anti-inflammatory compound, MN-166 (ibudilast), targets several phosphodiesterases and macrophage migration inhibitory factor and is currently in clinical trials (NCT02238626 and a biomarker study NCT02714036). An *in vitro* study has shown that an ibudilast treatment could clear TDP-43 and SOD1 aggregates and prevent TDP-43-induced neurotoxicity in cells [281]. Its efficacy has been demonstrated, particularly in the SOD1[G93A] and FUS[R521C] mouse models, in prolonging survival, reducing spinal glial neuroinflammation and MN degeneration [282].

In a recent study, a high-throughput pharmacological screen using human induced motor neurons (hiMNs) identified a compound (F6260933) that inhibits MAP4 kinases (MAP4ks), previously implicated in neurodegeneration in ALS [283], and is able to improve the survival of ALS hiMNs and their ability to form NMJs [284]. Importantly, a neuroprotective effect was observed *in vivo* in SOD1[G93A] mice after MAPK4 inhibition, with prolonged survival, an increase in the number of motor neurons compared to untreated control mice, and a significant decrease in nuclear mislocalization of TDP-43 protein [284]. A clinical study is currently underway for a similar compound, a MAP4k inhibitor named Prosetin and Phase 1 has been completed (NCT05279755). There are many other examples of therapeutics identified in the ALS rodent models that are currently in clinical trials. Among these, the tyrosine kinase inhibitor masitinib (AB1010), now in clinical study (NCT03127267), has emerged as a promising compound in the SOD1[G93A] rat model, preventing microglia-associated inflammation, particularly the number of aberrant glial cells, in the degenerating spinal cord, extending lifespan and improving motor neuron pathology [285]. Similarly, the compound SAR443820, an inhibitor of receptor-interacting serine/threonine protein kinase 1 (RIPK1), which is involved in neuroinflammation [286], was found to be beneficial in both SOD1[G93A] mice and *Optn*^{−/−} mice (Yuan et al., 2019), preventing oligodendrocyte loss, glial inflammation and axonal degeneration of motor neurons [287]. A phase 2 clinical trial is currently ongoing (NCT05237284).

Pridopidine, a sigma-1 receptor (S1R) agonist, in addition to promoting autophagy, would be able to compensate for TFEB nucleoplasmic transport deficiency in C9orf72-associated pathology [288]. It reduces SOD1 aggregation and ameliorates muscle fiber atrophy in the SOD1[G93A] mouse model [289], making it a pharmacological compound of choice, now in clinical study (NCT06069934).

5.2. ASOs and other gene therapy approaches

Mouse and rat models have played a decisive role in preclinical efforts to develop new therapeutic strategies based on gene therapy, particularly ASO knockdown of SOD1 [290,291] and C9orf72 [292,293]. Among these studies, preclinical data obtained in SOD1[G93A] mouse and rat models provide a strong rationale for SOD1 reduction as a therapeutic approach. Indeed, novel SOD1 ASOs reduced mRNA and protein, extended survival and preserved neuromuscular innervation in treated animals [291]. Following a clinical trial [12], the antisense oligonucleotide tofersen was approved by the FDA in 2023 for the treatment of ALS patients with SOD1 mutations. Encouraged by this success, ASO ION-363 is currently being evaluated in a phase III trial (NCT04768972), for instance for FUS mutations, based on its efficacy in the mouse model [294].

TDP-43 plays a key role in RNA metabolism and other cellular functions. Targeting TDP-43 toxicity as a therapeutic approach is increasingly being explored, including in translational studies using the mouse model. A very recent study shows that genetic expression of a fragment of RGNEF (NF242) or injection of AAV9/NF242 into a TDP-43 overexpressing fruit fly ALS model and a TDP-43 mouse model (rNLS8), respectively, improved lifespan and motor phenotype and reduced neuroinflammation markers. These are promising results for a protein that directly interacts with TDP-43 and acts as a modifier of TDP-43 toxicity *in vivo* [295].

Additionally, depletion of ataxin-2 in animal models, via genetic approaches, reduces TDP-43 toxicity. For instance, inhibition of ataxin-2 in a mouse TARDBP model using ASOs improves motor function and prolongs survival [296]. Recently, it was reported that targeting ataxin-2 using the CRISPR-Cas13 system in a mouse model of TDP-43 proteinopathy significantly improved motor deficits and neuronal survival, increased survival and reduced abnormal TDP-43 pathology [297]. Consequently, ataxin-2 has received much attention as a potential therapeutic target for treating ALS. Currently, BIIB105, an ASO targeting *ATAXIN-2* gene to lower its protein levels, is in a phase I clinical trial for ALS (NCT04494256).

Viral vectors derived from adeno-associated virus (AAV) has emerged as the lead vector for CNS gene therapy delivery tool. However, to address the limitations of mRNA- and RNA-based drugs in practice, including those associated with

safety, efficacy, and bioavailability, various non-viral vectors are being investigated for drug delivery in ALS therapies [298]. This research focuses on improving the pharmacokinetic profiles of individual drugs, using physical or chemical systems such as nanoparticles, to overcome the clinical failure of some ALS therapies. For instance, Chen and colleagues developed an innovative way of delivering ASO encapsulated in calcium phosphate (CaP) lipid nanoparticles [299]. This alternative approach improves the delivery of the gene construct compared with the free form of ASO and reduces SOD1 levels in HEK293 cells. *In vivo*, the lipid encapsulation improves bioavailability in the zebrafish bloodstream, for better diffusion into the brain and spinal cord after direct injection. These encapsulation approaches could offer a considerable improvement in gene therapy for ALS in the future.

5.3. Antibody-based therapy approached

The use of antibodies targeting misfolded proteins is a promising approach for the treatment of ALS. Among the large number of antibody-based interventions tested for fALS (see [300] for review), a few stand out. For instance, Minamiyama and colleagues designed a new monoclonal antibody targeting misfolded SOD1 (D3–1) [301] which delayed disease onset and extended lifespan in treated-SOD1^{H46R} rats. Others stand out for their anti-protein aggregation mechanism. One example is the AP-101 antibody (NI005) directed against SOD1 protein, currently in Phase IIa clinical trials (NCT05039099). It has been found to attenuate motor symptoms and increase overall survival in mouse models (Neurimmune).

Tegoprubart (AT-1501) is an antibody that targets the CD40 ligand (CD40L), previously shown to be upregulated in the blood of ALS patients. Inhibition of CD40L delayed paralysis and prolonged survival in the SOD1^{G93A} mouse model [302]. Tegoprubart is now in Phase II clinical trial (NCT04322149). Finally, a Phase IIa study is underway evaluating ANX005 (NCT04569435), a recombinant anti-C1q antibody capable of preserving NMJs in SOD1^{G93A} ALS mice [303].

6. Conclusion

As ALS research continues to evolve, the model organisms presented here are valuable assets as versatile genetic models to study the pathogenesis of ALS and to identify new disease-modifying drugs to treat this devastating neurodegenerative disease. There is currently no sufficiently effective treatment to slow the progression of the disease or to compensate for the pathophysiological defects that have been identified and are yet to be discovered.

7. Expert opinion

Over the past decade, there has been a marked increase in interest in using simple organisms (*C. elegans*, Drosophila and zebrafish) to model ALS. Due to the key advantages of the simple models (Figure 3), such as their relatively short lifespan, low cost, ease of genetic manipulation and assay throughput, they have provided important insights into the understanding of ALS pathogenesis. Simple models stood out, offering the possibility of developing new tools for translational research. Their ease of use, small size and, in some cases, transparency have led to the development of new techniques for *in vivo* imaging, automated low to high-throughput screening of small molecules and genetic screening for human mutations. These developments have been instrumental in therapeutic discovery with the identification of lead compounds such as pimozide, α-lipoic acid and HA-114, which are currently being tested in ALS clinics.

Admittedly, the motor behavior and organization of the neuromuscular system are simplified in these animals. However, this is a real advantage in quickly and easily identifying new therapeutic targets before confirmation in larger animal models or cellular models. Translational studies in simple models have so far focused on the identification of small molecules, particularly as part of a drug repositioning strategy. However, we expect to see emerging proposals for new gene therapies. In zebrafish, ASOs can be administered to the embryo by microinjection or immersion in embryonic water or directly to the brain in adult fish. Moreover, CRISPR-based and cytosine base editing [153], is a powerful method for efficient and rapid insertion of ALS-associated mutations. Compared with traditional genome-editing methods, it can faithfully reproduce the exact point mutations found in ALS patients and could improve model reliability without double-strand DNA cleavage or a donor DNA template.

Likewise, several mouse models have been developed to study the different genetic forms of ALS, and they have provided key insights into pathological pathways implicated in ALS. They have also allowed the development of therapeutic strategies for ALS. The strength of the mouse model lies in its ability to provide multiple therapeutic strategies, ranging from small molecules to antibodies, gene therapy approaches and ASOs. Unlike simple models, the rodent model offers a wide range of behavioral and cognitive tests that allow for a more detailed assessment of the safety and efficacy of a therapeutic intervention.

However, preclinical studies using mouse models face a number of difficulties due to the cost of raising animals and the difficulty of obtaining animal models reflecting critical ALS pathophysiology [304]. As a result, most of the compounds identified in these models have unfortunately failed in the clinic [305]. Some translability issues seem inherent to the model. For example, we can question the robustness of the SOD1^{G93A} mouse model or other commonly used preclinical mouse models that do not exhibit TDP-43 pathology.

But also, the design of preclinical experimental therapy, which now lacks sufficient protocol uniformity, requires improvement. It is crucial to clarify if the experimental drug or therapy was given during the pre-symptomatic or symptomatic stage. The development of symptoms in mouse and rat models varies significantly, with a delayed and progressive pattern. This variability must be carefully taken into consideration for all *in vivo* models. This allows for the distinction between prospective disease-modifying therapies and those that will have a preventative impact.

Some therapeutic compounds have failed in clinical trials likely due to their inability to cross the blood-brain barrier
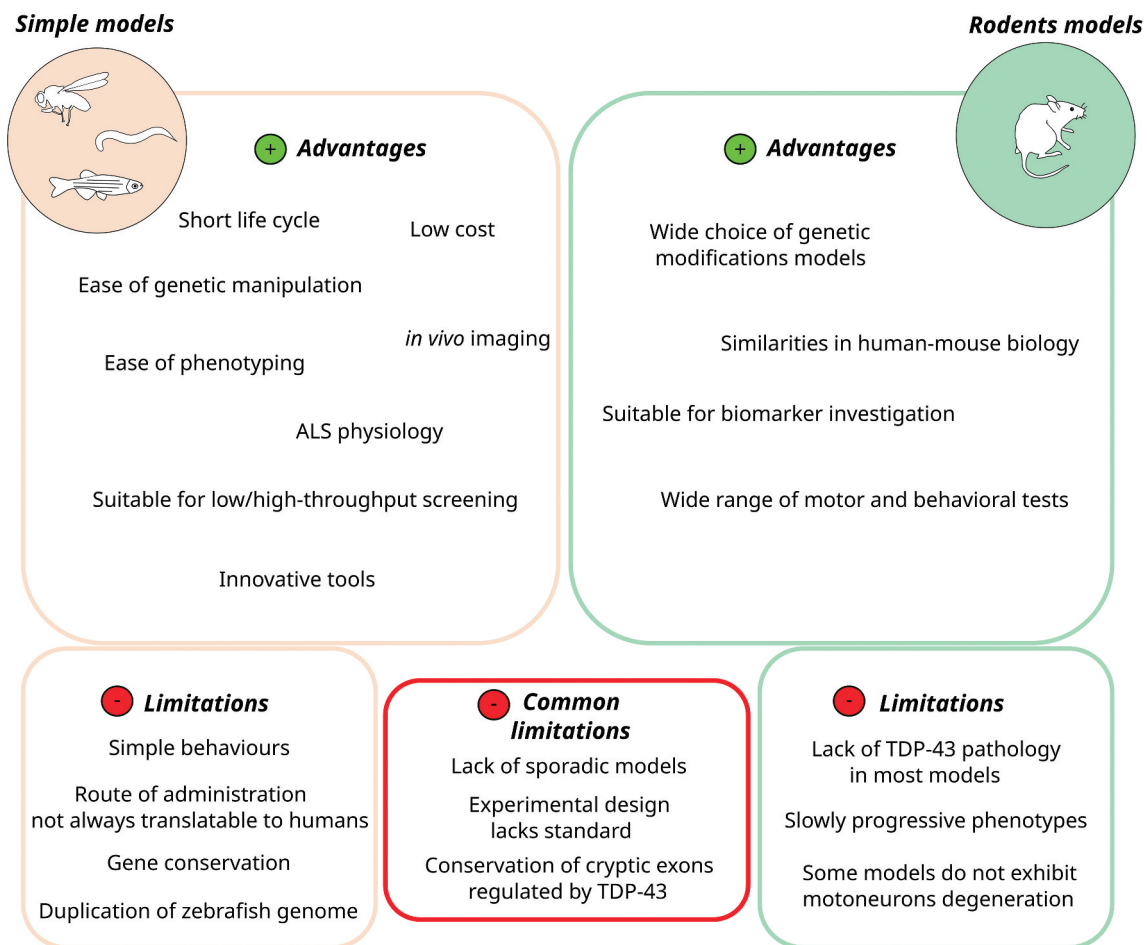
Figure 3. Advantages and limitations of simple models (zebrafish, *C. elegans* and *Drosophila*) and rodent models for the translational research of new therapeutics for ALS.

(BBB), as well as their poor pharmacokinetic profiles (including absorption, bioavailability and metabolism). Research efforts are aimed at overcoming these challenges, notably by developing new strategies for delivery of therapeutics using nanotechnologies [298]. For example, the encapsulation of a therapeutic cocktail composed of Leptin and Pioglitazone (already successfully tested in mice [277–279]) in a mesoporous silica nanoparticles (MSNs) has been explored to optimize the cocktail's *in vivo* delivery [306]. Moreover, recently, the formulation of intranasal edaravone-loaded poly(lactic-co-glycolic acid)-poly(ethylene glycol) PLGA-PEG polymeric nanoparticles improved the biodistribution of the preexisting pharmacological compound [307]. In addition, chronic treatment of SOD1^{G93A} mice with the retinoid adapalene encapsulated in similar PLA-PEG nanoparticles has a neuroprotective effect, improving motor performance and prolonging lifespan [308].

Liposomes have the potential to cross the BBB due to their lipophilic properties, and guarantee good bioavailability in the CNS. They are an excellent ally for neurodegenerative diseases. One study showed that they improved the therapeutic potential of steroid compounds methylprednisolone. When intravenously injected in SOD1^{G93A} mice, the anti-inflammatory compound encapsulated in PEGylated liposomes (2B3–201)

significantly improves histopathological outcomes in brainstem motor nuclei, resulting in reduced gliosis and reduced neuronal loss [309]. Similarly, the anti-inflammatory agent minocycline, when contained in modified lipopolysaccharide liposomes, has better bioavailability and can ameliorate microglia-dependent neuroinflammation [310]. Thus, the development of new delivery and administration options offers a promising prospect to address the inefficiency of some chemicals in the clinic. Importantly, animal ALS models are essential for testing novel drug delivery approaches in preclinical studies.

Furthermore, it is important to highlight the significant contribution of animal models in the identification of biomarkers for ALS [179]. The exploration of novel biomarkers is widely encouraged. This might enhance our comprehension of the heterogeneity of the disease. Effective biomarkers can also function as surrogate endpoints in clinical trials, offering early indicators of the efficacy of a treatment.

A common constraint in the ALS field is that research is focused on developing animal models of the familial form of the disease, omitting most cases which are sporadic. Developing non-genetic animal models of ALS is one of the major current challenges in the field. Our current knowledge of environmental and genetic ALS risk factors is

limited [311]. The disease exhibits significant genetic heterogeneity, with over 150 genes that have been identified as either linked or associated with ALS [8]. This diversity is also reflected in the diversity of pathophysiological mechanisms, for which we have yet to identify the main cellular pathway responsible for the progression, severity and onset of the disease. In this sense, much of the fundamental biology of ALS is still not understood. Consequently, many pathophysiological pathways remain unknown, and we are probably overlooking many therapeutic targets. It is therefore important to focus efforts on a convergent pathway, which remains one of the biggest challenges to be overcome by the community toward developing an effective treatment.

As with many of the examples discussed earlier in this review, the strategy of combining *in vivo* and *in vitro* models has been very successful in moving promising compounds along the translational pathway. The modeling of ALS motor neuron phenotypes using human induced pluripotent stem cells (iPSCs) is rapidly expanding [312], and has the advantage of covering both major familial and sporadic forms [313] of the disease. The iPSC motor neuron model, for example, is proving very effective in combination with simple organisms for identifying new compounds and new genetic targets in high-throughput screening studies. We believe that the combination of these two models could be a real asset in overcoming the challenges that remain for the translatability of therapeutic interventions for ALS.

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